

*Electrophysiological Analysis of human P2X3 Receptors for Biophysical
Characterization of the Structural-function Relationship*

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Abstract

Goal To clarify how defined regions of the human P2X3 receptor govern channel opening and ion passage, using mainly electrophysiological measurements.

Method The cytoplasmic cap was probed by controlled heteromer formation: channels carrying only one C-terminus or lacking the N-terminus were expressed. Two-Electrode Voltage Clamp (TEVC) recorded current size and desensitisation. Ionic and hydrophobic contacts inside the cap were weakened or removed. Biochemical assays tracked the approach of the termini on activation. A cysteine-scanning series (I323–V334) in TM2 was made and tested with TEVC. Charges at three pore fenestration levels were changed, and a rat P2X5 TM2 domain was introduced into the hP2X3 channel.

Results A single C-terminus still allowed opening, but loss of the N-terminus terminated it, showing the cap's asymmetrical control. Breaking key contacts in the cap accelerated desensitisation, confirming cap stabilisation. I323, T330, and V334 acted as gate points; V326 contributed to partial dehydration of ions, and G333 helped keep the pore wide. TM2 showed marked flexibility, matching molecular-dynamics data and its link to the cap. Positive clusters at outer fenestrations enhanced cation entry; Replacing the corresponding TM2 residues of P2X3 with those from P2X5 resulted in varied changes in ion permeation.

Discussion These observations give a biophysical picture of how the cap, TM2, and fenestrations together shape P2X3 activity, mapping structural features directly onto gating and permeation behaviour.

Zusammenfassung

Ziel Ermitteln, wie definierte Domänen des humanen P2X3-Rezeptors Kanalöffnung und Ionentransport steuern, hauptsächlich mittels Elektrophysiologie.

Methode Kontrollierte Heteromerbildung, Deletion einzelner Cytoplasma-Termini, TEVC-Aufnahmen, Cystein-Scanning in TM2 (I323–V334) und Austausch ausgewählter P2X5-TM2-Reste; Auswertung von Stromhöhe, Desensitisierung und Selektivität.

Ergebnisse Ein einzelnes C-Terminus ermöglichte Öffnung, N-Terminus-Entfernung verhinderte sie. Aufbrechen zentraler Kontakte beschleunigte Desensitisierung. I323, T330, V334 erwiesen sich als Gatepunkte; V326 erleichterte partielle Ionendehydratation, G333 stabilisierte den offenen Zustand. TM2 zeigte ausgeprägte Flexibilität. Positive Ladungcluster an äußeren Fenestrationsen förderten Kationeneintritt; Einbau von P2X5-TM2-Resten veränderte die Permeation.

Diskussion Kap, TM2 und Fenestrationsen wirken zusammen und bestimmen die P2X3-Funktion; die Daten liefern eine biophysikalische Grundlage für strukturgeleitete Modulation.

1. Introduction

The biomembrane is one fundamental component of normal physiological activities, where various proteins reside, function, and participate in life cycles. Among the abundant membrane proteins, membrane receptors perform crucial tasks in signaling, allowing communication between cells and each other. Ionotropic and metabotropic receptors are two categories into which membrane receptors can be divided. Ion channels are transmembrane ion channels that allow the passage of ions such as Na^+ , K^+ and Cl^- as a result of the binding of specific chemicals (known as ligands), while metabotropic ones can be further divided into enzyme-linked receptors, which are receptors that are or associate with enzymes, and G protein-coupled receptors, which activate G proteins in response to external signals and modify the level of second messengers, or thereby alter the function of ion channels (Luckey, 2008).

The following part will focus on the ligand-gated ion channels, more specifically, the P2X receptors that belong to the purinergic receptor family.

1.1 The Purinergic Receptors

Nucleosides, particularly adenosine, and nucleotides, notably *adenosine diphosphate*, ADP, and *adenosine 5'-triphosphate*, ATP, are best known for their role in DNA/RNA synthesis and energy metabolism, fundamental processes in all biological systems. In addition, since the article about purinergic signaling was published in the 1970s, it has become clear that ATP also serves as a neurotransmitter (Burnstock, 1972, 1976, 1990). Over the years, the narrative about the purinergic

receptor family emerges and deepens from an outline to gradually enriched details as research expands.

Three distinct receptor families mediate the effects of purine nucleotides and nucleosides. Those are: (Ritter et al., 2019)

- **Adenosine receptors (P1 receptors):** G protein-coupled receptors that respond primarily to endogenous adenosine. The P1 receptors are, up to date, divided into 4 subtypes, namely A1, A2A, A2B, and A3. Those subtypes take on various responsibilities, including mediation of immunological and central nervous system (CNS) activities (Burnstock, 2018).
- **P2Y metabotropic receptors (P2Y1–14),** G protein-coupled receptors often prefer ATP over various adenine nucleotides among the endogenous ligands they bind to. Eight P2Y receptors have been specifically identified in humans so far: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14. The depth of the studies demonstrates the complexity of their physiological roles. The P2Y receptors are crucial components of the delicate regulation that is active throughout life, controlling processes like cell proliferation, platelet aggregation, immunological response, and ion transport (Burnstock, 2018).
- **P2X ionotropic receptors (P2X1–7),** ATP-gated ion channels. Seven subtypes of P2X receptors have been identified, from which homotrimeric or heterotrimeric channels can be assembled. Similar to their close relatives, those receptors are widely distributed and perform functions such as signaling modulation, immunological response, and cell-death triggering in sensory neurons, immune cells, and smooth muscle cells (Burnstock, 2018). The relevance shall be described in detail in a later chapter.

1.2 The P2X receptors

The P2X receptor (P2XR) family is a group of proteins that includes mostly cation-permeable ligand-gated ion channels that open in response to extracellular ATP binding. The P2X receptors are divided into seven subtypes according to their

different pharmacological characteristics. These receptors function at the interfaces between neurons as well as between neurons and smooth muscle cell junctions upon the ATP released by exocytosis from various neurons (Saier Lab, 2023). ATP-evoked currents were observed on neurons dissociated from several regions of the mammalian brain, spinal cord, glial cells, primary sensory neurons, and other positions, implying a wide range of overall expression (North, 2002). mRNA that encodes specific P2X subtypes was detected in several retinal cell types too (Franke et al., 2005). Though different tissues seem to have a preference for some subtypes over others (Vial & Evans, 2000). The overlap of different subunit distributions seems to be more common.

1.2.1 Molecular properties

Similarities of more than 35% in sequences can be found across the different P2X receptors, despite variability in amino acid composition and differences in overall length (North, 2002). While sharing a similar topology, all subtypes start from a cytoplasmic N-terminus followed by the first transmembrane domain, namely TM1, to a bulk extracellular domain and span through the membrane the second time via the TM2, the second transmembrane domain, and end with a cytoplasmic C-terminus. The C-termini, among all domains, carry the most amino acid variability as reported, while the identity is mostly manifested in the extracellular domains, where several glycyl residues and more unprecedentedly, 10 conserved cysteine residues that form 5 disulfide bonds have been found (North, 2002; Saier Lab, 2023).

Overall Architecture

As the first successful attempt at the crystallization of zebrafish P2X4 receptors (zfP2X4Rs) (Kawate et al., 2009), and the following release of the crystal structure of human P2X3 channels (hP2X3Rs) (Mansoor et al., 2016) as well as the full-length human P2X7 channels (hP2X7Rs) (McCarthy et al., 2019), the piling up researches have been providing more and more enriched details on the molecular properties on the P2X receptors, also allows the crossing structural comparison aligned with structure-function study within the different subtypes.

From crystallization of the N- and C-termini deleted homotrimeric zfpP2X4 (Δ zfpP2X4) in apo state, which means the receptor is without ligand binding and thereby inactivated, one receptor was observed to be constructed by 3 “dolphin-like” single subunits with a large extracellular domain above the membrane plane and a smaller hourglass-shaped TM region that consists of six helices spanning through the membrane. The TM helices are placed antiparallel to one another within each subunit, and wrapped into a left-hand twist before being inserted into the cytoplasm at a 45-degree angle close to the membrane. On the contrary, the extracellular regions from single subunits are grouped in a right-handed twist with extensive subunit-subunit contact interfaces. The extracellular domain has an equilateral triangle shape and a rigid surface. Due to a weak electron density of the TM domain, not all residues were able to be correctly attributed (Kawate et al., 2009). In the later released hP2X3 structure spanning from D6 to T364 (hP2X3-MFC) in apo, activation state, which is defined as a ligand-bound and activated state, and desensitized state, which is ligand-bound yet inactivated state induced by prolonged ATP application, were identified. In addition to the hydrophilic extracellular domain mentioned above and the six helices that formed the TM domain, a part of the intracellular termini, including D6-I23 prior to the TM1 and N345-T365 after

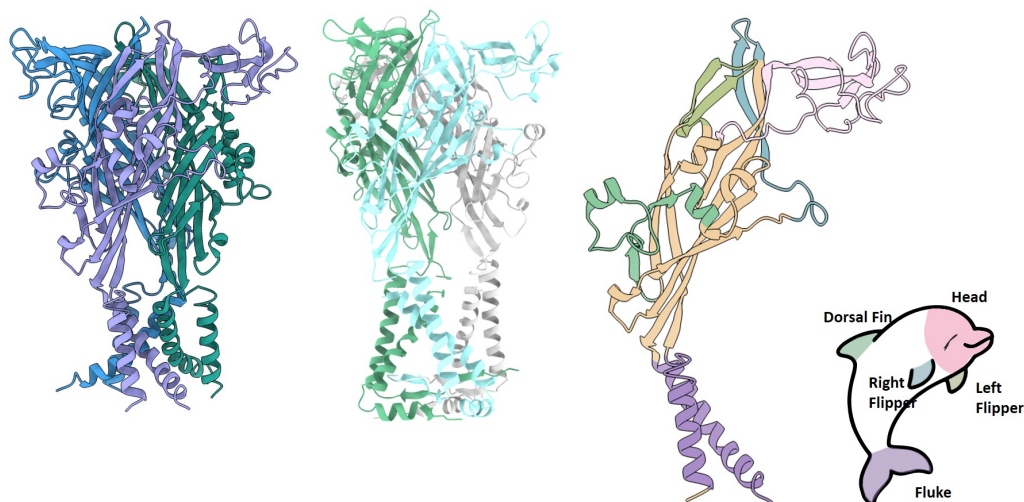


Figure 1.1: The left panel shows a zebra fish P2X4 receptor in the apo state, the three monomers are color-coded (PDB:4DW0); the middle panel shows the human P2X3 channel in the open state, the monomers are color-coded. Notice the cytoplasmic cap below the TMs (PDB: 5SVK). The right panel depicts the P2X monomer resembling a dolphin. Each function domain is color-coded and specified.

the TM2, were also established.

A striking observation was the presence of a single “cytoplasmic cap”, as shown in Figure 1.1 during the activation state formed from the folded intracellular termini, implying that the intracellular domain is vital for establishing the open channel. Additionally, special extended TMs and the cytoplasmic domain of hP2X3-MFC were characterized (Mansoor et al., 2016). The crystal structure of rat P2X7 (rP2X7) declared a large cytoplasmic domain of P2X7, also a permanent cytoplasmic cap in contrast to the activation-specific one from the hP2X3 (McCarthy et al., 2019).

The dolphin-shaped protomer conveniently provides a proper and illustrative way to describe its structure, as detailed in Figure 1.1. The dolphin rises above the membrane, emerging the curved upper body that can be further broken down to the head, beak, body, dorsal fin, and flipper domains, while leaving the fluke domain under the “surface of the ocean” and in reality, penetrating the cell membrane and conventionally referred to as the transmembrane domains. Three subunits curled up along an axis of symmetry that projects perpendicularly from the cell membrane (Khakh & North, 2012).

The Extracellular Domain

The crystal structure of Δ zfP2X4 demonstrates approximately 70Å of which the extracellular domain extends above the membrane (Kawate et al., 2009). It shows an exact composition of thirteen β -strands and four α -helices. The β -sheets establish the rigid backbone crossing the subtypes. At the same time, the peripheral α -helices and loops define the variances with three out of five suggested disulfide bonds localizing on the head domain and stabilizing the loops and short β -strands in this area (Khakh & North, 2012).

From this stabilized head domain, the three subunits extend downwards, creating a continuous cavity with three sections referred to as upper, central, and extracellular vestibules defined by narrowing in pore-lining along the axis of hP2X3 (Mansoor et al., 2016), as visible in Figure 1.2. Each subunit provides a 2-stranded β sheet to build the wall of the upper vestibule, following the constrictive opening formed from the loops at the head domain. The three-stranded β -sheets wall the central and extracellular vestibules, define the lower body, and end at the membrane from

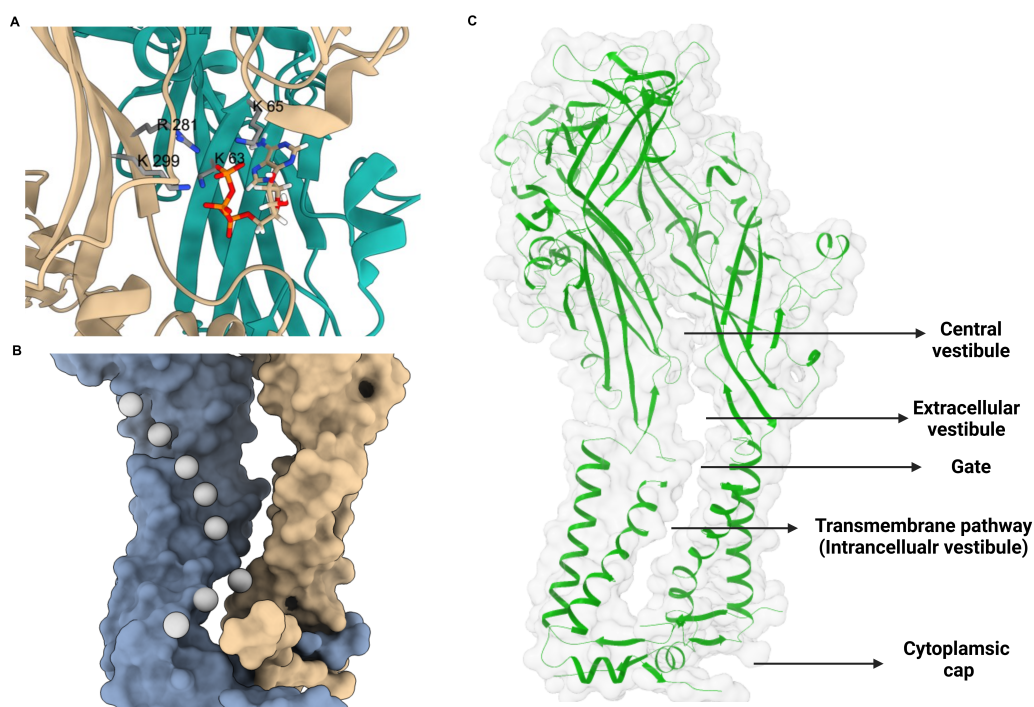


Figure 1.2: Panel A zooms in the ATP binding pocket, with an ATP molecule docked. The binding pocket is cradled by two subunits in different colors. Elements O (red), P (yellow), and N (blue) are noted. The essential residues K63, K65, R281, and K299 are shown in bone and sticks and labeled. Panel B shows the proposed ion path, from the extracellular fenestration to the cytoplasmic cap. The crystal structure is shown as surfaces, with one chain hidden for better illustration. The rest 2 chains are colored differently. The grey balls indicate the ions traveling to the cytoplasm. Panel C shows two chains out of 3, in ribbons and surfaces for a better boundary definition. From top to bottom, the central, extracellular, and intracellular vestibules are indicated. Additionally, the residue defining the extracellular boundary is noted as the gate. The cytoplasmic cap is included and indicated. (All structures obtained from hP2X3R in the open state, PDB code 5SVK)

where the transmembrane domains originate from the extension of β -sheets.

The extracellular domains cradle binding pockets for the endogenous ligand ATP as well as other chemicals that function as orthosteric agonists/antagonists or allosteric modulators. The ATP binding pockets formed by adjacent subunits' interfaces localized on large extracellular loops are generally conserved but still show subtype-specificity to a certain extent (North, 2002).

Exploring the zfp2X4 reveals that, to adapt the negatively charged triphosphate tail of ATP, and to coordinate the α -phosphate group, the binding pockets carry residues with positively charged side chains, most essentially, K70, K72, K316, and R298 (zfp2X4 numbering)(Chataigneau et al., 2013), whose substitutions of corresponding residues in crossing subtypes have been proven to greatly disrupt the ATP potency Ennion et al., 2000; Hausmann et al., 2012; L. H. Jiang et al., 2000. This finding also indicates how P2X receptors favor ATP considerably over ADP, which lacks the γ -phosphate group. The hydrophobic T189 and N296 (zfp2X4 numbering) that potentially participate in the coordination of adenine moiety and β -phosphate group in respect also appear to be crucial as well (Chataigneau et al., 2013). The non-conserved residues, on the other hand, had been proposed to endow subtype-specific features (Kasuya et al., 2017; Wang & Yu, 2016).

As observed in the dolphin-shaped structure, the extracellularly marked subdomains cooperate and initiate the activation of the P2X receptors by binding ATP and falling into a changed conformation. The head, flipper, and body domains are responsible for ATP binding and conduct the conformational changes, while the dorsal fin contributes to the latter (Wang & Yu, 2016). Symmetrically placed lateral portals (fenestrations) at the interfaces of neighboring subunits, between β -strands of the lower body and closing up to the membrane, have been proposed to be the ion pathway during channel activation based on both crystallized structure and function studies(Samways et al., 2011), as depicted in Figure 1.2. This unobstructed pathway is deemed the most obvious route of ion entry instead of the previously speculated central vestibule (central pathway), even though fewer charged residues that could potentially facilitate ion permeation are sitting on the former (Samways et al., 2011).

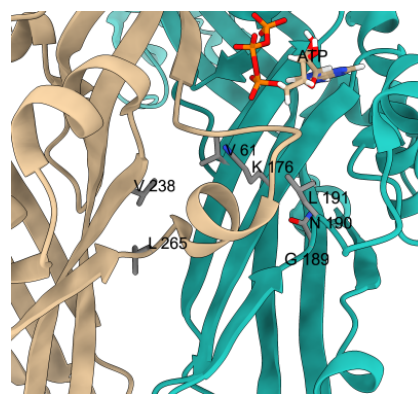


Figure 1.3: Zooming the allosteric modulator binding pocket (PDB: 5SVK). Notice that ATP is bound and the AM pocket is below the ATP binding pocket. The binding pocket is cradled by two subunits in different colors. Elements O (red), P(yellow), and N (blue) are noted. The essential residues V61, V238, L265, G189, N190, L191, and K176 in bone and sticks and labeled.

Another mention-worthy fact is the confirmed existence of allosteric binding sites

on the P2XR, seen in Figure 1.3. A negative allosteric site of hP2X3 was identified as contributed by one left flipper and the adjacent lower body domain (Wang et al., 2018), as shown in Figure 1.3. In panda P2X7 (pdP2X7), another allosteric site must be identified that is formed by two neighboring upper body domains (Karasawa & Kawate, 2016). It is promising that more allosteric sites are expected to be uncovered in other subtypes, as they could potentially serve as clinical drug targets.

The Transmembrane Domains

The dolphin's tail, noted as the fluke domain or transmembrane domain, is embedded in the lipid bilayer, providing a channel pore for ion flow during activation.

The transmembrane domain is composed of the peripheral TM1 and the pore-lining TM2, two α -helices that span the membrane while connecting the extracellular and cytoplasmic domains. The TM2s are widely separated from each other viewing extracellularly, and approach until crossing and reaching down the cytoplasm. Each subunit devotes two helices, and together, the ion permeation pore is formed. While the TM2 defines the ion transmembrane pathway, the TM1 tilts and contacts the membrane (Oken et al., 2022).

Both TM1 and TM2 are connected directly to the low body from the extracellular domain, thereby receiving the mechanical force induced by the changed conformations above the membrane, and undergoing conformational changes accordingly (Wang & Yu, 2016). The stretching from the extracellular domain induces an iris-like movement of TM2, thus dilating the channel into an ion-permeable size (North, 2016). Studies have shown that TM1 still lines the larger portion of the extracellular pore opening, even though its contribution to ion penetration is min-

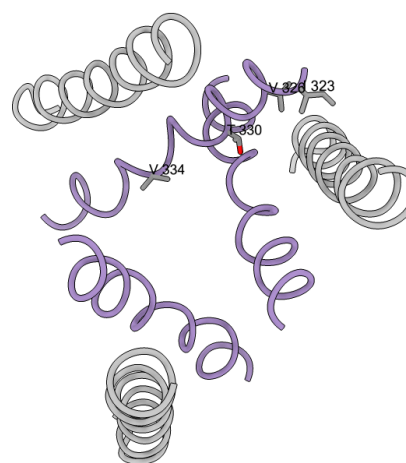


Figure 1.4: Transmembrane helices of the hP2X3 receptor in the open state (PDB: 5SVK). TM1 helices are shown in grey, TM2 in purple. Residues I323, V326, T330, and V334 are highlighted on a single subunit in bone representation.

imal (Heymann et al., 2013).

By capturing the hP2X3 in the apo, activated, and desensitized state, it has been suggested that 4 residues on the TM2 helices that are one helical turn apart are placed as pore occlusion (Mansoor et al., 2016). Among all, I323 gates the channel from the extracellular side, V326 contributes as a barricade, and the narrowest position is defined by T330, which potentially serves as an ion filter, as what has been reported previously where the substitution of its equivalence in hP2X2 altered the ion selectivity remarkably (Browne et al., 2011). Another residue, V334, constricts the ion permeation during desensitization.

The Intracellular Termini

Though entire structures of the N- and C-termini have not been fully resolved except for work of crystallization of rat P2X7 receptors (McCarthy et al., 2019), relevant studies have elaborated to a certain extent.

It has been reported that the N-termini consist of approximately 30 amino acids, while the C-termini are formed by β -strands of varying lengths across different subtypes (Burnstock, 2000). Two sequential β -strands from the N- and one from the C-terminus come together to create a structure known as the "cytoplasmic cap" that caps the pore from the cytoplasmic surface. The interaction between neighboring N- and C-terminal β -strands results in a compact β -sheet structure, integrating the receptor's subunits by incorporating strands from different protomers (Mansoor et al., 2016). The stability of this structure is crucial for channel opening, as has been observed directly in the captured open state of P2X3, and implied so in previous studies (Hausmann et al., 2014).

Mutagenesis on the N-terminus has resulted in a great impact on the P2XR behaviors, especially on desensitization. An early study suggested that a complete replacement of the P2X1 receptors' N-terminus with the non-desensitizing P2X2's ones diminished the fast-desensitization of the P2X1 Rettinger and Schmalzing, 2004. Later on, it was revealed that N-termini contain a highly conserved protein kinase C consensus site, whose mutations ended with changed desensitization kinetics (Boué-Grabot et al., 2000). By mimicking the hydrophobic interaction in the cytoplasmic domain by mutating three P2X3-specific residues into the corresponding ones within the N-terminus of the P2X2 receptor, a fully non-desensitizing

phenotype of P2X3 was successfully created (Hausmann et al., 2014). Besides the contribution of desensitization kinetics, N-termini seem to play roles ion permeation as well. A mutation between lysine and glutamic acid at the position of 17 on mouse P2X5 dramatically alters the cation-anion selectivity (Tam et al., 2023). A special uncharged region of the N-terminus of P2X6 has also been reported causing ER retention of P2X6 homomeric receptors (Ormond et al., 2006).

Studies suggested that the modification of the C-termini also affects the desensitization phenotype. For example, the rP2X2A variant desensitizes more slowly compared to its C-terminally modified splice variant, rP2X2B (rP2X2B) (Smith et al., 1999). The C-terminus is also involved in receptor trafficking. Studies proved a conserved trafficking motif YXXXXK (tyrosine – three variant amino acids - lysine) within the C-terminus that is vital for surface expression, as it stabilizes the P2X receptors on the cell membrane and avoids being internalized (Chaumont et al., 2004). As one with a uniquely long C-terminus among all the subtypes, the P2X7 receptor intrigues a specific interest. An 18-amino acid cysteine-rich region was identified on P2X7 by crystallography, termed “C-cys anchor”, which anchors the cytoplasmic cap to the membrane through palmitoylation (McCarthy et al., 2019). By stabilizing and anchoring the cytoplasmic cap to the membrane, the C-cys anchor creates a permanent version of the cap, preventing the receptor from falling into a desensitization state. Additionally, a sequence of approximately 200 amino acids forms a structure known as the “cytoplasmic ballast,” which is believed to participate in downstream signaling (McCarthy et al., 2019).

Subunits Assembly

To form a functioning pore, the P2X receptors need to assemble into multi-protein complexes termed oligomers, to enable insertion into the hydrophobic bilayer cell membrane (Nicke et al., 1998b). This process can be done within identical subunits as homooligomeric complexes, or among different subtypes to form heterooligomeric complexes (Torres et al., 1999). Most P2X receptor subtypes can form heteromers with other subtypes. However, P2X7 subunits tend to form homomers, which are not directly related to their lengthy intracellular domain. Meanwhile, P2X6 cannot form a functional homotrimer due to sequence-specific ER retention (Torres et al., 1999). The specificity of subunit assembly is not identified, though it is suggested that P2X receptors might have extensive intersubunit inter-

actions through their extracellular and TM domains, while the effect intracellular termini play a minimal role (Murrell-Lagnado & Qureshi, 2008).

1.2.2 The Function Profile

Functional studies on the P2X receptors have been evolving over the decades, especially upon the obtaining of multiple crystal structures. Not only have the general activation-deactivation mechanics been elaborated, but subtype-specific features have also been clarified to an extent.

Ligand Binding

Electron density maps from crystalized Δ zfp2X4-C-ATP indicate the localization of binding sites for ATP, illustratively (Kawate et al., 2009). Those pockets sit at 45Å from the extracellular boundaries of the transmembrane domains and are cradled by the head domain, upper body, lower body, left flipper, and dorsal fin from two neighboring subunits. To form one open-jaw-shaped binding pocket in the zfp2X4R, one subunit donates K70, K72, F188, and T189, while the other one contributes with N296, F297, R298, and K316. These residues interact extensively with ATP through hydrophilic interactions, initiating the action of the receptors. The orientation of these residues suggests how ATP is accommodated within the binding pocket, similar to the binding pockets observed in hP2X3 in Figure 1.2.

To define the open jaw structure, the head, left flipper, and upper body domain seem like the upper jaw; the lower body and dorsal fin compose the lower jaw (Hattori & Gouaux, 2012). Analyzed by molecular dynamics study (MD) and confirmed by the voltage clamp fluorometry (VCF) experiments (Fryatt et al., 2016) along with crystal structure (Hattori & Gouaux, 2012), it was revealed that the ATP molecules adopt an unusual U-shape when bound. This conformation is favored by the flexible head domain, causing β and γ -phosphates to fold towards the adenine ring instead of extending, while the adenine ring and the ribose ring are facing away from each other. This shape is stabilized by hydrogen bonds between γ -phosphates and one hydroxyl group on the ribose ring (Hattori & Gouaux, 2012). The negatively charged phosphate group establishes hydrogen bonds with positively charged and polar residues described above in the jaw. As crucial par-

ticipants, those residues exhibit highly conserved and coordinate ATPs similarly across different subunits.

For example, the ammonium group from K70 is placed in the ATP's U-turn, enabling its interaction of the oxygen atoms on the three phosphates groups from the latter; R296, and K316 from the same subunit interact with β -phosphate, while K72, R298 and another K316 contact with γ -phosphate (Hattori & Gouaux, 2012). The adenosine ring of ATP is facilitated by L191, L217, and I232 via hydrophobic interaction to be stabilized in the correct position (zfP2X4 numbering). Compared with other artificial ways of placing, it seems that only such a position of the adenosine ring tightens the jaw and induces downward movement of the head domain (Kawate, 2017). Although less conserved, hydrophobic residues at certain positions remain consistent across all subtypes, indicating a similar binding profile (Kawate, 2017).

Though ATP is primarily bound in complex with Mg^{2+} under physiological conditions, the Mg -ATP $^{2-}$ activates only fast-desensitizing phenotypes such as P2X1/P2X3, while the free ATP $^{4-}$ acts as a more universal agonist (Li et al., 2013). This is further confirmed by the free ATP-bound Δ zfP2X4 (Hattori & Gouaux, 2012) and the Mg -ATP $^{2-}$ -hP2X3 (Mansoor et al., 2016). Overall, the mode and conformation of ATP binding were also confirmed in the crystal structure of human P2X3Rs (Mansoor et al., 2016).

Multiple studies have investigated how ATP binding influences the activation of P2X receptors, revealing complex cooperative interactions. Ding and Sachs proposed that, during activation, P2X2 receptors exist in multiple closed and open states rather than simply switching between being open and closed, and the distribution of these states changes with the concentration of ATP (Ding & Sachs, 1999). In the same study, they found out that, the associations of ATPs are not independent but modify each other, the first ATP in the binding pocket included transient conformational changes that accelerate the binding of the second one (Ding & Sachs, 1999). Later experiments proved that the entering of the first ATP molecule inside of the binding pocket likely induced an intermediate closed conformation (R. Jiang, Taly, Lemoine, Martz, Specht, & Grutter, 2012), facilitating the binding of the following ATP, referring to "positive cooperativity" (Bhargava et al., 2012). Therefore, the occupation of at least two ATP-binding pockets enables activation of the channel (Chataigneau et al., 2013; Hattori & Gouaux, 2012), but the binding of one ATP is by no means pointless.

From ATP Binding to Channel Gating

Once ATP molecules bind to the P2X receptors, the receptors undergo conformational changes that transition the channel from closed to open states. Conformational changes take place once the binding of the first ATP molecule initiates, to couple the positive cooperativity taking place simultaneously within separated binding pockets (Moffatt & Hume, 2007). As more ATP molecules occupy the binding pockets, the receptor stabilizes in the open state, while trapping the agonist with even higher affinity (Bhargava et al., 2012).

Figure 1.6 illustrates the gating process. Upon entering ATP, the head domain moves down and the dorsal fin domain moves up, manifesting as the closure of the open jaw. During such a process, the presence of ATP not only triggers the approaching of the upper and lower jaw by intermolecular force but also breaks the formally established stable conformation defining the apo state. For example, it was experimentally confirmed that E167 and R290 (P2X2 numbering) in the binding pocket maintain bound, and the initiation of channel opening confirmation requires the rapture of such interaction from ATP insertion and usurps the interaction with R290, thereby disturbing the closed state (Hausmann et al., 2014; R. Jiang, Taly, Lemoine, Martz, Cunrath, & Grutter, 2012; Marquez-Klaka et al., 2007).

Upon the jaw closure, the coupled lower body domain thereby moves outwards, and the left flipper domain is pushed away. This movement of the lower body encourages the dorsal fin to move outward and widen the lateral portals localized at every subunit-subunit interface, allowing the subsequent ion flow (Kawate, 2017). The flexibility of the low body is thus vital for the transition of conformational changes (Habermacher et al., 2016). Linked to the β -sheets of the lower body, the transmembrane domains undergo expansion, where the transmembrane helices move outward and create a dilated pore that finally allows the passage through of ion flows (Kawate, 2017).

Both of the transmembrane helices, pore-lining TM2 and surrounding TM1 rotate upon the flexing of the lower body domain, to different extents (Kawate, 2017). Comparing the crystal structures of apo and open states, it was observed that TM2 moves counterclockwise while the exact angle of rotation differs among the subtypes. Details are described from the crystallized hP2X3. The gating residue defines the extracellular boundary (I323 in hP2X3) moves toward the extracellular

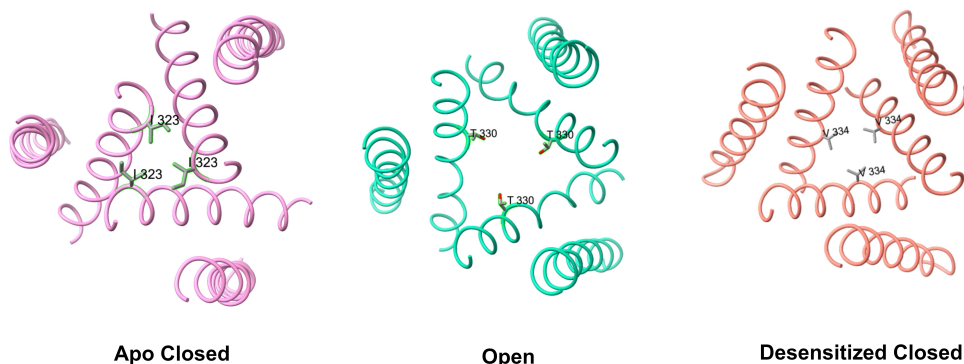


Figure 1.5: Comparisons of different TMs arrangements during the apo closed (5SVJ), open (5SVK), and desensitized closed (5SVL) state of the gating process. Each state is labeled and colored differently. The restriction sites in different states are noted, shown in bone and sticks, and colored by elements.

surface and positions itself away from the ion pathway. The gate is now open (Mansoor et al., 2016). This “iris-like” movement of the TMs turns the helices away from the center and produces an ion-conductive pore. In addition to that, the G333-V334-G335 sequence as part of the α -helix composing channel of hP2X3 transmits it into a 3_10 -helix with a more compacted structure, which allows the transition from opening to desensitization (Mansoor et al., 2016). Many native intersubunit contacts that keep the pore in the closed state diminish as the TMs rotate, while new contacts form to stabilize the channel in the open state (Hattori & Gouaux, 2012). An hourglass shape- determine residue, as T330 in hP2X3Rs, defines the narrowest part of the channel, and it must be pulled away to make the channel large enough for the hydrated ions to pass through. Moving down to the cytoplasmic side, a highly conservative residue, the G333 (hP2X3 numbering) raises interest as it was suggested to be the gating hinge (Hattori & Gouaux, 2012; Khakh & North, 2012).

It is suggested that the cytoplasmic end of the TMs rearranges and establishes both intersubunit and intrasubunit interactions, in contrast to the rotation of the upper TM (Kawate, 2017). During the crystallization of hP2X3Rs, the cytoplasmic cap was suggested to exist only during the open state, while the termini remains disordered without activation (Mansoor et al., 2016). When the P2X3 channel is activated, the intracellular domains are linked and undergo further conformational

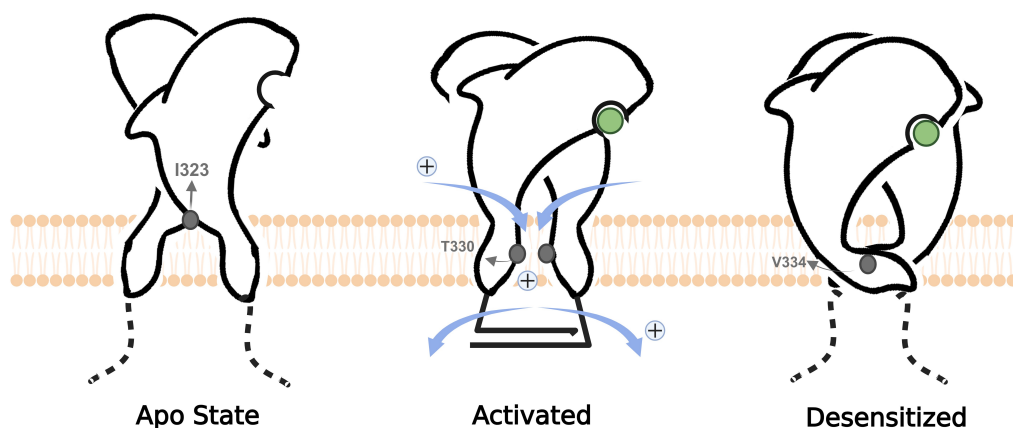


Figure 1.6: Diagram depicts the process of gating. For simplification, only two monomers are shown. The orange layers represent the membrane. During the apo state, the binding pockets are available, and the TM2s close the pore via the restriction of the I323 that defines the extracellular entrance of the pore. The termini are simplified and shown in dashes because of a lack of crystallography evidence. The green balls in the middle and right panels represent one ATP molecule. Upon the binding, the head domain descends due to the closure of the binding pocket, triggering a series of conformational changes and eventually leading to the rearrangement of the TM. The pore thereby opens. The blue arrows stand for the ion flow, along with "+" representing positively charged ions. Notice the lateral fenestration and the T330 within the pore that defines the narrowest gate during the open state. The cytoplasmic terminus folds and forms the cytoplasmic cap. While the ATP remains bound, the cap collapses and leads to further rearrangements of the TM2, during which the V334 becomes the gate of the desensitized pore and closes the transmembrane pathway.

changes due to the stretching conducted from the TMs above. Specifically, two N-termini and one C-terminus from the third subunit each donate one β -strand and knit into a β -sheet. These β -sheets further assemble into the cytoplasmic cap structure, whose stability helps maintain the open state of the channel. These conformational changes fix the cytoplasmic end of TM2, inducing the α -helix of TM2 to transition into a 3_{10} -helix, whose vertical straightening creates a more compact structure and aids in opening the channel pore (Kawate, 2017; Mansoor et al., 2016).

Termination of Activation

There are two macro states that the receptors reach for the termination of activation, depending on whether ATP molecules escape from the binding pockets

or not. This is also where two different types of P2X receptors diverge: the slow desensitizing ones, exhibiting almost no visible fast desensitization within the second's scale, such as P2X2 and P2X4; the fast-desensitizing ones, such as P2X1 and P2X3.

Once the ligands dissociate from slowly desensitizing P2XRs, the lower body domain reverts quickly back to the resting conformation. Without extracellular stretching, the TMs relax, narrow, and eventually close the open pore (Kawate, 2017). The desensitization, on the other hand, entails many more complex issues. Instead of simply reverting to the conformation of the apo state, the desensitization describes a distinct movement, initiated by the rotation of TM2's cytoplasmic portion and relaxing into an α -helix of the intensive 3_{10} -helix, described as a "helical recoil", an upwards translation of the promotor is prompt, as well as an inward rotation of the V334 sits on the later part of the TM2, as shown in Figure 1.5 and 1.6. The three subunits hereby approach and narrow the ion pathway, with V334 creating a constrictive site deeper in the membrane bilayer than the constriction in the apo state (Mansoor et al., 2016).

As mentioned in the section ?? extensive studies proposed that both N- and C-termini have a great impact on fast desensitization, which agrees with the later uncovering of the cytoplasmic cap in P2X3R. Identification of two conservative glycines on both the N- and C-terminus that could serve as hinges, G24 and G347 in respect (hP2X3 numbering), suggests the high flexibility of the termini and the possibility of their converging and the construction of a cytoplasmic cap (Mansoor et al., 2016). The cause of TM2 recoiling is the collapse of the cytoplasmic cap. In contrast, the C-cys anchor captured in the crystallized non-desensitizing rP2X7, which extends from the end of TM2 to the cytoplasm, forms a loop that acts as a hinge, keeping the cytoplasmic cap attached to the membrane while allowing its flexibility. The recoiling of the TM2 is prevented in the presence of the C-cys anchor, and further desensitization is stopped from there (McCarthy et al., 2019).

However, the desensitized receptors will be reset into the apo states eventually as ATP molecules dissociate. The dissociation of the ATP is required for the recovery, and this is considered the rate-limiting step of the long-lasting process (Rettinger & Schmalzing, 2004), which is partially credited to the changes in the ligand binding domain leading to more tightly held ATPs (Roberts et al., 2006). Further studies on the hP2X3 reveal that, when the dissociation of ATP is taken out of consideration, the conformational changes between desensitization and apo state are rapid (Pratt

et al., 2005).

Besides the structural variants of different ligands, the participation and presence of divalent cations is another factor that affects agonist dissociation. The hP2X3 inhabits a divalent cation-binding chamber that enables the ATP to access the binding pocket in the special form, for example, Mg^{2+} -ATP, which is a special feature that distinguishes such receptors from those subtypes that can only be activated by ATP^{4-} (see “**Ligand binding**” section). The bound divalent cation increases the ATP affinity, thus preventing the receptors from recovery, in which Mg^{2+} shows higher potency than Ca^{2+} . It can be concluded that those divalent cations act as an allosteric modulator (Li et al., 2019).

1.2.3 Ion Access And Selectivity

In the earlier subsection, the lateral portals were highlighted as the extracellular ion pathway through which the ions enter the channel. Despite the extracellular vestibule appearing to be a more constricted path, this counter-intuitive finding has been demonstrated (Kawate et al., 2011a; Samways et al., 2011). Such a pathway is constructed by Q52 and Q321 on one chain, and I328, D57, E59, and K195 on another (zfP2X4 numbering), blocked partially by lipids. Even though constructively existing as wide open pathways, the lateral portals expand more significantly during the activation (Khakh & North, 2012). The ion flows go through the three lateral portals and merge at the central vestibule, where negatively charged residues are localized and facilitate the passage of cations, creating a high concentration of permeating ions locally.

As the ion flow moves downward toward the cytoplasm, it reaches the channel region clustered by TM2. During the activation, the constrictive residue determines the narrowest part of the channel switch from the extracellular gate (T336 in P2X2 and I323 in P2X3), to central residues within the TM2, forming an hourglass-shaped constriction. Mutation of T339 in P2X2, which is such a kind of equivalence, into positively charged lysine, resulted in a significant increase in chloride permeability in contrast to the cation-selectivity of P2X2R. This provides direct evidence that such residues extend their side chains and interact with permeation ions (Browne et al., 2011).

Finally, ions exit into the cytoplasm. Instead of escaping directly along the receptor's central vertical axis, which is too narrow, the cytoplasmic cap and TM2 helices from adjacent units form the borders of a triangle-shaped opening, or 'cytoplasmic fenestration,' located within the lipid membrane. This mechanism was confirmed by MD simulation (Mansoor et al., 2016). Overall, ions enter through lateral fenestrations on the extracellular side, travel through the TM2s cradled channel, and exit through lateral fenestrations on the cytoplasmic side.

One way used commonly to describe ion selectivity quantitatively is the "relative permeability". In terms of "relative", the results are often presented as a ratio between the permeability of one ion X and another particular ion, usually sodium, as in $P(X)/P(\text{Na})$. The calculation is based on the experimental reverse potential or comparing how conductive a channel is in different ion environments, with Goldman-Hodgkin-Katz (GHK) voltage and current equations. Though the ion permeation that happens in an ion channel, in reality, might not strictly follow the assumption of the GHK equation, the calculation still offers an approximate estimation (Samways et al., 2014). What should be considered as a factor influencing ion permeability can be roughly concluded as the size of the channel, electrical environment, and ion interaction with the pore (Hille, 2001).

Generally speaking, P2XRs are mostly seen as non-selective cation channels, though it may not be the most accurate description. It is considered that P2XRs do not discriminate between K^+ and Na^+ , while Ca^{2+} , on the other hand, seems to be favored by P2XRs compared to other non-selective channels, as has been shown in the studies on rat P2X1 and P2X4 receptors (Egan & Khakh, 2004; Samways & Egan, 2007). Another notable receptor is the P2X5 with an unusually high Cl^- permeability. A recent study has found that a single lysine on the N-termini might be the key to this behavior, indicating the presence of ion pathways of cytoplasmic fenestration (Tam et al., 2023). Besides small ions, some members of the P2X receptor family were thought to adopt different conductive states where large organic ions can also be allowed to pass. The theory of "pore dilation", which implies a gradually dilating pore during continuous activation, was proven wrong by single-channel analyses of P2X7R (Riedel et al., 2007) and more recent studies on the accumulation of ion species during prolonged activation (Li et al., 2015).

1.2.4 The Agonists, Antagonists and Allosteric Modulators

In the previous section, ATP was mentioned as a standard and most important endogenous agonist for the P2XRs. Here, other compounds that bind to the P2XRs as agonists, antagonists, and allosteric modulators will be discussed. The detailed P2XR pharmacology was recently updated in the IUPHAR review no. 30 Illes et al., 2021.

Angonists The structural differences of binding pockets among variants of subtypes define their different binding profiles. Compounds structurally derived from ATP, such as 2-meSATP (*2-Methylthio-ATP*), and ATP γ S (*adenosine-5'-O-(3-thiotriphosphate)*), have been reported as metabolically more stable P2XR agonists (Illes et al., 2021). BzATP (*2',3'-O-(4-benzoyl benzoyl)*), an ATP analog, has been reported as the most potent unselective agonist. α,β -meATP (*α,β -methylene ATP*), on the other hand, is selectively favored by P2X1 and P2X3, while demonstrating low potency for the other (Saul et al., 2013). β,γ -meATP (*β,γ -methylene ATP*) exhibits similar selectivity, as a potent partial agonist for P2X1 and P2X3 (Syed & Kennedy, 2012). Besides the ATP analogs, P2X1 exhibits sensitivity towards diadenosine polyphosphates, though with very low efficiency (Conant et al., 2000).

Antagonists Derivatives of the antiparasitic agent suramin were among the firstly reported antagonists. Suramin *per se* is a potent non-selective P2X antagonist, with the exception of P2X7 with a much lower potency. Derived compounds such as NF279 and NF449 are extremely potent and quite selective for P2X1 only (Syed & Kennedy, 2012). PPADS and Reactive Blue 2 are other mentionable non-selective antagonists, except for P2X7 (Bennetts et al., 2022; Illes et al., 2021; North & Jarvis, 2013).

Nucleotide derivatives negatively modulate P2X receptors as well. TNP-ATP (*trinitro phenyl-ATP*), as one of these variants, blocks P2X1, P2X3, and P2X2/X3 heteromers at the nanomolar range (North & Jarvis, 2013). A-317491 was one of the first developed P2X3 and P2X2/3 selective antagonists, showing the capability of reducing chronic inflammatory and neuropathic pain in the rat (Jarvis et al., 2002). P2X7 has been the most extensively targeted object for the antagonist's development, mainly allosteric ones (Illes et al., 2021; North & Jarvis, 2013).

Allosteric Modulators Trace metals such as zinc and copper, are the first reported P2X allosteric modulators with subtype-specific effects, among which, distinct effects might occur within the same receptors. For example, P2X2 and P2X4 are potentiated by low-concentration zinc, while suppressed by a high concentration (Coddou et al., 2011). Other metal ions can behave as allosteric modulators as well. Magnesium and calcium are seen as ligand-facilitating for P2X3 (Li et al., 2019). while these divalent cations inhibit P2X7 activity (Coddou et al., 2011).

pH affects the ATP-induced currents, indicating that hydrogen ions modulate the receptors allosterically. The rP2X2 is potentiated by acidification, but all of the P2XRs are inhibited by low pH. (Coddou et al., 2011). Alcohol, as another common factor of channel activation, also affects P2XRs, by inhibiting P2X2 and P2X4 while potentiating P2X3 (Coddou et al., 2011; Dhuna et al., 2019).

Specific allosteric modulators have been developed. Ivermectin, an antiparasitic agent, is proven to be a positive allosteric modulator (PAM) at P2X4Rs. Moreover, it also targets hP2X7 (Illes et al., 2021). Another established P2X4 PAM reported is ginsenosides, an active substance extracted from ginseng (Dhuna et al., 2019). An anthraquinone sulfonic acid derivative, Cibacron Blue, is suggested to be PAM for P2X3 and P2X4 while disrupting P2X4 at a higher concentration (Stokes et al., 2020).

High-throughput screenings and later identification of an allosteric binding pocket on P2X3Rs led to the discovery and development of a series of negative allosteric modulators (PAM) for P2X3, such as AF-219 (Wang et al., 2018) and AF-353 (Ford et al., 2011). One P2X3 NAM named Gefapixant, previously as compound MK-7264 (Illes et al., 2021), has been approved for relieving chronic cough in the EU in September 2023 (European Medicines Agency, 2023).

2. Aims of the Study

Since the crystal structure of the human P2X3 receptor has been revealed, significant progress has been made in understanding its overall architecture. However, such structural data largely reflect fixed snapshots of the receptor and offer limited access to the dynamic processes.

The present study was to investigate human P2X3 receptor under functional recording conditions, focusing on the conformational behavior of the cytoplasmic domains during gating transitions, the functional role of TM2 residues in channel activation, and the structural contributions to ion permeation.

Firstly, the cytoplasmic cap domain was examined. The formation of a cytoplasmic cap structure plays a critical role in channel activation and deactivation. In particular, the crystal structure of human P2X3 revealed a well-formed cap conformation in the open state. In the present study, the structural determinants supporting cap formation were investigated, with a focus on how the N- and C-terminal elements contribute to the gating process and overall channel function.

Following this, the study moved upwards to the transmembrane domain, focusing on the directly adjacent TM2 domain. A systematic cysteine scanning was performed to evaluate the functional role of individual residues within TM2. The goal was to characterize the contribution of specific residues to channel activity, therefore to understand how TM2 participates in the gating process.

Following detailed analysis of the TM2 helix, a key component of the ion channel, the next aim was to comprehensively characterize the ion permeation and selectivity of the P2X3 receptor, including both the central pore and possible lateral fenestrations. The focus was to move beyond the identification of single decisive

selectivity filters and instead to characterize the general ion permeation strategy of the channels.

Two-electrode voltage clamp (TEVC) recordings performed in *Xenopus laevis* oocytes served as the primary method throughout this study, supported by biochemical assays and whole-cell patch-clamp recordings in HEK293 cells. This combination of approaches enabled the analysis of receptor behavior under conditions that preserve its activity in a controlled, functional context, thereby allowing a more physiologically relevant understanding of the human P2X3 receptor.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents

General solutions and buffers are listed below. Others that were not mentioned here were obtained from Sigma-Aldrich (St. Louis, MO, US) in analytical grade. All restriction enzymes were purchased from New England Biolabs, USA.

Table 3.1: Overviews of compounds, reagents and kits

Compound / Reagent / Kit	Manufacturer / Supplier
ARCA Cap	Jena Bioscience, Germany
rNTP mix (ATP/CTP/UTP/GTP)	Jena Bioscience, Germany
SP6 RNA Polymerase	Biozym Scientific GmbH, Germany
RNasin Inhibitor	Promega, USA
RQ1 DNase	Promega, USA
TurboFect Transfection Reagent	Thermo Scientific, USA
Zeocin (100 µg/mL)	InvivoGen, USA
Blasticidin (15 µg/mL)	InvivoGen, USA
Hygromycin B (100 µg/mL)	InvivoGen, USA
QIAquick Nucleotide Removal Kit	Qiagen, Germany
Oligonucleotides (cloning/sequencing)	Eurofins Genomics, Luxembourg
Gateway BP Clonase II Enzyme Mix	Invitrogen, Thermo Fisher Scientific, USA

Compound / Reagent / Kit	Manufacturer / Supplier
Gateway LR Clonase Enzyme Mix	Invitrogen, Thermo Fisher Scientific, USA
Collagenase Type I	Worthington Biochemical Corporation, USA
Phusion High-Fidelity HF PCR MasterMix 2×	New England Biolabs, USA
Dulbecco's Modified Eagle Medium (DMEM), high glucose, L-Glutamine, phenol red	Gibco, Thermo Fisher Scientific, USA
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific, USA
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Carl Roth, Germany
Sodium chloride (NaCl)	Carl Roth, Germany
Trypsin/EDTA (100 mL)	Pan Biotech, Germany
6× DNA gel loading dye	Thermo Scientific, USA
Super Optimal broth (SOC) medium	New England Biolabs, USA

3.1.2 Buffers and Solutions

The composition of general buffers and solutions used in this study is listed in Table 3.2. Other specific solutions will be described in the corresponding section.

Table 3.2: Overview of buffers and solutions

Buffer / Solution	Components
Agarose gel loading buffer (5x)	0.05% Bromophenol blue, 40% Sucrose, 0.5% 0.1 M EDTA pH 8.0
Oocyte ringer with calcium (Ori ⁺)	90 mM NaCl, 1 mM KCl, 1 mM CaCl ₂ , 1 mM MgCl ₂ , 10 mM HEPES, NaOH (pH adjustment), autoclaved
Oocyte ringer without calcium (Ori ⁻)	90 mM NaCl, 1 mM KCl, 1 mM MgCl ₂ , 10 mM HEPES, NaOH (pH adjustment), autoclaved
TAE buffer (50x)	40 mM TRIS, 1.14 mM acetic acid, 1 mM EDTA, pH 8.5
TE buffer	5 mM TRIS, 0.5 mM EDTA, pH 8.0 (adjusted with HCl)
Intracellular solution (patch clamp)	140 mM NaCl, 2 mM MgCl ₂ , 1 mM CaCl ₂ , 10 mM HEPES, 11 mM EGTA, pH 7.4 (adjusted with NaOH)
Standard extracellular solution (patch clamp)	140 mM NaCl, 1.5 mM CaCl ₂ , 1 mM MgCl ₂ , 10 mM HEPES, pH 7.2 (adjusted with NaOH)

3.1.3 Water

Demineralized water was produced from tap water using ion exchange and further purified to ultrapure grade (conductivity: 0.055 $\mu\text{S}/\text{cm}$) via ultrafiltration. This ultrapure water, nominally DNase- and RNase-free, was used for all buffer and solution preparations.

3.1.4 Equipment and Software

General equipment and software are listed below.

Table 3.3: General equipment, software and tools

Equipment / Software / Tool	Manufacturer / Developer
Incubator Function Line	Heraeus, Hanau, DE
Centrifuge 5415D	Eppendorf, Hamburg, DE
Gel documentation system	Phase, Lübeck, DE

Equipment / Software / Tool	Manufacturer / Developer
MasterCycler (PCR)	Eppendorf, Hamburg, DE
Microsoft Office	Microsoft, Redmond (WA), US
pH meter 605	Metrohm, Herisau, CH
Shaking incubator Minitron	Infors, Bottmingen, CH
VectorNTI Software	Invitrogen, Karlsruhe, DE
Vortex Genie 2	Bender & Hobein, Zurich, CH
PC-100 puller	Narishige Group, Japan
Nanoliter 2000 injector	World Precision Instruments, USA
Borosilicate glass with filament ($1.20 \times 0.69 \times 80$ mm)	Science Products GmbH, Germany
EVH-9 valve system	Bio-Logic, France
VM-8 valve system	ALA Scientific Instruments Inc., USA
Zeiss Axiovert 135 TV inverted microscope	Zeiss, Germany
Power Supply EA-PS 3016-10	ElektroAutomatik, Germany
P-1000 pipette puller	Sutter Instrument, USA
PatchStar Micromanipulator	Scientifica, UK
PatchMaster v2x90.5	HEKA Electronic, Germany
HEKA EPC 10 USB Patch-Clamp Amplifier	HEKA Electronic, Germany
Turbo Tec 05X amplifier	npi electronic GmbH, Germany
Cellworks 6.2.1	npi electronic GmbH, Germany
Cellworks Reader 6.2.2	npi electronic GmbH, Germany
FITMASTER version 2x92	HEKA Electronic, Germany
Marienfeld glass coverslips	Marienfeld GmbH, Germany
R Statistical Software (v4.2.3)	R Core Team
RStudio (2022.07.2)	Posit, PBC, Vienna, Austria
GraphPad Prism 10.0.0	GraphPad Software, Boston (MA), USA
tidyverse, dplyr (R packages)	R community
Swiss-Model (web service)	Swiss Institute of Bioinformatics, Switzerland
UCSF ChimeraX	University of California, San Francisco, USA
BioRender	BioRender, Toronto, Canada

3.2 Molecular and Cell Biology

3.2.1 Vectors and Plasmids

Various vectors were used to fit different purposes in this study.

- **PNKS2/ PNKS4:** One of the commonly used vectors to adapt the *Xenopus laevis* oocytes expression system. The PNKS2 vector targets the *X.laevis* oocytes expression system, which is embedded with a restriction enzyme cut site after the mRNA-protecting poly-A tails, in our case mostly XhoI (Gloor et al., 1995). Compared to the PNKS2 vector, the PNKS4 carries a different enzyme cut site. Since this difference has no significant impact in this context and considering that new mutants were built on the previously established plasmids, no distinction was made between these two vectors in later analysis or discussion.
- **pDONR:** The donor vector for Gateway recombinational cloning carries attP sites flanking the ccdB gene, whose successful substitution by the entry clone during the BP reaction allows the growth of the positive clones. The attP sites serve as recognition sites for the identification and localization of entry clones with corresponding attB sites. Besides, pDONR provides kanamycin resistance, allowing the selective growth of *E. coli* that accepted this plasmid on kanamycin-containing agar plates.
- **pNKS-GW:** One of the destination vectors used in Gateway recombinational cloning. This is a modified PNKS vector that is Gateway-cloning compatible with both oocyte expression and plasmid shuffling (Fallah et al., 2011). The pNKS-GW features flanking attR sites to recognize the entry clone of pDONR. Unlike the pDONR, PNKS-GW is characterized by its ampicillin resistance, marking a selection on different plates.
- **pcDNA5/FRT/TO** (invitrogen, 2012): Inducible expression vector designed for use with the Flp-In System. It contains a Flp Recombination Target (FRT) site for Flp recombinase integration of the vector into the Flp-In host cell line. Upon the successful integration of the target gene, the hygromycin resistance is activated, thereby rescuing positively transfected cells during hygromycin selection.

- **pOG44** (invitrogen, 2012): Flp-recombinase expression vector designed for use with the Flp-In System. It codes the Flp recombinase enzyme during the cotransfection with pcDNA5/FRT and facilitates the integration of the desired vector.
- **pFRT/lacZeo** (invitrogen, 2012): Flp recombination target site vector designed for use with the Flp-In System. This vector houses the lacZ-Zeocin fusion gene as well as a single integrated FRT site. It provides a cell line generation technique combined with Zeocin and screening by β -galactosidase activity assay.

3.2.2 DNA preparation

Site-directed Mutagenesis All desired mutants are introduced to previously established plasmids via PCR (Polymerase chain reaction)-mediated site-directed mutagenesis according to the Quick Change Protocol (New England Biolabs, 2012), which provides a neat and accurate modification of DNA. The steps of the method are as follows:

1. Primer design: Primer designing followed the general guidelines of primer construction (New England Biolabs, 2012). The sequences of the primers are designed via vectorNTI and synthesized accordingly by Eurofins Genomics.

2. PCR Amplification. In the following table, precisely controlled components are described.

Table 3.4: Components for PCR

Template DNA	150ng
Primer (Oligonucleotides)	125ng
Dimethyl sulfoxide (DMSO)	3 μ l
2x Phusion High-FidelityHF PCR MasterMix	25 μ l
Distilled water	add until the final volume of 50 μ l

The DMSO is sometimes incorporated as an optimization of the PCR processes. The Phusion High-Fidelity HF PCR MasterMix contains the Phusion DNA Polymerase as the synthesis tool, dNTPs as raw materials, and reaction buffers. The

thermocycler protocols vary, but all involve three basic steps: denaturation, annealing, and extension. Examples are given below.

Table 3.5: Basic Conditions of Different PCRs

	Single-point Mutation	BP-PCR	Large Insertion
Initiation	95°C, 5 min	98°C , 30s	98°C, 5min
Denaturation	95°C , 30s	98°C , 10s	98°C , 30s
Annealing	55°C , 1 min	55°C , 10-30s	55°C , 1 min
Extension	68°C , 2min/kb	72°C , 15-30s/kb	68°C , 2min/kb
Final Extension	72°C, 10 min		
4°C for cooling			

The process of denaturation, annealing, and extension is repeated over multiple cycles, with 25–35 cycles typically being sufficient, as outlined in the Quick Change protocol (New England Biolabs, 2012). However, for large insertions, more cycles are often required (GenomicsAgilent, 2007). The annealing temperature depends on the primer length: the longer the primer, the higher the temperature needed. For primers of average length, a temperature range of 55–60°C was used. Although the protocol suggests 30 seconds per kilobase for extension, a longer time of 2 minutes per kilobase was routinely used to ensure a more robust yield.

DpnI digestion Plasmids served as templates for the PCR process are methylated, due to their replication in *E. coli*, while the PCR products are not. Thereby, DpnI, which recognizes and cleaves methylated sites, can be used to digest the template once the PCR is finished while leaving the desired products intact (Addgene, 2023). 2–3µl of DpnI was added to the PCR tube and incubated for 2–3 hours, followed by agarose electrophoresis to verify the degradation of parental plasmid but not products.

The three different PCR schemes mentioned above were designed for different purposes: the single-point mutation was the most universal plan, which was used to introduce a point mutation into the template DNA. The BP-PCR is the initiating step of Gateway Cloning (GW), which is a method aiming to incorporate DNA sequences into different vectors. Following the instructions, the following steps of BP-PCR, BP, and LR reactions are achieved via the Gateway BP Clonase II Enzyme Mix and LR Clonase Enzyme Mix. The Large Insertion technique introduces a large insertion in plasmids (GenomicsAgilent, 2007). The two-step technique includes standard PCR that copies the desired fragment and the noted

PCR procedure of inserting such a fragment into the new plasmid. All procedures are performed according to the instructions, with plasmid-specific modification.

3.2.3 Transformation, Plasmid Isolation and Examination

Plasmid or PCR products are introduced as foreign DNA into bacteria via transformation, to replicate in this study. 1-5 μ L of the prepared sample, either from DpnI digestion or a ready plasmid, was transformed into *E. coli*. Two strains of competent bacteria, DH5 α and XL1-Blue, were used for transformation. All steps were conducted in a clean environment, with disinfection using ethanol and a lit flame to maintain sterility. A routine procedure is easily completed by adding the prepared DNA to the defrosted competent cell and evenly spreading it onto the warm agar plate with a sterilized tool. For higher transformation efficiency, a heat shock step and incubation with SOC medium were also employed. The plates were then incubated at 37°C for approximately 16 hours to allow colony growth.

After overnight incubation, transformed bacterial colonies appeared on the agar plates. To avoid overgrowth, plates were transferred to a refrigerator. Selected colonies were subsequently inoculated into LB medium and incubated at 37°C for an additional 16 hours. Following incubation, the cultures were harvested by transferring them into 2 mL centrifuge tubes for further plasmid isolation.

Plasmid isolation was carried out using the GenElute Plasmid Miniprep Kit according to the manufacturer's instructions. The quality of the collected plasmids

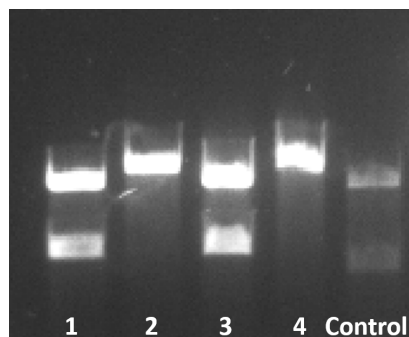


Figure 3.1: Example of test digestion results. The first 4 lanes were 4 samples of different digested plasmids from the same mini-prep extraction, where the control is the digested template plasmid. Notice the different pattern between samples 2&4 and 1&3&control. The results suggested that samples 2 and 4 are more likely to be successfully mutated at the desired position, and should be picked for further sequencing verification.

was assessed using agarose gel electrophoresis and Sanger sequencing.

During the design of the mutation, a distinct pattern of restriction enzyme digestion between the template and the mutation site was used as one of the guiding principles. All procedures were carried out according to the specific instructions provided for each enzyme. Once the catalytic reaction was complete, samples were verified using electrophoresis to visualize and compare the cutting pattern between the mutated DNA and templates. Positive plasmids identified by electrophoresis were subsequently sent for sequencing to confirm the results.

3.2.4 Linearization and RNA Synthesis

In the scenario where such confirmed plasmids are generated directly for the oocyte expression system, it requires subsequent DNA strand linearization and linear DNA template-based RNA synthesis.

Linearization and Purification An XhoI site serves as a linearizing site for each of the plasmids used in this study. The XhoI catalyze procedure is finished according to the enzyme instructions. The finished products were sampled and proceeded with agarose electrophoresis in parallel with the un-linearized DNA as a control.

After linearization, the remaining enzyme and any unincorporated nucleotides were removed from the sample before proceeding with downstream applications. Nucleotide removal was performed using the QIAquick Nucleotide Removal Kit. The resulting product was further precipitated with sodium acetate (NaAc). The next day, the sample was purified using ethanol to precipitate and collect the DNA plasmid, which was subsequently resuspended in TE buffer.

RNA Synthesis A kit-free RNA synthesis method was used, whose recipe is described as follows:

The TR buffer forests are an optimized environment for transcription. The rNTP solution is a mixture of raw materials that constitute the new RNA chains, specifically 100 mM each of rATP, rCTP, rUTP, and rGTP. These elements are mixed in

Table 3.6

Template DNA	10µl (varied according to the concentration)
Transcription buffer (TR buffer)	5µl
Free ribonucleoside triphosphates (rNTPs)	5µl
Anti-Reverse Cap Analog (ARCA Cap)	6µl
SP6 RNA polymerase	1µl
Recombinant RNasin Ribonuclease Inhibitor	3µl
RNAase-free water	add until the final volume reaches 45µl

volumes of 120µl, 120µl, 120µl, and 24µl respectively, along with 16µl of RNAse-free water; the ARCA Cap, also known as $m_2^{7,3''-O}GP_3G$, is a modified cap analog that serves as a variant of natural GTP, whose incorporation into the newly synthesized RNA strands provides higher transcription efficiency and chain stability (Addgene, 2023); SP6 RNA polymerase is the crucial enzyme that undertakes the synthesis task, adapting the SP6 promoter embedded on the template chain; Lastly, Recombinant RNasin Ribonuclease Inhibitor is RNAase inhibitor that protects RNAs from any potential RNAase presence.

The synthesis procedures were carried out according to the provided instructions. Midway through the process, a sample of the mixture was taken and tested via electrophoresis to monitor the ongoing transcription and determine whether termination was required in case of failure. After 2 hours of incubation, the reaction was terminated by further incubation with RQ1 RNase-Free DNase to remove the template, followed by the addition of 0.5M EDTA to deactivate the synthetic enzyme. The resulting RNA was precipitated with LiCl and stored overnight at -20°C, followed by cleaning the next day using RNase-free 70% ethanol. The final products were resuspended in 5 mM Tris-HCl buffer and stored at -80°C. All processes and materials were

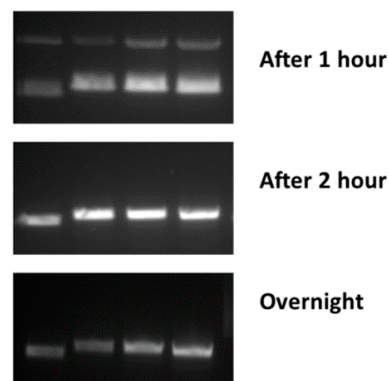


Figure 3.2: Examples of electrophoresis results from the RNA products during the RNA synthesis. The time points of the sample taken are noted.

strictly controlled to be RNase-free.

The very unstable nature of RNA due to the ubiquity of RNase requires rigorous quality controls of the products. Three samples were drawn during one synthesis for the electrophoresis tests. Besides the one sampled during and after the procedures, an extra aliquot is kept in an RNase-free environment at room temperature overnight, to examine whether the final resuspension is clean enough for long-term RNA storage. The concentration and purity of the final RNA products are evaluated by UV spectroscopy. Ideally, the yield RNA has a 260/280 OD ratio of around 2 and a concentration of no less than 1mg/mL.

3.2.5 Generation of Stable Expression Cell Lines

In contrast to the transient cRNA injection performed on the frog's oocytes, stable P2X receptor-expressing cell lines were generated via cDNA incorporation into the Flp-In System built within Human Embryonic Kidney (HEK) cells. All procedures outlined here were carefully executed within a sterile biosafety cabinet except for a microscope examination.

Cell Cryopreserving and Revive An established cell line was preserved as a seed stock, as continuous culturing might have an undesirable impact on the health of the cells, such as genetic drift or contamination. The method described here is referred to as “cryopreserving”. A standing freezing medium utilized here is a mixture of 20% FBS, 10% DMSO, and 70% antibiotics-free DMEM. The medium was filtered and cooled down on ice before use.

Before transfection, an aliquot of the cell seed stocks was retrieved from liquid nitrogen storage and revived according to the provided guidelines. The cells were then cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose and L-Glutamine, supplemented with 10% fetal bovine serum (FBS), 100 µg/mL zeocin, and 15 µg/mL blasticidin.

The details of the technique are described by Thermo Fischer (invitrogen, 2012).

Routine Culturing and Kill Curve The native Flp-In 293 cell lines are embedded with a zeocin resistance gene, which is “replaced” by a hygromycin B resistance following successful transfection. This substitution is the foundation for the selection, and determining an appropriate hygromycin concentration is crucial. The procedure is described by Mirusbio (Mirus Bio, 2023).

For a precise determination of the kill curve, it is essential to establish an approximate cell count before plating the cells. The desired cell amount to perform a kill curve test is 5×10^4 cells, to be placed in 500 μ l per well in a 24-well tissue culture plate, which usually could be reached by diluting the cultured cells until the optimal cell density is achieved.

Here, the optimal killing dose was determined as 200 μ /ml, while the sustaining dose for the stable cell lines is 100 μ /ml.

Transfection, Culturing, and Cryopreserving The transfection was performed using a TurboFect Transfection Reagent (Thermo Scientific). While the manufacturer’s instructions served as a basic guideline, certain modifications were implemented. The optimized protocol used 4 μ g of mixed DNA, consisting of the transfected plasmid and pOG44, along with 4 μ l of transfection reagent per group. After successful transfection, verified by cell survival in the presence of selection antibiotics, routine culturing for experimental purposes was conducted, and excess cells were cryopreserved according to Thermo Fisher protocol for cell freezing (Thermo Fisher Scientific, 2019).

Routine culturing required regular inspection of cell growth. The growth medium, DMEM+10% PBS supplemented with 100 μ g/mL hygromycin B and 15 μ g/mL blasticidin, was replaced with a fresh medium every 1-2 days to control cell colony expansion. During the study, cells were split and diluted when confluence reached 70-80% to avoid an overly crowded population.

3.3 Electrophysiology

The electrophysiology experiments conducted in this research employ two methodologies: two-electrode voltage clamp (TEVC) and whole-cell patch clamp. TEVC

is utilized to explore proteins expressed in *Xenopus laevis* oocytes, while the whole-cell patch clamp is applied to stable cell lines derived from HEK cells.

3.3.1 Two-electrodes voltage clamp

As a conventional electrophysiology technique, TEVC has been extensively used to study the properties of membrane proteins, especially ion channels.

Working Principle (Axon Guide, 2021) By applying voltage to the cell membrane, the technique enables the examination of the cells' electrical characteristics, primarily driven by their membrane proteins. When two points where an electrical potential difference (**Voltage**, abbreviated as **V**) exists in between, are separated by an insulator such as a lipid membrane, the charges cannot move freely and the established "transmembrane potential" is maintained. When such a barrier turns to or is swapped to a conductor, in this context, the ion channels on the membrane open, and electric charges flow under the potential, reducing the difference in potential energy. This signal can be recorded as the flow of electrical charge passing a point per unit of time, denoted as **Current** represented by **I**.

Two key principles establish the foundation of TEVC. In line with Kirchhoff's Law, which states that the current entering a circuit node equals the current leaving it, the first principle infers that the measurement of injected ion flow into the cell during the channel opening monitors also the ion channel flow, assuming that these channels are the only conductive pathway. Secondly, a continuous path between the potential differences is needed to allow the current to flow. Resistance, denoted as **R** in **Ohms (Ω)**, barricades current flow, while conductance, denoted as **G** in Siemens (**S**), facilitates it.

The potential difference between two points where current flows through is defined as :

$$\Delta V = IR = \frac{I}{G} \quad (3.1)$$

This is the well-known Ohm's Law. In a voltage-clamp experiment, the **total conductance (G)** is the sum of the conductances of all open channels. ΔV is the electrochemical driving force; it multiplies the total conductance to determine the current flow. The number of active channels changes as channels open and

close, affecting the I measured by voltage-clamp. This is fundamental to observing current changes and concluding changes in ion activity from that.

In electrophysiology, the Ag/AgCl electrode is commonly used to transmit current from electrons on the wire into the solutions. By converting between Ag and Ag⁺, the electrode makes a reversible circuit where currents can flow in both directions. Despite being a perfect candidate material, one phenomenon needs to be noted. When two Ag/AgCl electrodes face different concentrations of Cl⁻, a steady potential difference occurs due to ions being driven by different electrochemical gradients, termed the **liquid junction potential**. The liquid junction potential can be calculated and then subtracted electronically from the measured potential. One of the electrodes connecting to a clamping amplifier senses and controls the voltage, while the other one measures the current. Besides the two measuring electrodes, two electrodes are placed in the bathing solution (Gamper, 2013). The bath electrodes ground the system and establish the robustness of the measurement, while the reference electrodes measure and control the solution side potential in a TEVC setup.

In practice, glass micropipettes are attached to the wire of the electrode. Those pipettes are filled up with 3M KCl, to minimize the liquid-liquid junction potential existing between the pipette solution and the extracellular solution.

***Xenopus laevis* Oocytes as an Expression System** The oocytes isolated from the South African clawed frog *Xenopus laevis* have been introduced as a heterologous expression system for proteins for decades. The researcher benefits from their two most outstanding merits: the large size and the steady expression of proteins induced by foreign RNA (Nathan, 1987). The large size allows easy handling, including dissection and microelectrode penetration. Moreover, the RNA-mediated expression simplifies the transfection process, often through direct RNA injection.

The oocytes obtaining and further follicle-removing procedures were performed as described before (Schmalzing et al., 1991). The procedure is approved by the governmental animal care and use committee of the State Agency for Nature, Environment, and Consumer Protection (LANUV, Recklinghausen, Germany) under reference number 81-02.04.2019.A355. This entire process complies with the European Parliament and Council's Directive 2010/63/EU.



Figure 3.3: Schematic demonstration of the TEVC employed on a *Xenopus* oocytes.

The collected oocytes are wrapped in follicle cells, requiring defolliculation with collagenase Type I before use. After surgery, the oocytes, still enclosed in follicle cell sacs, were placed in Petri dishes containing Ori⁺ solution. Before collagenase treatment, the sacs were carefully opened using forceps to expose individual

oocytes in the solution. Collagenase was dissolved in Ori⁺ with gentamycin (1 mg/mL), making the final concentration of the collagenase solution 1.5 mg/mL. The solution was then filtered and aliquoted into Petri dishes.

The oocytes were rinsed in fresh Ori⁺ and transferred into the collagenase solution. Care was taken to ensure a suitable density of oocytes in each dish, allowing for effective digestion of follicle cells while minimizing potential damage to the oocytes. The Petri dishes were placed on a slow-moving shaker inside a 19°C incubator for 16 hours of digestion.

The following morning, the collagenase solution was removed, and the oocytes were rinsed with Ori⁻ solution. During this process, damaged cells and residual material were carefully removed, leaving only intact *Xenopus* oocytes.

On the day of the injection, RNAs were retrieved from the -80°C freezer and kept on ice. If necessary, the RNA was diluted to the required concentration. Oocytes that were intact and properly sized were selected for the injection process. Glass pipettes were pulled from borosilicate glass with filament of size 1.20x0.69x80 mm using a PC-100 puller. Under a microscope, the tip of each pipette was carefully pressed against a clean, RNase-free surface, such as the side of a pair of tweezers, to open the needle end to a size that allowed the RNA to pass through under the pressure of the injector without being wide enough to cause permanent damage to the cells during injection. To ensure a proper seal, each pipette was filled with silicone oil, and any air bubbles were removed. The pipettes were then attached to the end of a Nanoliter 2000 injector. RNA was dropped onto the lid of a clean 1.5 mL tube, and under the microscope, the injector was used to fill the pipette with the desired RNA. Subsequently, the oocytes were positioned on a dish with grooves to limit their movement. Each oocyte was injected with approximately 41 μ L of RNA solution. After injection, the oocytes were either placed in gentamycin-containing Ori⁺-filled Petri dishes or transferred to 96-well plates, with one oocyte per well when over-expression was expected to damage the cells potentially. The containers were then placed in a 19°C incubator for overnight incubation or longer, depending on experimental needs.

Noticeably, an oocyte is composed of two distinct hemispheres, termed the dark “animal” and the white-yellow “vegetal” hemispheres. It is suggested that microinjection performed on the animal hemisphere would result in better expression (Aguero et al., 2018). The principle is adopted here.

Recording Procedures The TEVC recording is done via an NPI Turbo Tec 05X amplifier controlled by Cellworks 6.2.1.. All perfusions are done via the magnetic valve system-controlling gravity flow connected to the recording chamber.

Before the measurement, the amplifier was turned on, and the Ag/AgCl electrode was oxidized in chlorine. While the instruments warmed up, the recording solution was prepared. The recording buffer, typically based on Ori⁻, was freshly prepared by diluting a 10x stock on the recording day. The composition of the buffer was deliberately formulated without calcium ions to prevent the activation of the oocytes' endogenous calcium channels, avoiding unwanted false signals that could arise from the flow of Ca²⁺ ions through the P2X channel.

Recording pipettes were pulled from borosilicate glass with filament, measuring 0.86x1.50x80 mm, using a PC-100 puller in a two-step protocol. This method created pipettes with shorter, wider tails and narrower tips compared to those used for injections. The pipettes were then filled with filtered 3M KCl and attached to the ends of the oxidized electrodes.

The system was perfused with a background buffer, and the flow speed was adjusted as required. Both electrodes were carefully positioned using manipulators, ensuring that the pipette tips were immersed in the buffer within the recording chamber. The pipette parameters were checked via the amplifier, and the offsets were zeroed. The ideal resistance was confirmed to be around 1 mΩ.

As various recording schemas were employed, here the basic section is described, while the detailed protocols will be elaborated in the **Results** chapter. Most protocols, though details may vary, typically include an activation step followed by an off-washing step. In many experiments, this process is referred to as a “single dose” session. During this session, ligands, most commonly ATP, are applied after a 10-second background wash. This is then followed by another wash step, all while maintaining a holding potential of -60mV.

Another key session that was incorporated in many experiments was the I/V (current-voltage) recording. This involves taking an I/V recording before activation, followed by another recording after the ligand is applied, once the steady state is reached (if any). Details of the I/V recordings will be introduced later.

Substituted Cysteine Accessibility Method In later experiments, cysteine scanning was adopted as a method to exploit the accessibility of residues. The substituted cysteine accessibility method (SCAM) is a highly versatile technique in ion channel studies. The designated cysteine substitution not only elaborates on the role the substituted residues might play in channel functioning but also reveals new characteristics the inserted cysteines bring. Additionally, it enables the further exploration of the thiol group, such as testing thiol group-active reagent reactivity. SCAM was employed in combination with TEVC recordings from *Xenopus laevis* oocytes expressing the indicated cysteine substitution mutants and their respective controls.

To optimize the results of the mutagenesis experiments, cadmium was selected as the cysteine-reactive reagent. The thiol group from cysteine can coordinate metal ions such as Ag^+ and Cd^{2+} , and when two or more cysteine side chains approach close enough to coordinate the same ion, a “metal bridge” is formed and stabilizes the conformation of the protein. Compared to direct disulfate bond formation, the participation of the metal ion allows more flexibility on the three-dimensional distance among the cysteine of interest, as the distance between Cd^{2+} and one of the sulfate ion of thiol groups is approximately $2.5\text{\AA}$, implying an S-S distance of $5\text{-}6\text{\AA}$, considering the size of one cadmium ions as around $1\text{\AA}$, while a disulfate bond is restricted to $2\text{-}3\text{\AA}$ (Linsdell, 2015a). Another commonly adopted accessibility probe is MTS reagents. Yet, the size of those chemicals limits their passage through the narrow part of one channel pore, which often makes them impermeable. Unlike these bulky chemicals, metal ions such as Cd^{2+} merit from their similar or even smaller size compared to conducted ions such as K^+ or Na^+ , allowing more accessibility through the channel, hence more throughout the screening of the cytoplasmic side of the ion channel (Linsdell, 2015a).

$CdCl_2$ and DTT, which were the oxidative reagents to reverse the Cd^{2+} -cysteine-specific bond, were prepared in Ori^- . During the experiments, the concentration of $CdCl_2$ was often set to $10\text{ }\mu\text{M}$, but also varied in different cases. The details will be described in subchapter 3.2 in the **Results** chapter. DTT was prepared in a concentration of 1 mM .

To apply the cadmium ions during the open state of the channels, a mixed solution from $10\text{ }\mu\text{M}$ ATP and Cd^{2+} was perfused onto the oocytes for 20-30 seconds after the pre-wash and ATP application described above. The solution was immediately switched back to pure ATP for extended activation before ending rinsing

with Ori^- . During the record, the endpoint of the amplitude of the first part activation to ATP is defined as “Current_{ATP}”, while the end of the Cd^{2+} + ATP application is “Current_{ATP+Cd²⁺}”. The term “**blocking efficiency**” here evaluating the Cd^{2+} effect describes the fraction of the current that was blocked upon the administration of the metal ions, calculated as follows:

$$\text{Blocking efficiency} = 1 - \frac{\text{Current}_{\text{ATP+Cd}^{2+}}}{\text{Current}_{\text{ATP}}}$$

During the experiments, it was observed that for certain mutants with leak current, the blocking sometimes exceeded the full response. In this scenario, the blocking efficiency was considered 100% to avoid a negative value for blocking. For the mutants exhibiting effective current blocking, a DTT + ATP solution was applied after the current reduction was settled. When a current rebound was observed during the DTT application, it was verified that the current reduction was caused by the Cd^{2+} -thiol group reaction.

In some cases, the current recovery after its reduction was evaluated, either under the influence of DTT or not. The TEVC measurements were initiated with baseline establishment achieved by short wash, proceeding with ATP activation for 20 seconds. The maximal current obtained here was recorded as Amplitude1. Subsequently, a 20-second perfusion with the combination of Cd^{2+} and ATP was introduced. The amplitude recorded at this endpoint was referred to as Amplitude2.

The following solution was either a switch back to solely ATP or a mixture of ATP and DTT. The amplitude obtained at the end was Amplitude3. The recovery is thereby defined as:

$$\text{Recovery} = \frac{\text{Amplitude3} - \text{Amplitude2}}{\text{Amplitude1} - \text{Amplitude2}}$$

To detect whether the cysteines were spontaneously crosslinked in the apo state, DTT screening was performed as well. 20 seconds of DTT + ATP activation following 20 seconds of ATP application. Any increase in the current amplitude after DTT addition was recorded as the DTT potentiation. Secondly, a DTT incubation of 30 seconds was applied before the ATP activation, immediately followed by

DTT + ATP solution perfusion, to determine whether the incubation altered the DTT potentiation effect. The DTT potentiation is calculated as follows:

$$\text{DTT Potentiation} = \frac{\text{Current}_{\text{DTT+ATP}} - \text{Current}_{\text{ATP}}}{\text{Current}_{\text{ATP}}}$$

Step-wise Ion Concentration Reduction Profiling the ion permeation generally requires changing the extracellular ion concentration to determine how the channel conductance changes accordingly. Theoretically, the P2X channels do not discriminate between K^+ or Na^+ , and the potential of chloride permeation adds to the complexity.

The common expression of the Goldman–Hodgkin–Katz equation is shown below:

$$V_{\text{rev}} = RT \times \ln \left(\frac{P_{\text{Na}}[\text{Na}^+]_{\text{out}} + P_{\text{K}}[\text{K}^+]_{\text{out}} + P_{\text{Cl}}[\text{Cl}^-]_{\text{in}}}{P_{\text{Na}}[\text{Na}^+]_{\text{in}} + P_{\text{K}}[\text{K}^+]_{\text{in}} + P_{\text{Cl}}[\text{Cl}^-]_{\text{out}}} \right) \quad (3.2)$$

where P stands for ion permeability. Traditionally, V_{rev1} and V_{rev2} , as obtained values from TEVC, can be used to evaluate the relative permeation (P_x/P_{Na}). Exponentiate both sides of the original equation after rearranging the constants, and divide equations of V_{rev1} and V_{rev2} , and the following equation is derived:

$$\frac{\exp \left(\frac{V_{\text{rev1}}}{RT} \right)}{\exp \left(\frac{V_{\text{rev2}}}{RT} \right)} = \frac{P_{\text{Na}}[\text{Na}^+]_{\text{out1}} + P_{\text{K}}[\text{K}^+]_{\text{out1}} + P_{\text{Cl}}[\text{Cl}^-]_{\text{in}}}{P_{\text{Na}}[\text{Na}^+]_{\text{out2}} + P_{\text{K}}[\text{K}^+]_{\text{out2}} + P_{\text{Cl}}[\text{Cl}^-]_{\text{in}}} \quad (3.3)$$

Observing the equation, it's clear that, when the chloride's conductance has to be considered, the term describing the intracellular chloride cannot be eliminated. In other words, the initial consideration of both extra- and intracellular anion concentration is required, thus making it impossible to calculate the relative permeability without knowing the intracellular details, for example, when recording on TEVC using oocytes as an expression system.

The variability of intracellular conditions can make even qualitative comparisons unreliable when the changes are subtle. Although the equation suggests a direct correlation between change in Vrev (exponential term) and P_{Cl} , this relationship becomes practically undetectable unless the mutation induces a large shift in chloride permeability. Moderate changes are often buried under the noise introduced

by oocyte-to-oocyte differences in intracellular ions' concentrations. This was observed in preliminary experiments here when attempting to assess change in Cl^- permeability solely based on changes in V_{rev} .

In addition to using whole-cell patch-clamp experiments, where intracellular conditions are strictly controlled, efforts were made to establish robust TEVC experiments to overcome the noise from varying intracellular environments in individual oocytes. This was achieved by setting up self-control for each recording:

Oocyte underwent I/V recording in standard 90 mM NaCl-based Ori⁺ first with the protocol described previously, followed by rinsing with the same 90 mM NaCl-based buffer for 1-2 minutes; subsequently, the perfusion solution was switched to a second buffer with an altered ion composition, with either decreased Na⁺ or Cl^- concentration, for a 10-second perfusion step, to rapidly change the chamber solution without potentially altering the condition of the cell due to long exposure. ATP prepared in the same solution was then applied, during which a second I/V recording was performed. Specifically, most activation sessions were kept brief for 1-2 seconds to avoid extensive channel opening that could alter local ion concentrations.

Two parameters were taken from one measurement: maximal current I during ATP application under standard clamping voltage -60 mV; current obtained in 90 mM NaCl-based Ori⁻ termed **I1**, the one in altered Ori termed **I2**, **I2/I1** was used for analysis; similarly, V_{rev} obtained from the first measurements termed **Vrev1**, the second termed **Vrev2**, $V_{\text{rev2}} - V_{\text{rev1}} = \Delta V_{\text{rev}}$ was used for analysis.

The use of relative values (**I2/I1** and ΔV_{rev}) serves to reduce the impact of the varied intracellular environment of the oocytes. The **I2/I1** ratio provides an indication of changes in how easily ions pass (related to conductance), and ΔV_{rev} indicates changes in the channel's ion preference (related to selectivity). For a better understanding of these observations, particularly how ions interact with the channel concerning diffusion resistance and electrostatic driving effects, the principles of the Poisson-Nernst-Planck (PNP) equation were applied:

$$\mathbf{J}_\nu = -D_\nu \nabla n_\nu + n_\nu \mu_\nu (-\nabla \phi) \quad (3.4)$$

where

$$\mu_\nu = \frac{D_\nu z_\nu e}{kT} \quad (3.5)$$

The J_ν stands for flux of each ion species; n_ν stands for ions number density, which could be seen as an analog of ion concentrations, therefore ∇n_ν could be seen as the concentration gradient; D_ν is the diffusion coefficient defining how easy ions diffuse inside of the pore. μ_ν stands for ion mobility, meaning ion's speed in an electric field, associated with diffusion coefficient and $z_\nu e$ (electrical charge carried by one ion, +1 for Na^+ and -1 for Cl^-) via Einstein relation, as shown in equation 1.5. $\nabla\phi$ stands for potential gradient.

Based on the principles of the PNP equation outlined above, the experimentally determined parameters $I2/I1$ and ΔV_{rev} can be interpreted as follows: The current ratio $I2/I1$, reflecting changes in ion conductance under different ionic gradients (at a constant voltage), is considered to be primarily influenced by the diffusional component of ion flux $-D_\nu \nabla n_\nu$. Specifically, alterations in this ratio are taken to indicate changes in the effective 'diffusion resistance' that ions overcome during diffusion, according to Ohm's Law. Similarly, the reversal potential V_{rev} , the point at which the net ion flux J_ν becomes zero, signifies a balance where the diffusional flux is exactly countered by the electromigrational flux. Therefore, shifts in ΔV_{rev} directly reflect changes in how the electromigrational term $n_\nu \mu_\nu (-\nabla\phi)$ must adjust to counter new chemical gradients, providing insight into the channel's selectivity and its interaction with the electric field.

As current was used for evaluation, rundown effects needed to be considered: most of the triple-mutant-based constructs exhibited moderate but varied levels of rundown, making the comparison between them and the triple mutant less trustworthy. To address this, a control group was included: each constructs were also recorded under the same 90mM NaCl-based buffer twice, and any intrinsic changes were caused by the repetitive activation rather than by changes in ion composition.

During the recording, Na^+ or the Cl^- were replaced by impermeable organic molecules, either with choline⁺ (⁺) or gluconate⁻ ($[\text{C}_6\text{H}_{11}\text{O}_7]^-$). In the first experiments where Na^+ or the Cl^- was swapped completely, it was noticed first that the liquid junction potential error was inevitable when swapping Cl^- entirely, causing reversal potentials to shift significantly towards the negative. Theoretically, this error is correctable by setting up an agar bridge; however, this added to the complexity and failed to reach the expected effect after the trial. Besides using a completely modified Ori⁻ resulted in abnormal activation, likely due to unpredictable channel behavior when exposed to these unnaturally concentrated organic ions. Subsequent tests showed that these disruptions wouldn't be noticeable until

the physical Na^+/Cl^- was replaced by more than 50% (45mM). To minimize the interference, the NaCl^- -based Ori^- was mixed with Choline-Cl or Na-gluconate in varying ratios to modify ion concentrations while maintaining osmolality, and the following solutions were prepared:

Table 3.7: Ionic compositions of solutions used for stepwise concentration alteration protocol

	70mM Na^+		50mM Na^+		70mM Cl^-		50mM Cl^-	
Cation (mM)	Na	Choline	Na	Choline	Na	Choline	Na	Choline
	70	20	50	40	90	0	90	0
Anion (mM)	Cl	Gluconate	Cl	Gluconate	Cl	Gluconate	Cl	Gluconate
	90	0	90	0	70	20	50	40

Detailed analysis method will be described in the later section **Two-way ANOVA**.

3.3.2 Whole-cell Patch clamp

Working Principle As another classical electrophysiology method, the principle introduced above still applies to the patch clamp technique. Patch clamp can be applied in various scenarios, while here, whole-cell measurements were adopted.

Only one electrode is utilized in the patch clamp technique. Similar to TEVC, a glass micropipette is attached to the electrode whose wire bathes in an electrolytic solution for conduction. Unlike the TEVC, the electrolytic solution mimics the intracellular environment of the cell. In comparison, the patch clamp pipettes require high uniformity and smoother edges, which are achieved by fire polishing after fabrication. Those prerequisites aim at forming a high-resistance($\text{G}\Omega$) seal between the pipette and the cell it is attached to, providing low-resistance electrical access to the cell's interior.

For whole-cell patch-clamp experiments, a pipette was brought into contact with the cell to form a high-resistance seal. After establishing the seal, the membrane patch in contact with the pipette was ruptured, typically using a pulse of suction, to gain electrical access to the interior of the cell. This whole-cell configuration allowed simultaneous voltage control and current measurement through the same electrode.

Cell Preparation The stable cell lines generated from HEK293 cells were used as experimental objects. The cell lines are embedded with a tetracycline-regulated mammalian expression system, allowing the on-and-off regulation of the protein expression upon the presence or not of the tetracycline (**ThermoFisher_InducibleProteinExpressionSystem**).

The day before recording, 12mm round glass coverslips were prepared and placed individually into each well of a 24-well plate. Cells were then diluted according to the previously described protocol, adjusting the ratio based on the specific cell growth conditions to ensure non-clustered, individual cell attachment after reattaching overnight. Tetracycline solution was added to the cell suspension to achieve a final concentration of 1 mg/mL, and the mixture was thoroughly pipetted up and down to ensure uniform mixing. Next, 2-3 mL of the prepared cell suspension was added to each well. This setup provided a movable surface for cell attachment, facilitating their relocation during patch-clamp measurements. The well plate was then returned to the incubator for overnight protein expression.

Recording The whole-cell patch clamp was performed by HEKA EPC 10 USB Patch Clamp Amplifiers, controlled by HEKA PatchMaster v2x90.5 software. The motorized PatchStar Micromanipulator is supplied by Scientifica, UK. A Zeiss Axiovert 135 TV inverted microscope is equipped with a supplementary light source controlled by Power Supply EA-PS 3016-10. The solution exchange is mostly done manually, with the EVH-9 mechanical valve system. The measurement setup is located on a vibration isolation table.

External solution stocks were prepared by dissolving individual components into highly concentrated solutions. These concentrated stock solutions were autoclaved and stored at 4°C. The stocks were discarded and prepared regularly to avoid possible contamination and alteration in salt concentration. The internal solution was prepared and aliquoted into 10 mL, which were later stored -20°C.

On the day of recording, stock solutions were removed from the fridge and allowed to reach room temperature. Each stock was pipetted into a volumetric flask based on its concentration and the desired final volume of the external solution. After adding distilled water, the solutions were titrated using a pH meter. The NaCl-based and Na⁺-based external solutions were titrated to pH 7.2 with NaOH, while the Cl⁻-based solution was titrated with [(CH₃)₃NCH₂CH₂OH]OH. The internal solution was titrated to pH 7.4 with KOH. Once the pH was adjusted, the volume

of each solution was brought up to the final mark using distilled water.

Micropipettes were pulled from 0.86x1.50x80 mm borosilicate glass using a P-1000 micropipette puller (Sutter Instrument, USA) equipped with a 3×3 mm box heating filament. After pulling, the pipettes were fire-polished. Before pulling, the ramp value was recorded three times to ensure the proper function of the filament and to determine the appropriate heating value. The puller program was designed to produce consistent pipettes with a resistance of 2-3 MΩ, as described in detail below:

Table 3.8: Parameter for P-1000 puller

Line	Heat	Pull	Velocity	Delay	Pressure
1	ramp value-30	0	19	1	600

The parameters were slightly adjusted for aged heating filament to obtain reproducibly consistent pipettes. The pipette was filled halfway with filtered, room-temperature internal solution to prevent outflow when inserting the wire. The installation of the pipette was meticulously performed to avoid any air bubbles. Cells were retrieved from the incubator, and one cover glass was carefully picked up using a sterilized tweezer and placed into the recording chamber. The cells were rinsed with external solution, ensuring enough solution was added to fully immerse them. Subsequently, the pipette's qualification was verified by checking its resistance in the software. Once confirmed, the pipette tip was gently attached to the cell, and a high-resistance seal was established. The cell membrane was then ruptured to achieve the whole-cell measurement configuration.

The protocol used on the patch clamp was a single activation supplied with multiple I/V curve recordings. The single activation segment started with a measurement of baseline and an I/V measurement before switching to ligand administration. After the ligand was applied, the I/V recording was performed again once a steady state was reached. Subsequently, the flow was rapidly switched back to a clean buffer. The amplitude record was performed at a membrane potential of -60mV, while the I/V record was done by step, starting with a constant voltage of -60mV and dropping to -120mV for 50ms, stepping to +10mV at a rate of +10mV/10ms, and finishing with returning to -60mV holding potential.

3.4 Data Analysis

To handle the raw data from electrophysiology measurement, Cellworks Reader 6.2.2 was used for TEVC experiments, and FITMASTER version 2x92 for patch clamp results. When further curve fitting was required, both GraphPad Prism version 10.0.0 for Windows and R Statistical Software were used. To process the I/V curves, the R package "tidyverse (Wickham et al., 2019)" and "dplyr (Wickham et al., 2023)" were used. Statistical analysis was performed with GraphPad Prism version 10.0.0 unless noted otherwise.

Representative traces were generated with RStudio version 2022.07.2. Additionally, traces shown in this dissertation may have been processed with a low-pass filter (8-pole Bessel, cutoff ranging from 1- 10 Hz) for clarity and visualization purposes. This filtering was used solely to enhance the presentation and did not affect the outcome of the data analysis.

3.4.1 Double-mutant Cycles

The double-mutant cycles method probes the potential interaction between two residues. Compared to the wild-type protein, when the sum of free energy changes from two different single-point mutants does not equal the changes brought by a protein that has mutations at both of these sites, it indicates that these two residues are coupled. The coupling further suggests the interaction between these two residues. Defining free energy differences between each mutant and the wild type as ΔG , while X and Y represent two residues respectively, $\Delta G_{0-XY} \neq \Delta G_{0-X} + \Delta G_{0-Y}$ suggests that, besides the mutant-altering energy changes, coupling energy from the X-Y interaction contributes as well (Horovitz, 1996).

The calculation originated from the equation for Gibbs free energy:

$$\Delta G = -RT \ln K$$

Where R is the universal gas constant equal to 8.314 J/mol·K, T is the temperature in Kelvin. 293K is generally used for a room temperature of 20°C. K represents the equilibrium constant and can be defined in various ways. In this study, the focus is on the desensitization kinetics, whereby K quantifies the balance between receptor activation and desensitization during prolonged agonist exposure.

Macroscopically speaking, the equilibrium between activation and desensitization inclines to the former and moves to the latter over time during the extended exposure of the receptor to the agonist, which visually manifests as signal inclination and decline on the electrophysiology measurement. The maximal activation was defined as the starting state, and the remaining amplitude in 20 seconds as the final state, so the equilibrium constant K could be defined as the ratio between them.

During the double-mutant cycles, different mutants are generated from the background protein His-T13P, S15V, P16I-hP2X3 (Hausmann et al., 2014). This non-desensitizing version of the hP2X3 receptor, referred to as the “triple mutant” later, was previously established in our lab, presenting a K value of around 1. Based on paired residues of interest, two single mutants (X and Y) and the corresponding double mutants (XY) were built in groups, whose desensitization kinetics were calculated, evaluated, and compared to the triple mutant (noted as 0):

$$\Delta\Delta G(X) = -RT\ln K(X) - (-RT\ln K(0))$$

$$\Delta\Delta G(Y) = -RT\ln K(Y) - (-RT\ln K(0))$$

$$\Delta\Delta G(XY) = -RT\ln K(XY) - (-RT\ln K(0))$$

Experiments also set up a threshold of ± 0.35 kcal/mol where non-interacting residues might possess (Hausmann et al., 2013). In other words, whether $\Delta\Delta G(X) + \Delta\Delta G(Y) - \Delta\Delta G(XY) > 0.35$ or < -0.35 implies potential interactions between residues. Simplifying the steps above, the criterion for the determination of the potential interaction between two residues was set up:

$$RT\ln \frac{K(0)K(XY)}{K(X)K(Y)}$$

When the results are significantly higher than 0.35, the interaction between X - Y destabilizes the structure, and *vice versa*. To overcome the small sample size and more precise estimation of P values, R software was utilized to estimate the difference from a specified value using a t-distribution, assuming that the sample size was large enough for this statistical model to be appropriate.

3.4.2 Correlations and Non-linear Curve Fitting

Non-linear curve fitting contains range statistical methods and is used to model complex relationships between variables. The curve fittings were performed extensively in this study to describe the dynamics in different scenarios.

To evaluate the different potencies of potential antagonists or negative modulators of the mutants, a modified Hill equation was used for the fitting to determine IC₅₀ values for comparisons. The equation is as follows:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log(\text{IC}_{50}) - X) \times \text{HillSlope}}}$$

The fitting was achieved by applying the "dose-response inhibition — log(inhibitor) vs. response — variable slope (four parameters)" model in GraphPad Prism. The "variable slope" accommodates the unique characteristics of the biological response observed. In the equation, X represents the logarithm of the compound concentration, while Y represents the percentage inhibition observed following the introduction of the antagonist during channel activation. The "bottom" and "top" represent the minimal and maximal inhibitory levels, in respect. The IC₅₀ value is the concentration of the modulator that induces a response halfway between the Bottom and Top levels. Hill Slope describes the steepness of the curves. For each mutant, full channel activation was considered as 0% inhibition. Upon the release of, e.g., negative modulators in different concentrations, the percentage of diminished response was recorded and fitted against the logarithm of the modulators' concentration. The fitting reported and IC₅₀ represent the efficacy of the modulators.

Determining the IC₅₀ values from several mutants led to further interest in the correlation study. The correlation was attempted between the IC₅₀ values and the proposed α -C distance between residues of interest (details will be described in the Results chapter). The Pearson correlation coefficient was employed to measure the strength and direction of the linear relationship between these two continuous variables. Assuming the data was distributed normally, the Pearson correlation provides a preliminary exploration of the potential co-dependency of the two variables. For this analysis, a two-tailed test with a 95% confidence interval was employed to determine the significance of the correlation.

After establishing a significant correlation between the two variables, further analysis was conducted to model their relationship. An exponential growth model was identified as the most suitable fit. The exponential growth or decay models suggested a relationship where changes in one variable exponentially affect the response of the other, typical of many biological processes where effects accelerate over a range of conditions.

In this study, exponential decay models were adopted to study the onset rate of negative modulators to the open channels. The exponential fittings are described by the equations:

$$Y = (Y_0 - \text{plateau}) \cdot e^{-k \cdot X} + \text{plateau}$$

for one-phase decay or

$$Y = Y_0 \cdot e^{-k \cdot X}$$

In both equations, "X" stands for the independent variable, which could be the time in kinetic studies or the alpha carbon distance in structural analyses. "Y" represents the dependent variable, such as IC50 values or the ratio of decayed response at a specific time. "Y0" is the initial value of "Y" when "X" is 0, indicating the starting level from which changes occur due to the influence of "X". In the decay model, the rate constant "K" determines how quickly "Y" approaches a stable value known as the "plateau". In the kinetics study, τ , which was computed as the reciprocal of k, was more commonly used. Conversely, in the growth model, "k" is the rate constant that dictates how rapidly "Y" increases.

Noticeably, one-phase decay was strictly used, despite occasions where two-phase decay fit better. Firstly, the focus was primarily on the rate of decay rather than a detailed description of the kinetic profile. The one-phase decay model, while less precise in capturing complex dynamics, provides a clear, singular rate constant that effectively summarizes the overall trend of the decay. Secondly, while the two-phase decay model might offer more precision by describing transitions in two distinct phases, traditional analysis often involves converting the constants from the two-phase model into a geometric mean. This approach aligns with the rationale of using the one-phase decay model—achieving a less precise but more comprehensive understanding of the decay dynamics.

Another non-linear regression model used was the second-order linear regression. Although an ideal I/V relationship is linear as described by Ohm's Law, the ion

channels often display non-linear characteristics that cannot be simply described by simple linear models. The channels go through different states during activation and inactivation, which adds to the complexity (Hille, 2001). To more accurately assess the I/V curve, second-order non-linear regression is employed. The solution of the functions provides an easy determination of the reversal potential. In the cases of some mutants' rectification that interrupts the attempting of regression, only when the I/V correlation around the x-axis ($y = 0$) could still be described by a 2nd order polynomial equation, and only this part of the recordings was used for the regression, as the rectifications happen at extreme voltages. In most cases, the r^2 of the regression is greater than 0.99. Meanwhile, as the recording results with great noise were not excluded, the degrees of fitting were not only decided by the r^2 , but most of the time also relied on individual inspection. Details of the rationale will be explained in the **I/V Curve analysis** part.

3.4.3 I/V Curve Analysis

As described above, a complete I/V recording session requires ramping or stepping scanning of the same cell before and during the application of antagonists. Later processing differed between the TEVC and patch clamp experiments, depending on different measurement protocols.

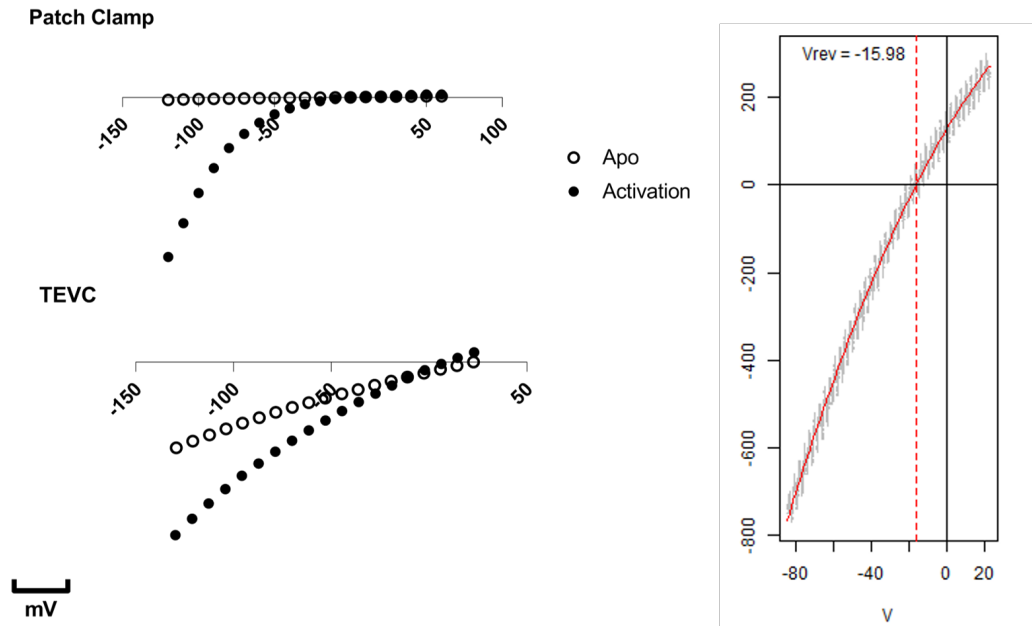


Figure 3.4: The left panel illustrates and compares the recording from the stepping protocol on both patch-clamp and TEVC. Noticeably, the activation and apo currents have the perfect overlapped voltage, while the values on the TEVC cannot match perfectly. The right panel shows one example of non-linear regression of the I/V curve. The grey curve describes the I/V profile, and the red curve is the fitting. The reversal potential is estimated from the fitting curve.

The stepping protocol resulted in fewer data points, depending on how fast the voltage increase was commanded to be, which points to a rougher but more manageable outlining of the I/V profile of the channels. To compare the change in current from the channel opening, subtract the current values measured successively at the same voltage to determine the net current. With the simplicity of objects measured on the patch clamp and the capability of the amplifier, the precise control of the voltage at each step was achievable. However, when the recording is performed using the TEVC, as shown in Figure 3.4, the data points collected in two sequences cannot achieve a perfect match at the clamping voltage. The degree of mismatch can sometimes reach a level that cannot be ignored and thereby makes the data unusable.

The ramping protocols significantly increase sampling density, from dozens to ten thousand levels, effectively overcoming previous technical limitations. Two

methods are used to process this large volume of data.

The first method involves setting a filter in Excel or GraphPad Prism to select one data point for every few hundred, ultimately yielding a more manageable number of data points. This approach makes it easier to align data points between the apo and activation phases due to the increased sampling density. However, despite this advantage, the method still provides a rough description of the I/V behavior due to the limitations in data size. It requires manual inspection of each dataset, significantly increasing the workload and difficulty. Lastly, this method is constrained by the signal-to-noise ratio. When the conductance of the channels is low or due to technical issues in the measurement setup, the noise is so pronounced that it becomes comparable to the actual signal; the filtered data might end up representing the noise, for example, a periodic wave, instead of the signal. The second method, more extensively used in subsequent experiments, employs R programming to process large datasets quickly. This method preserves detailed and complete data points, allowing for a more precise description of channel behavior. It also lowers the quality threshold required for raw data, enabling the observation and interpretation of channels with very low conductance.

The logic of handling each dataset is as follows: loading the complete 2 datasets from the same experiment with both ends of 1000 data points cut off to avoid capacity currents, then subtracting the apo current from the activation ones. The I/V current will be fitted with a 2nd-order polynomial regression, whose solution when $I=0$ defines the reversal potential, as illustrated in Figure 3.4.

In the cases of extremely high noise, the data is processed without a low-pass filter. This decision was based on the understanding that the least squares regression method used for curve fitting in general acts as a form of noise reduction. By seeking to minimize the sum of squared deviations, the regression naturally finds a line that represents the central tendency of the data points, effectively averaging out random fluctuations and thereby emphasizing the underlying signal. This principle of identifying a central trend amidst noise is conceptually similar to the operation of many dedicated signal filters, such as those that might determine a median or other representative value by considering the distribution and spread of the data.

I/V curve fitting was performed using least squares regression in R. A subsequent visual inspection of each fit served as a quality control step. Data from individual

recordings were included in the final analysis only if the fitted curve was considered a good representation of the data points, so that the obvious outliers would not be included and bring significant distortions.

3.4.4 Statistical Analysis Methods

All statistical results presented in later chapters are derived from data processed through the procedure described below unless specified otherwise.

General Statistics

Before performing any test, all results must be detected for potential outliers with the ROUT test, with the threshold being set at $Q = 5\%$ to remove likely outliers. The ROUT test does not assume a normal distribution of the data and can identify multiple outliers, thereby being chosen over tests such as Grubb's test. The removal procedures were performed solely once for each dataset, and the rest of the data points were kept for further analysis.

To detect significant differences, t-tests and one-way ANOVA tests were performed, depending on whether comparisons were made between two groups or among more than two groups, respectively. When comparing two sets of data with t-tests in GraphPad Prism, an F-test is always conducted at the same time, which aims to determine whether the variances between the two groups are significant. When the F-test shows no differences, an unpaired t-test will be conducted; otherwise, a Welch's t-test is chosen instead, as it does not assume equal variances and provides higher reliability under this condition. Similar consideration was adopted during one-way ANOVA tests. Here, Brown-Forsythe tests were performed to assess the equality of group variances. When a significance of variances is found, Welch's ANOVA test is used for further statistical analysis; if not, an ordinary one-way ANOVA is chosen instead. All tests were two-tailed tests with a confidence interval of 95%. Meanwhile, Dunnett's T3 multiple comparisons were performed as the recommended method, detecting significant differences between two groups, usually between the control and a test group.

When the comparisons were meant to be noticed in the graph, the results were

represented as follows: "ns" stands for "no significant differences", "*" stands for significant differences with $0.01 < P \text{ values} < 0.05$, "**" stands for P values between 0.001 and 0.01, "***" for P values between 0.0001 and 0.001, and "****" stand for $P < 0.0001$.

Two-way ANOVA

During sections of ion permeability evaluation, two-way ANOVAs were used for analysis. The design included two fixed factors—external ion concentration (90/90, 90/70, 90/50 mM NaCl) and channel construct (wild-type plus each mutant). The 90/90 group allows for better control of rundown-related variance and incorporates that data in the same model to effectively reduce error. Therefore, the comparisons always included at least 2 constructs and 2-3 different ion compositions, and two-way ANOVA adapts well for such a kind of analysis. The two-way ANOVA is explicitly suited when analyzing the simultaneous effect of two independent variables on a dependent variable. In the following section, the impacts of both ion concentration and different constructs on the channels' conductance were studied to see how specific mutations within the P2X3 channels affect ion permeability under varying conditions.

Although the focus was always on the differences brought by constructs, the ion concentrations were incorporated as factors in a two-way ANOVA analysis. This approach allowed better control of individual differences brought by oocytes and standardization of measurements, as it allows including more results obtained from multiple concentrations, including control construct and control concentration in parallel in each comparison. By analyzing the two factors simultaneously, the true impact of constructs was revealed by relative values, instead of absolute values that highly varied among different individuals. Details are described in the experimental setup sections.

In the two-way ANOVA tests, all values obtained under different ion concentrations from two different constructs, usually the control and one mutant, were compared. The tests were meant to find whether the factors have contributed significantly to the differences in values between the two constructs. A mixed-effects model was chosen for the two-way ANOVA, which is recommended for biological data involving both fixed and random effects. The fixed effects are the controllable variables (constructs and ion concentrations), while the random effects

include random error and sample differences. The mixed-effects model allows for the analysis of both these effects simultaneously, providing a more accurate and generalizable understanding of the data. Sphericity tests to evaluate the variances and effectiveness of matching were automatically performed at the same time as the test. These tests were not as much considered due to the limitation of sample size, though most comparisons passed these two tests.

After determining significant effects from the two-way ANOVA, further analysis is conducted using Sidak's multiple comparisons test. It was used to compare the groups while taking one factor out of the considerations. It is particularly useful for a detailed examination of how each mutant and condition differs from others, providing a more detailed observation of the specific effects.

4. Results

4.1 Characterization of the Cytoplasmic Cap

In this study, experiments on the TEVC were designed and performed to test the predictions from the MD simulation, aiming to profile the structure and dynamics of the cytoplasmic cap of hP2X3.

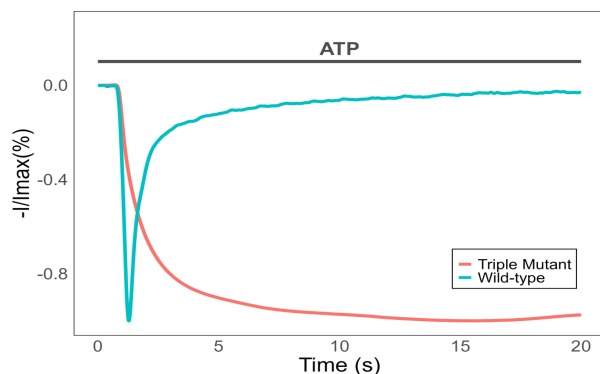


Figure 4.1: Overlapping of normalized traces from the WT and the triple mutant on TEVC. Traces are normalized to the maximal amplitude * (-1), and color-coded, ATP applications are noted.

4.1.1 The MD Simulations Suggest Roles of the N- and C-terminus

As has been identified for P2XRs, the presence of the cytoplasmic cap seems to be crucial for keeping the channel open during activation. As the wild-type P2X3 receptor is fast desensitizing, the existence of a transient cytoplasmic cap is deducible. The establishment of its non-desensitizing phenotype, T13P,S15V,P16I-hP2X3, referred to as the **triple mu-**

tant, provides a tool for an investigation of cytoplasmic cap rearrangements during the functional cycle. The comparison between the kinetics of these two constructs is as in Figure 4.1. All simulations were based on the triple mutant, and so were the generated mutants unless specified.

The first hint from the simulation results indicated that during activation, in other words, with the presence of the cytoplasmic cap, the N-terminus plays a crucial role in cap folding. In case there is either one of the three N-termini lacking from the structure, there was no sign of a cytoplasmic cap forming, which meant no opening of the channel, as described in the left panel of Figure 4.2. However, the participation of only one out of the three C-termini within a single receptor appears to be sufficient for maintaining cap stability. While the channel achieves optimal performance with intact termini, the absence of one or two C-termini is tolerated and does not significantly impair channel opening. Yet, the absolute absence of all of the C-terminus still eliminates the possibility of an open pore. The left panel in Figure 4.2 also suggests that the C-N term contacts, reported by the distance between paired residues located on C- and N-termini, decrease during the activation. underlining a partial unfolding of the cap during deactivation.

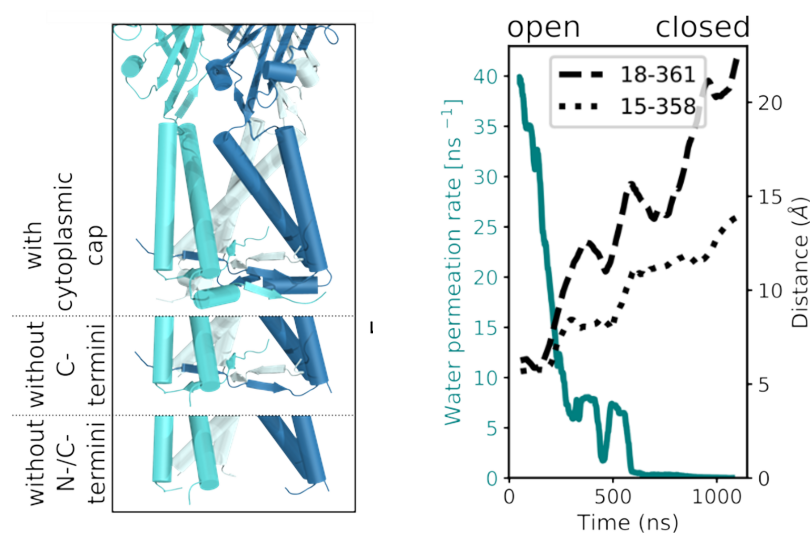


Figure 4.2: The left panel describes the simulation of the cytoplasmic cap with a twist in participation of the N- or C-terminus and depicts the partial disassembly of the cap. The different subunits are color-coded. While the N- and C-termini are both folded, the cap is present. The lack of the C terminus does not affect the integrity of the cap structure, while the absence of both termini disrupts the cap. The right panel shows the changes in the relative distances between V18-V361 and V15-F358. The water permeation rate is equivalent to the pore opening, which decreases along with the channel closure. Meanwhile, the interactions between these two residues are reduced or increasingly separated, indicated by the increasing distance.

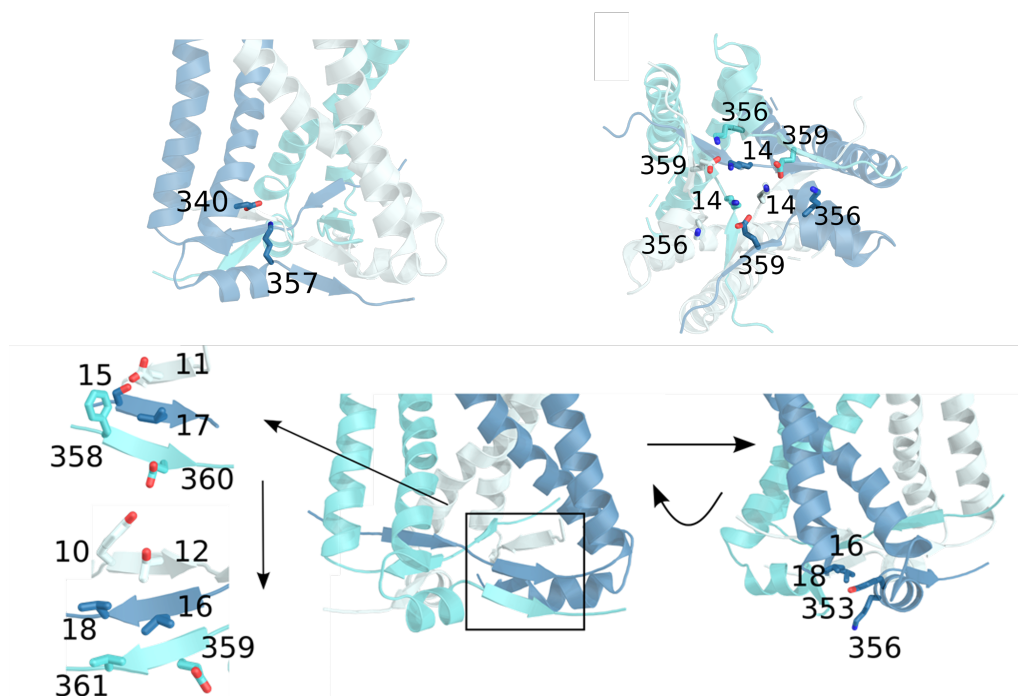


Figure 4.1.3: Residues of interest are located on the termini. Different subunits are color-coded, and specified residues are represented using a ball-and-stick model with the oxygen atoms colored in red. In the upper left panel, viewed vertically, D340 and K357 from two subunits are labeled on the cap. The right upper panel illustrates parts of TMs and the cap in a trimer viewed from the bottom, marking K14, K356, K357, and E359 from the bottom. The middle lower panel gives an example of the partial trimer without the extracellular domain. Adjacent to it, the left panel zooms in on the approaching sheets from the terminus, where 10Y, E11, T12 from one subunit, and residues 15-18, all valines, located on another subunit, are noted. Residues 358-361 (FEEV in order) sit on the third subunit. The right panel focuses on helices coming close during cap folding, where 16V, V18, and 353K, K356 from two subunits approach.

To probe in detail, the attribution of the specific residues was introduced. These residues are located on the terminus and are suggested to be crucial for maintaining a steady cap. The residues of interest are noted in Figure 4.1.3. Both salt-bridge interactions and hydrophobic interactions were proposed to play a part in hinging the cap in a steady state. Primarily, D340-357K, K14-E359, K356-E359, and K357-E359 were the four pairs of residues that were suspected to build salt bridges and therefore enhance the cap structure. Moreover, the possible hydrophobic interactions between F8 and W21 might also contribute to stabilizing the cap. Additionally, how single residues assist the stability of the cap was quantified by the calculation of the free energy differences performed on single-point mutation,

termed $\Delta\Delta G_{\text{stability}}$. In these calculations, energy differences between the folding and unfolding of the cap were defined as the ΔG , and mutating the residues of interest led to changes in these values as $\Delta\Delta G_{\text{stability}}$. Comparing the magnitude and the sign (positive or negative) of the $\Delta\Delta G_{\text{stability}}$ values reveals how the residue network contributes to the global state of the cap. The importance of those single mutants takes them into account for desensitization kinetics. In a series of simulations, mutations on F8, T12, 14E, V17, W21, D340, K356, K357, F358, and E359 raised further interest in detailed explorations and later TEVC experiments, where the residues were mutated into suggested amino acids and the resulting changes of desensitization were studied.

Besides the calculations of the energy differences, distances between certain residues were also estimated, as in the right panel in Figure 4.1.3. These simulations focus on residues going through relocation during the folding of the cap, enabling observation of the relationship between the relative distances between two amino acids and the opening of the pore. By attributing the residues, the travel trajectory of the parts of the terminus, where the studied residues are embedded, could be depicted during the functioning of the receptors. The distance relationships between V15–F358 and V18–F358 were recorded during the closure of the pore, where they departed from a distance of around 5 Å to 15–20 Å. By experimentally capturing the distances between two paired residues during different states of the receptors, one can infer the movements of the terminus along with the activation.

The MD simulations extensively characterized the cytoplasmic cap of the triple mutant. Essentially, the findings demonstrated that the N- and C-termini played varying roles in establishing the cap; a combination of salt bridges and a hydrophobic network contributed to the stability of the cap structure, and particular residues marked the movements of the N/C-termini. In the later study, TEVC experiments were performed to evaluate the simulation results.

4.1.2 Heteromer Assembly Defines the Incorporation of N- and C-termini

As mentioned in the **Introduction** and shown in the MD simulations, it was highlighted that the N-terminus determines the stability of the open state extensively, thereby playing a central role in the kinetics of desensitization. This is also con-

firmed by the successful generation of the N-terminus-mutated triple mutant, where the N-terminus has been altered, resulting in significantly slower desensitization and thus cap stability. Here, the special feature of the triple mutant enabled the investigation of the amount of mutated N-terminus to sustain a structurally stable cap.

Triple Mutant Contributing N^M and Wild-type N^{WT} The RNA from wild-type hP2X3 and the triple mutant was first diluted at a ratio of 1:20. WT RNA functions well without dilution; however, the triple mutant requires dilution to reduce its expression level. For parallel expression, it is critical to ensure that both the RNA quantity and protein expression levels are comparable. Therefore, all RNA samples were diluted to achieve better control. In later experiments where the two RNAs were mixed, they were combined based on volume, ensuring that the overall RNA amount in the mixture remained comparable to the two individual controls. Prepared RNA dilutions were injected into oocytes with the same volume of 41nL,

Prepared RNA dilutions were injected into oocytes with the same volume of 41nL, aiming to create various synthesized amounts of each protomer and alter the percentages of trimers containing either the wild-type N-terminus (noted as N^{WT}) or the mutated N-terminus (indicated as N^M) or various combinations of both. Provided that both cRNAs lead to a comparable amount of synthesized amount of the corresponding protomer, changing the ratio for WT and triple mutant RNA from 1: 2 to 1:1 and 2:1, ideally the ratio for $N^{WT}/N^{WT}/N^{WT}$: $N^{WT}/N^M/N^M$: $N^{WT}/N^M/N^M$: $N^M/N^M/N^M$ change accordingly, from 8:8:4:1 to 1:3:3:1, to 1:4:8:8. The construct in majority should be the phenotype dominating the overall kinetic.

As noted in the representative traces in Figure A.1.4 (Appendix), the recording routine went through three repetitions of the same sweep. Each sweep starts with 10 seconds of washing with Or^- buffer, proceeding with 10 seconds of 10 μ M ATP administration, and wrapping up with further washing of 80 seconds. During the repetitive ATP application in intervals of 90 seconds, the signal from the fast-desensitized WT should be significantly decreased and buried under the later recording due to desensitization and run-down. This is crucial in the case where the WT gives a supremely high amplitude.

The comparison of the first amplitude among different groups of injected cRNA

ratios is shown in Figure A.1.1 (Appendix). As depicted in the figure, the wild-type group possesses small signals of $147.4 \pm 33 \text{ nA}$, compared to -3583 ± 623 , -2582 ± 355 , -3657 ± 504 , and $-4213 \pm 501 \text{ nA}$ yielded respectively from the 2:1, 1:1, 1:2, and the complete triple mutant group. The huge differences ruled out potential interference of wild-type homomers during the first ATP application, as the signals from WT homomers were most likely buried, which allowed the direct comparison of desensitization kinetics of the biggest signal acquired from the first activation without concerning the WT.

The conventional quantitative method to compare the desensitization kinetics is to determine the kinetics (decay) time constant τ obtained from regression fitting, mostly exponential decay. In this case, the triple mutant, as one of the most important controls, desensitizes too slowly to be depicted by an exponential decay within one minute of recording. Thereby, the differences between the maximal amplitude and the decay amplitude after 10 seconds were adopted, which were noted as “**remain**”. By comparing how much the remaining current is away from the maximal response within a certain time course, the desensitizing speed is described in such a way that a larger remaining current implies a slower desensitization.

The right lower panel in Figure A.1.3 (Appendix) demonstrates the differences in the remains of all groups. The wild-type receptors significantly desensitize within a 10-second time course, with a remaining current of 0.16 ± 0.03 , while the RNA-mix groups exhibit a similar speed of desensitization compared to the control group of the triple mutant, which exhibits the largest remaining current with 0.91 ± 0.04 of the maximal response in 10 seconds. The WT: triple mutant = 2:1 group has shown a great deceleration of desensitization, with a 10-second remaining of 0.74 ± 0.03 . It is still significantly faster in contrast to the triple mutant, with a P value of 0.0289. The other two groups exhibited slow enough desensitization to show no significant differences from the triple mutant groups, with the remaining 0.85 ± 0.03 and 0.88 ± 0.02 , respectively. Qualitatively speaking, this experiment seemed to aid the theory that the stability of P2X3Rs can be varied by substituting N^{WT} with N^M , judging from the increasing levels of remaining current along with the increasing amount of triple mutant RNA. The existence of perfectly functioning triple mutant homomers generates observable signals that potentially persist, even when efforts are made to suppress them, which limits the interpretation, as it may interfere with experimental outcomes. Additionally, the study faces a significant shortcoming in that it not only fails to distinguish between the two heteromers with different stoichiometries but also fails to determine whether the observed

receptor behaviors are due to the presence of these heteromers or added/combined properties of homomers, requiring further improvements. Together, it is intriguing if the responses of the triple mutant homomers could be eliminated from the results.

N^M -K63A Contributing N^M and Wild-type or ΔC -WT N^{WT} To obtain a non-functional phenotype of the triple mutant, the lysine residue in position 63 was substituted by alanine within the triple mutant. The lysine 63 is located in the binding pockets and is vital for ATP activation. Former studies showed that K63A in P2X2/X3Rs retards the $\alpha\beta$ me-ATP activation as its EC50 value increases from 4 μ M to >300 μ M (e.g. (Hausmann et al., 2012)). By introducing alanine to lysine 63 (K63A) in the triple mutant, the response of the receptors to 10 μ M ATP was eliminated. Another advantage of K63A is that it helps to identify the two heteromers of the $N^{WT}/N^M/N^M$ and $N^{WT}/N^M/N^M$ receptors. In the same study (Hausmann et al., 2012), it was suggested that a trimer containing two neighboring binding pockets without function shows no response to ATP, which agrees with the conclusion from previous research stating a singular ATP-bound receptor is not activated (Ding & Sachs, 1999). One can conclude that when the N^M -K63A-hP2X3R monomer is incorporated into the trimer assembly, signals from the $N^{WT}/63A-N^M/63A-N^M$ and $63A-N^M/63A-N^M/63A-N^M$ are diminished in TEVC analyses.

Other improvements were the generation of N-terminus and C-terminus truncated receptors. Those constructs were generated by the entire removal of either the N or C-terminus of the WT receptors, thereby referred to as ΔN -WT and ΔC -WT. As determined, the N-terminus possesses the first 20 amino acids of the protein sequence, while the C-terminus is composed of the last 50 amino acids, numbering from 348 to 397. One purpose of these mutants was to determine the importance of the C- and N-termini for the receptors' function.

In this part of the experiment, RNA-mixing groups were tested. Details are listed in the table:

The three groups contain components: N^{WT} -K63A RNA mixed with components of either ΔN -, ΔC -WT, or WT RNA. Besides the RNA-mixing experimental groups, all the RNAs were also tested using sole injections as controls.

The concentration differences between the two RNAs were increased to further

Table 4.1: RNA mixing ratios in volume between indicated constructs for injection

	N^M -K63A			
Group 1	ΔN -WT	4:1	1:1	1:4
Group 2	ΔC -WT	4:1	1:1	1:4
Group 3	ΔC -WT	4:1	1:1	1:4

amplify the differences among various heteromers, without the need to consider the potential overexpression of homomers. Each group includes controls of the corresponding sole-injected RNA. All oocytes were injected with 41nL RNA in total. The recording protocols on TEVC were kept in accordance with the previous design, except for prolonged ATP application from 10 seconds to 20 seconds, to allow a more comprehensive presentation of the desensitizing process.

During the experiments, none of the ΔN -incorporated groups exhibited functional activity. Specifically, the efforts to express ΔN -wt homomers or heteromers in combination with N^M -K63A did not yield detectable currents. Likewise, neither the sole-injected ΔC -WT nor the N^M -K63A control groups produced any current responses following the application of 10 μ M ATP. Except for the previously presented sole WT group, representative traces from the rest groups are shown in Figure A.1.4 (Appendix). The results in the left panels state a few possible results. Firstly, the mixing groups from WT and N^{WT} -K63As appear to have better functions. In other groups, where ΔC -WT substitutes WT RNA, it was surprising that the ΔC -WT and N^{WT} -K63A, despite failing to generate functional homomers respectively, can assemble into activatable heteromers.

The right panels of Figure A.1.5 (Appendix) map the desensitization kinetics of each group. Within the WT + N^{WT} -K63A groups, the first observation is that the WT desensitizes faster than the mixed groups, specifically the WT:63A = 1:1 and 1:4 groups. At the first activation, WT desensitizes to a remaining current of 0.25 ± 0.05 of the maximal response in 10 seconds, while the 4:1, 1:1, 1:4 groups showed 0.17 ± 0.01 , 0.39 ± 0.05 , 1.1 ± 0.17 remaining current in respectively. Intriguingly, the differences among groups become more pronounced in subsequent activations. The WT stays within a similar range, of 0.25 ± 0.06 and 0.25 ± 0.08 . The WT:63A = 4:1 group appears to desensitize slower, as 0.26 ± 0.02 and 0.34 ± 0.03 . The WT:63A = 1:1 group shows a higher suppression of desensitization, of 0.81 ± 0.09 and 1.05 ± 0.03 . The WT:63A = 1:4 group, as it has been showing non-desensitizing features in the beginning, the fashion sustains, as in

0.98±0.08 and 1.08±0.05. The fact that the WT keeps the same desensitization rates implies that the heteromers account for the deceleration of desensitization in the other groups. In these groups, the phenotypes of the heteromers remained efficient after the WT homomers were suppressed after repeated activations. Noticeably, though the 4:1 group showed no significant differences in the desensitization, it performed better compared to the WT.

The $\Delta C + N^{WT}$ -K63A groups provide more evidence for those conclusions. The $\Delta C:63A = 4:1$ is the only combination whose desensitization kinetic changes during repetitive activations, from 0.31±0.04 to 0.65±0.08 and 0.60±0.06. The faster-desensitized, as well as the bigger-in-size current obtained from the first activation, reveals that part of the receptors that desensitize fast is suppressed. This phenomenon is quite similar to the WT, whose “rundown” is stated as a decreased response to repetitive ATP activation. Here, the $\Delta C/\Delta C/K63A$ dominates over $\Delta C/K63A/K63A$ and is most likely responsible for the rundown. The $\Delta C/\Delta C/K63A$ contains one mutated N-terminus and only one C-terminus, proving that one C-terminus is sufficient to support an open channel. Remarkably, the $\Delta C:63A = 4:1$ group appears to be more stable than the WT:63A = 4:1 group, revealed by the t-test performed between them, giving a significant difference with P value = 0.01. Although the WT homomers interfere with the interpretation, at least it could be one piece of evidence that the lack of the C-terminus does not contribute to altered desensitization kinetics. The results from the $\Delta C:63A = 1:1$ and 1:4 groups are more persistent, with the remaining from 0.56±0.06 to 0.60±0.06 and 0.55±0.05, and from 1.13±0.20 to 1.31±0.20 and 0.85±0.22. While it agrees with the previous statement that increasing the N^{WT} -K63A accompanies a decreasing signal and more stable response, the decreased C-terminus, the fact that there are no significant differences during the subsequent activations between the $\Delta C:63A = 1:1$ and 1:4 groups proves that one C-terminus is enough to ensure a similar kinetics compared to an integrated construction.

A lower concentration of ATP was also used to test the WT+K63A group. In these experiments, WT contains N^{WT} , and K63A with N^M . The initial intention was to verify that the $N^{WT}/N^{WT}/N^M$ remained inactive in the previous experiments. Otherwise, a difference would be expected to be observed, likely a fast desensitizing profile under a lower concentration of ATP, as the $N^{WT}/N^{WT}/N^M$ is insensitive to ATP compared to the WT homomers due to a decreasing in ATP binding pockets, and should be more and more unlikely to be activated while ATP concentration drops. In these recordings, ATP concentration was dropped to 1μM, and the rest of

the conditions remained unchanged. Representative traces are shown. By comparing these recordings to what was shown in Figure A.1.2 (Appendix), it is observed that the amplitudes no longer decrease when less WT RNA was injected. On the contrary, the WT: K63A = 4:1 group, which had the best performance under 10 μ M, lost the feature when less ATP was used. The 1:1 group now appears to have the best function, and the 1:4 group presents a similar response regardless of the ATP concentration. Another observation was that desensitization kinetics barely change with the different RNA ratios between the wild-type and K63A.

In conclusion, here heteromers that differ on N- and C-terminus were generated, to prove that in a hP2X3 trimer, complete loss of all three N-termini or retention of only one resulted in non-functional channels, while one C-terminus is enough for channel opening. When mutations on one out of three N-termini are introduced, it is sufficient to alter the desensitization kinetics. This effect appears to be enhanced with the increasing amount of the mutated N-terminus. All of these observations are similar to what was seen during the MD simulation.

4.1.3 Mutagenesis of Charged Residues Confirms Salt-bridge Interactions

The stability of the cytoplasmic cap determines the desensitization kinetics to a great extent. Generally speaking, the more stable the cap during activation is, the slower the desensitization will be. The residue interactions within the cap were investigated according to the possible salt-bridge networks within the cytoplasmic cap suggested by the MD simulation. In this experiment, the charged residues possibly forming salt bridges on the N- and C-terminus were mutated, either into non-charged or reverse-charged amino acids, to examine whether they are keys to maintaining a stable cytoplasmic cap.

The MD simulation was performed on the triple mutant and triple mutant-based constructs. Free energy differences were calculated from caps of the triple mutant-based single-point mutant and caps of the triple mutant in respect. The differences between the constructs with and without mutations of interest were determined as $\Delta\Delta G_{\text{stability}}$. A positive $\Delta\Delta G_{\text{stability}}$ indicates a destabilizing effect of the mutations, while a negative one implies the opposite. The observed changes in stability reflect how much the analyzed residues help keep the cap structure intact.

Further investigations focus on the potential interactions of amino acid residues within the cap. Mutations were grouped, into two single mutations with reverse-charged mutations and a double mutation containing both of these point mutations, manifesting as a charge-swapped mutant. In the case where the two mutated residues interact, single mutations are expected to exhibit changed desensitization kinetics as the salt bridge is broken. If the desensitization is restored on the double mutation, where the crucial mutants are introduced while the salt bridge is theoretically maintained between two swapped charged residues, it is further confirmed that the salt bridge contributes to the stability of the cytoplasmic cap. A double-mutant cycle analysis is introduced as well, as a method to quantitatively determine the interaction between two residues (described in **Materials and Methods**).

The following point mutations were suggested based on MD simulation results:

1. A potential interaction between **D340-K357**, where mutations **D340A**, **K357A**, and **K357R** are predicted to destabilize the cap.
2. A potential interaction between **K14-E359**, where mutations **K14E** and **E359K** are predicted to destabilize the cap.
3. A potential interaction between **K356-E359**, where mutation **K356R** is expected to further stabilize the cap.
4. A potential interaction between **K357-E359**.

The TEVC experiments were initiated with the single activation of the mutants. All RNA of the mutations was injected one or two days before experiments, with a volume of 41nL. The RNA was undiluted, except for a control group of the triple mutant with a dilution of 1:20, to avoid overexpression and subsequent cytotoxicity, due to the non-desensitizing phenotype and the high ATP affinity.

Charge neutralization and reversal were first tested on the D340 and K357. As is represented in Figure A.1.8 (Appendix), all mutations damage the function to a great extent. Alanine or lysine substitution of D340 dramatically decreased responses of -39.27 ± 12.05 and -30.77 ± 4.97 nA individually, with no significant differences between. This similarity between the two mutants, and the large reduction in comparison to the triple mutants in the same experiments, which has an overall amplitude of -8753 ± 1101 nA, suggests a crucial role of the aspartic acid in position 340. The K357 was also mutated into aspartic acid and alanine, and additionally,

into arginine. Even though these mutants all showed reduced current amplitudes, differences among them still drew attention. Charge reversal of K357 to K357D appeared to have the greatest impact on the function, with a remaining current amplitude of only -22.04 ± 4.48 nA. The K357A and K357R exhibited current amplitudes of -837.9 ± 222.5 nA and -3545 ± 408.2 nA, respectively. The combination of D340K and K357D with an amplitude of -14.14 ± 1.73 nA does not rescue the function, indicating the crucial importance of the residues themselves rather than the ionic interaction.

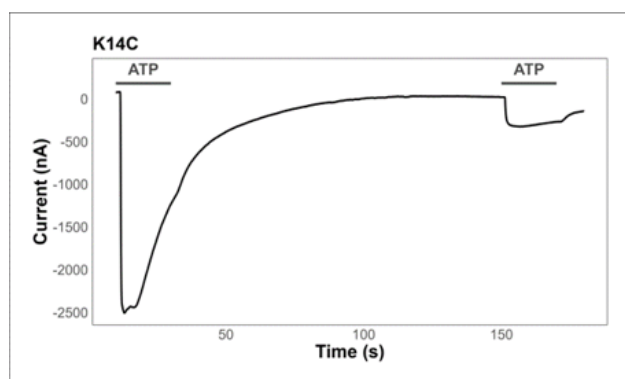


Figure 4.1.4: Representative trace of K14C during a double activation. ATP applications are as noted.

The triple mutant barely desensitizes, as shown in Figure 4.1, maintaining a remaining amplitude of 0.94 ± 0.01 compared to the maximal current, after 30 seconds of constant exposure to ATP. The D340K mutation accelerates this process, though not significantly, with a remain of 0.76 ± 0.05 in 30 seconds. Interestingly, though the extent of current reduction is similar for both, the changes in desensitization kinetics differ between D340K

and D340A. The alanine substitution of D340 unstabilized the opening of the channel, with a remain of only 0.18 ± 0.05 . The K357D, on the other hand, exhibits a remaining of 0.56 ± 0.09 after 30 seconds, significantly different from the triple mutant. The comparison within the K357 mutants suggests that K357A or K357R accelerates the channel closure extensively, remaining 0.22 ± 0.04 and 0.09 ± 0.03 respectively. Finally, the combination of the D340K, K357D mutations recovers the remaining into 0.85 ± 0.03 . Results are shown in Figure 4.1.4.

There are multiple residues suggested to be interacting with the glutamic acid at position 359: K14 within the N-terminus; K356 and K357 located on the C-terminus. The reversed mutations inhibit the current greatly for K14E, K357E, and E359K, as their average currents were reduced to -23.17 ± 9.43 , -34.65 ± 11.67 , and -2158 ± 566.9 nA, respectively. The K14E remains 0.71 ± 0.15 during constant ATP exposure, despite poor function. E359K, on the other hand, remains only

0.31±0.07 within the same time course. The charge-swapping between K14 and E359 (K14E/E359K) reduces the function to -278.8±79.7nA, which is between K14E and E359K and appears not to be able to completely rescue the fast desensitization, as its remaining ratio of 0.32±0.02 significantly differs from the triple mutant. A charge neutralization mutation K14C was generated (not based on MD simulation). The disappearance of the charge affects the desensitization kinetics but not the function, with an amplitude of -4029±1028nA and a remain of 0.40±0.05. Another observation from the K14C is that the second activation always exhibits a fairly slow desensitization, with a rather low amplitude, in contrast to the first signal evoked by ATP, see in Figure A.1.9 (Appendix).

Energy calculations from MD simulations suggested that a substitution of arginine (K356R) will further stabilize the cap. As in this study, the manifestation of cap stabilization is slow desensitization, and the control group of triple mutants has already shown extremely slow desensitization; it is imaginable that even if this stabilizing effect of K356R exists, it is not observable or masked by the already very slow desensitization. The results agree with this presumption since the K356R yields enormous amplitudes of -14563±2337nA and remains 0.9621±0.01 current in 30 seconds, both were not distinguishable from the triple mutant. To probe into this mutation, the same alteration was also introduced to the wild-type, aiming to exaggerate the possible stabilization effect.

However, the simulation was constructed based on the background of the triple mutant and might not hold when experiments are done on the WT receptors instead. The results, as expected, also failed to prove the effect of arginine, as shown in Figure A.1.6 (Appendix). The replacement of the K356 by glutamic acid also keeps the high performance of the triple mutant, indicating that its side chain might not be participating in the cap network. Its amplitude, -15093±4984 nA, does not differ from the triple mutant. Meanwhile, unlike arginine, K356E accelerates the desensitization into a remain of 0.41±0.03, outlining the importance of a positive charge at this position on stabilizing the cap. The double mutation of K356E and E359K shares similarities, with a non-significantly smaller amplitude of -6284±1570 nA, and 0.34±0.06 of remaining current, which is faster than the triple mutant.

The lysine at 357 was mutated into a glutamic acid to investigate its potential pairing with the E359. This mutant shows an extremely suppressed function, leaving an amplitude of only -34.7±11.7nA. Its desensitization also exhibits facilitation with a remain of 0.44±0.06 of the maximal current, significantly faster than the

triple mutant. By combining the mutation of K357E and E359K, the function is further impaired, to a level of -18.9 ± 4.8 nA. On the other hand, the slow desensitization kinetics is slightly recovered, rising to 0.69 ± 0.08 , from where the significant differences between the mutant and the triple mutant are erased.

For verification, the pairing residues were examined by the double-mutant cycles method. Details of this analysis have been described correspondingly in **Methods**. The remaining current ratio from each construct was adopted for the calculation. Denoting the K value of the triple mutant as K0, the two single-point mutants as K(X) and K(Y), and the combined double mutant as K(XY), taking both the mean and SEM values into consideration, the $\Delta\Delta G$ values of all the constructs were calculated. Differences between $\Delta\Delta G(X) + \Delta\Delta G(Y)$ and $\Delta\Delta G(XY)$ were further compared to a threshold of ± 0.35 kcal/mol with a t-test. When a significant difference is raised, a potential interaction is hinted at. The calculation was processed with the values listed in Table A.1.1 (Appendix).

The double-mutant cycle analysis indicates that the D340-357K, K356-E359, and K35-E359 are potentially interacting with each other, while K14-E359 does not show enough evidence for the same suggestion.

In summary, the point mutation technique was applied in this project, and TEVC experiments were subsequently carried out. The main focus was on how the mutation alters the maximal amplitude and the desensitization rate during consistent ATP-induced activation. The mutations consist of removing or reversing of charges of the amino acids, and in the case of two paired residues whose interaction was suspected, charge-swapping. The manipulation of the charges reveals the role that these charged amino acids play. It was established that 14E, D340, K356, K357, and E359 are crucial for channel functioning and for holding a stable cytoplasmic cap, and there are pieces of evidence that suggest interactions between D340 and K357, K356 and E359, 357 K and E359, but not K14-E359.

4.1.4 Investigation of Hydrophobic Network

Besides investigating the potential salt bridges, similar methods were also applied to investigate the hydrophobic networks that potentially affect the stability of the cytoplasmic cap. The MD simulation suggested a potential hydrophobic inter-

action between F8 and W21. Residues T12, 17D, and 358A might contribute to the overall constructs as well. All simulations and experiments were constructed on either the triple mutant or the it-based mutants. In this part of the study, the residues of interest were mostly substituted by alanine, because of its rather small side chain (Ennion et al., 2000). By manipulating the size of the hydrophobic side chains, the possible roles that these hydrophobicities play can be revealed. The same scheme of experiments described above was applied here as well. Except for the paired F8 and W21, all other mutants were studied individually.

During the investigation of F8A and W21A, the first intriguing observation was that the replacement of amino acids with a rather big side chain with alanine appears not to be interfering with the current amplitudes. Nevertheless, the alanine on position 8 further increases the amplitude to -20215 ± 2890 nA, while the W21A retains a similar function to the original triple mutant with an amplitude of -18201 ± 2589 nA. The double mutant F8A, W21A gains even more improvement on the conductivity of the channels, as it yields a current response of -29167 ± 3049 nA in response to ATP. The changes appear in the desensitization kinetics. While the W21A successfully maintains the slow desensitizing feature, with a remain of 0.92 ± 0.02 , the F8A shows a faster desensitization with a remain of 0.26 ± 0.03 , and the desensitization of the double mutant is further accelerated, with remain of 0.19 ± 0.03 . The double-mutant cycle analysis method was also conducted to investigate the relationship between these two residues, with the triple mutant denoted as K(0), F8A as K(X), W21A as K(Y), and the double mutant as K(XY). The results are shown in Table ?? (Appendix).

The calculation suggests that a potential interaction between F8A and W21A might be responsible for supporting the cytoplasmic cap.

Other single amino acids were suggested to contribute to the cap stability as part of the hydrophobic networks. The T12, V17, and F358 were highlighted during the MD simulation, and their roles were studied by single-point mutation. The T12A and V17D showed severely impaired function, yielding current responses of only -72.4 ± 20.1 and 26.2 ± 4.8 nA, respectively. Both of them showed accelerated desensitization, with remains of 0.21 ± 0.03 and 0.29 ± 0.03 , respectively, significantly different from the triple mutant. The alanine on F358, on the other hand, didn't largely reduce the current to the least, with an amplitude of -13681 ± 2457 nA. However, the stability of its opening was also affected, as it exhibits a remain of 0.24 ± 0.03 of the maximal current after 30 seconds.

In short, the outlining of the hydrophobic networks was elaborated by single- and double-point mutation, examined by the TEVC. By mutating the hydrophobic residues and evaluating the induced changes in function and desensitization kinetics, the importance of W21, T12, and V17 for supporting a stable cytoplasmic cap during the open state was elaborated. Moreover, the positive results from the double-mutant cycle analysis confirmed that the interaction between 8F and 21W affects the stability of the cytoplasmic cap.

4.1.5 Outlining the Conformational Rearrangement of the N- and C-terminus during the Functional Cycle

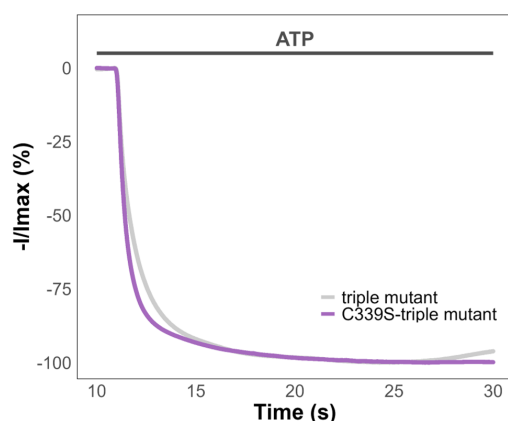


Figure 4.1.5: Normalized representative traces of the C339S mutant and the triple mutant. Traces are normalized to their maximal amplitude. ATP applications are noted. The C339S is in purple while the triple mutant is grey. Notice that though the overlapping is not perfect, the two constructs share a resemblance in desensitization kinetics.

Suggestions on details of the terminus movement during the channel functioning have been made from the MD simulation. To determine the movement trajectory, several representative residues within the termini were identified, whose relative distances from one another could be evidence for the conformational changes that the termini undergo during the transition between the apo closed, ATP-bound activated, and desensitized states. In this study, we took advantage of cysteine-reactive crosslinkers with different lengths to determine the change of distances between cysteine-substituted residues in positions of interest during different states of the receptor.

The MD simulations suggest distances between the C α atoms of two residues of interest. A proper distance hints at the possibility of interaction between these residues, while changes in distance indicate their relative relocation during the

gating cycle. The following table details the estimated distance that is close enough to interact and labels those too far apart as "no interaction":

Table 4.1.2: Suggested distances between the paired residues on cytoplasmic termini during different states of the channel

Residue	Location	Apo-state	Open-state	Desensitization
E11 V15	N-term N-term	No interactions	5Å; interactions	10Å, possible interactions
V18 V361	N-term C-term	No interactions	5Å; interactions	No interactions
V15 F358	N-term N-term	No interactions	5Å; interactions	No interactions

In this table, three pairs of residues were identified as reporter residues to estimate C-/N- relevant distances and their changes during channel gating. The relationship of E11-V15 represents an interaction within the N-termini. MDS provided evidence that both residues approach each other during activation (transition from the apo closed state to the ATP-bound open state) and separate upon desensitization. The changes in the distance between V18-V361 and V15-F358 pairs, respectively, describe a similar movement between adjacent C- and N-termini during the transition from the apo closed state to the ATP-bound open state, but these residues might move apart from each other during desensitization.

The designated cysteine-substitution mutants were generated based on a variant of the triple mutant, containing a serine substituting 339C within the distal TM2 helix (C339S-triple mutant), to avoid possible spontaneous crosslinking between C339 residues of adjacent subunits, as well as possibly newly inserted cysteines. This mutation does not bring observable changes to the triple mutant, as shown in Figure 4.1.5. Both single and double cysteine mutations, according to suggestions in Table 4.1.2, were generated.

Three crosslinkers with desired lengths were selected: MTS-2-MTS, BMOE, and BMB. MTS-2-MTS is expected to be able to crosslink two cysteines within 5Å, while BMOE and BMB have a spacer arm length of 8.0Å and 10.9Å respectively. To verify the hypothesis proposed by the simulation, the constructs were grouped as shown in Table 4.1.2, each containing two single mutants as controls and a combined double mutant. By applying different crosslinkers to the intact (non-denatured) receptors within the plasma membrane of the oocytes, either before

or after activation, it was expected that in double mutants, crosslinking would trap the cytoplasmic cap in a specific conformation, where two cysteine residues approached the optimal crosslinking distance for the applied crosslinker. In all cases, this was predicted to correspond to the open state, as suggested in the table above, through successful crosslinking between cysteines on the C- and N-termini. This kind of trapping is expressed as 1) a steadily constructed cap, which could be seen as a consistent channel opening from TEVC recording, and 2) covalently crosslinked subunits, after protein-labeling, affinity purification, and SDS-PAGE analysis.

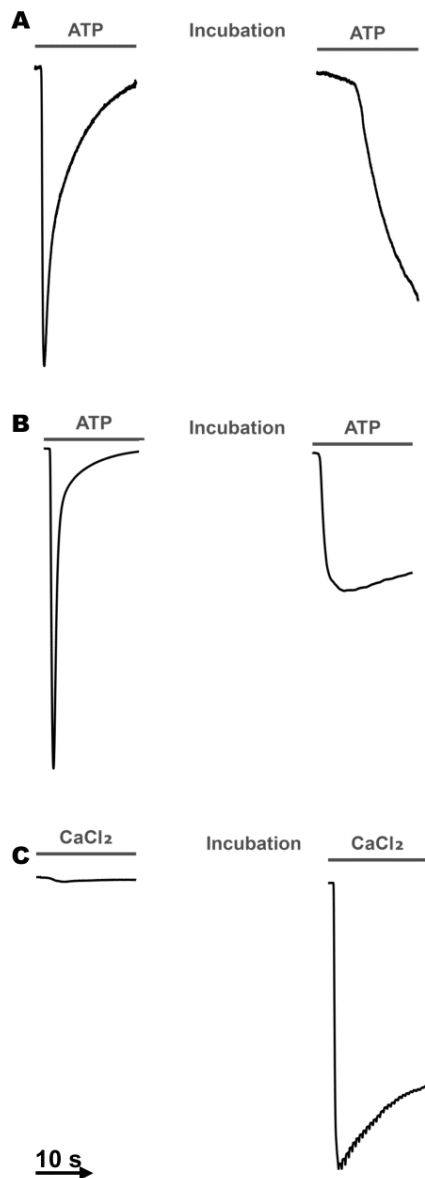
The first attempt was to see whether the successful results could be recorded via the TEVC.

RNA from each group was injected in dilutions from 1 to 10 to avoid damage caused by overexpression, as neither introduced cysteine had shown evidence of extensive damage to the channel expression level or overall conductance. After 1-2 days of incubation, the oocytes were tested for activation on the TEVC. The standard measuring scheme starts with 10 seconds of washing with the background buffer, proceeding with 10 seconds of 10 μ M ATP administration, and finishes with further washing.

The representative traces are shown in Figure A.1.11 (Appendix). Among three constructs of interest, the E11C,V15C mutants exhibited no affected desensitization kinetics, which erases the possibility of observing a further stabilization of the cap, if any. The V15V, F358C, and V18C, V361C, on the other hand, exhibited accelerated desensitization compared to the C339S-triple mutant. A successful crosslinking within the cytoplasmic cap during activation is expected to lock the cap in the open state, thereby halting the desensitization.

In the crosslinking experiment, the mutants were divided into two groups: 15C-358C and 18C-361C. Each group included single-point cysteine mutants, a

mixed RNA sample containing both single mutants in a 1:1 ratio, and the corresponding double cysteine mutant. The mixed RNA group was used to rule out the possibility of inter-oocyte crosslinking. Oocytes from the same injection batch were either pre-incubated in a 0.5 mM crosslinker solution (prepared in the background buffer) or in the buffer alone before TEVC recording. Meanwhile, oocytes that had not undergone pre-incubation were instead exposed to the crosslinker so



lution for 10–15 minutes after the first activation, during the wash-off phase. Following this brief exposure, the crosslinker was washed out, and a second activation was performed. The goal in both conditions—pre-incubation before activation and post-activation exposure—was to determine whether crosslinking induced kinetic changes.

Ideally, successful crosslinking during activation of the double mutants will result in noticeable stabilization of the open state. However, the specificity of this crosslinking was questioned.

While some double mutants exhibited altered desensitization kinetics after crosslinker incubation, seen in the example trace of Figure 4.1.6, changes of kinetics in 18C,361C after BMOE incubation, similar changes were observed in single cysteine mutants and even in WT receptors (Figure 4.1.6, panel B). The WT receptor contains an intrinsic cysteine residue (C339). The fact that incubation with the crosslinker non-specifically modified kinetics in both mutants and WT, despite the absence of designed crosslinking sites in the latter, strongly suggested that the procedure lacked specificity, suggesting the crosslinking approach was unsuccessful.

Figure 4.1.6: Representative traces during the attempt to crosslink. All applied compounds are indicated, and BMOE incubation processes are indicated. A, traces of V18C,V361C activation. Comparing the activations, the one that comes after crosslinker incubation exhibits slower opening and no desensitization; B, traces of the hP2X3R WT activation. After incubation, the desensitization is dramatically slower; C, application of solely CaCl_2 onto the WT. Only after crosslinker incubation, CaCl_2 induces an extensive current.

It was preliminarily proposed that the incubation in the crosslinker might have changed the membrane permeability of the oocytes, further altering the intracellular environment and affecting the ion channel activity. To initially investigate whether the crosslinker incubation had an impact on the membrane integrity, a testing experiment was conducted using calcium-activated chloride channels that the *Xenopus* oocytes intrinsically express, which are activated by calcium ion influx (Barish, 1983). Normally, calcium is excluded from the bathing buffer used in TEVC measurements to prevent activation of these chloride channels, as intracellular calcium levels can be elevated upon the opening of Ca^{2+} -permeable P2X3 channels. In this experiment, 5mM of CaCl_2 was prepared using Ori^- and applied as an agonist with the same recording protocol. The upper trace in panel C of Figure 4.1.6 shows the recording under CaCl_2 , which exhibits a negligible current.

Together, these experiments suggested that the change in hP2X3R desensitization kinetics might not be specific to the cross-linking of the target cysteine residues. The crosslinker incubation could change the membrane status of the oocytes, potentially affecting channel behaviors. Consequently, further experiments using the TEVC with the same protocol may not be reliable and thus cannot be continued.

The other approach to verifying crosslinking was through biochemical experiments, aiming to directly detect crosslinked dimers or trimers formed when the crosslinker reacted with the termini that came into proximity, specifically during the open state. The mutants constructed based on the triple mutant exhibited a relatively stable open state, as shown in Figure 4.1.6, providing sufficient time for the crosslinker to react and accumulate, facilitating polymer formation under

continuous ATP exposure.

The oocyte injection followed the protocol described above. Additional incubation of the oocytes with 5U/ml apyrase after the injection was included, aiming to secure an absolute close state. On the day of the experiments, all oocytes were washed three times with oocytes' PBS (pH 7.0), and treated with either 10U/mL apyrase in Ori⁺ or pure Ori⁺ for 10 minutes. The apyrase-treated groups were divided into two groups and incubated in either 500 μ M crosslinker solution or DMSO as controls for a specified period. ATP was then added to reach a final concentration of 300 μ M for 1 minute, followed by a 5-minute incubation with a 10 mM cysteine solution to saturate the excess crosslinker. After crosslinking, the membrane fraction of hP2X3Rs was fluorescence-labeled, affinity-purified, and samples were denatured by SDS sample buffer and separated by ready SDS-PAGE gel electrophoresis. This treatment should have abolished the natural non-covalently assembled dimers or trimers, leaving only the dimers and trimers formed from covalent crosslinking intact.

In the biochemical study, the analysis of 11E-15V was no longer pursued, as the suggested interactions are within the same subunit.

The result given in Figure A.1.11 (Appendix) demonstrates an experiment with variable incubating time (5 or 10 min.) of oocytes with the crosslinker BMOE. During the experimental optimization, it was found that 10 minutes of incubation with the crosslinker BMOE achieved the maximal efficacy. Lanes 1-4 demonstrate the ATP-treated double mutants 15C/358C and 18C/361C groups, containing strong bands showing a large amount of dimers and some trimers in comparison to the single mutant 15C and 18C (compare lane 1 or 3 with 2 or 4, respectively). Lanes 5-8 are from oocytes without ATP treatment after crosslinker incubation. Strong signals were also raised from the double mutant group 15C/358C. However, the 18C/361C double mutant group shows markedly less strong bands of dimers and trimers, as compared to the corresponding bands after ATP-treatment (compare lane 7 to lane 3). These results indicate less (efficient) crosslinking in the absence of ATP (and thus in the apo closed state) as compared to crosslinking in the presence of ATP (and thus the ATP-bound open state). Lanes 9-12 gave examples of how less crosslinker incubating time of BMOE with the receptors leads to a little lower crosslinking efficiency. Lanes 13-16 are the negative controls in the absence of any crosslinking (but in the presence of a similar concentration of DMSO as in the crosslinker buffer). Please note that only monomers were present

in the absence of BMOE, showing that no spontaneous crosslinking occurs.

The biochemical experiments visualized the crosslinking among the subunits when channels are opened, providing evidence of the residues on the terminus drawn near to each other in this process, from which the approach of N- and C-terminus during activation is imaginable.

In summary, a few pairs of residues located on the terminus, E11-V1, V1-F358, and V18-V361, were suggested to come close during the receptor activation, as the cytoplasmic terminus contacts and knits the cytoplasmic cap. Those amino acids were mutated into cysteines, and with a size-appropriate crosslinker, BMOE, the converging of two cysteines could be locked, accompanied by a steady cap and covalently bound dimers/trimers. These multimers were captured on the gel where the natural multimerization was abolished, proving the N- and C-termini move towards each other during the open state.

4.2 Investigation of the transmembrane domain (TM2)

The TM2 regions are known to form the pore of the P2X receptors. As shown in the upper panel of Figure 1.1, the pore is composed of the 3 α -helices donated from the three subunits. In addition to the crystal structure, which represents only a static snapshot, TEVC experiments provide a dynamic approach for the investigation of receptors at work.

In the following section, the TM2 of hP2X3R will be characterized in detail by utilizing the substituted cysteine-accessibility method analysis, combined with the TEVC. MD simulation based on the distance among residues located on the three protomers, providing additional evidence on the ion pathway of the receptors

4.2.1 MD Simulations Estimate the α -carbon Distance of Residues on the TM2

To dynamically analyze the conformational changes that the TM2 undergoes during activation, the C α -C α value was chosen for the readout. C α -C α describes the distance between every two α -carbons of the residue at the same position from different monomers. It conveniently provides an illustrative way to describe the size of different parts of the pore.

Preliminary simulations were based on the triple mutant in the open state as described in the previous section. As shown in the middle-left panel of Figure 4.2.1, the C α -C α distances between residues at each position were estimated, and their frequencies were analyzed. The higher frequency indicates a higher likelihood of that specific distance occurring. The peak values were thereby collected, as a reference for future characterization.

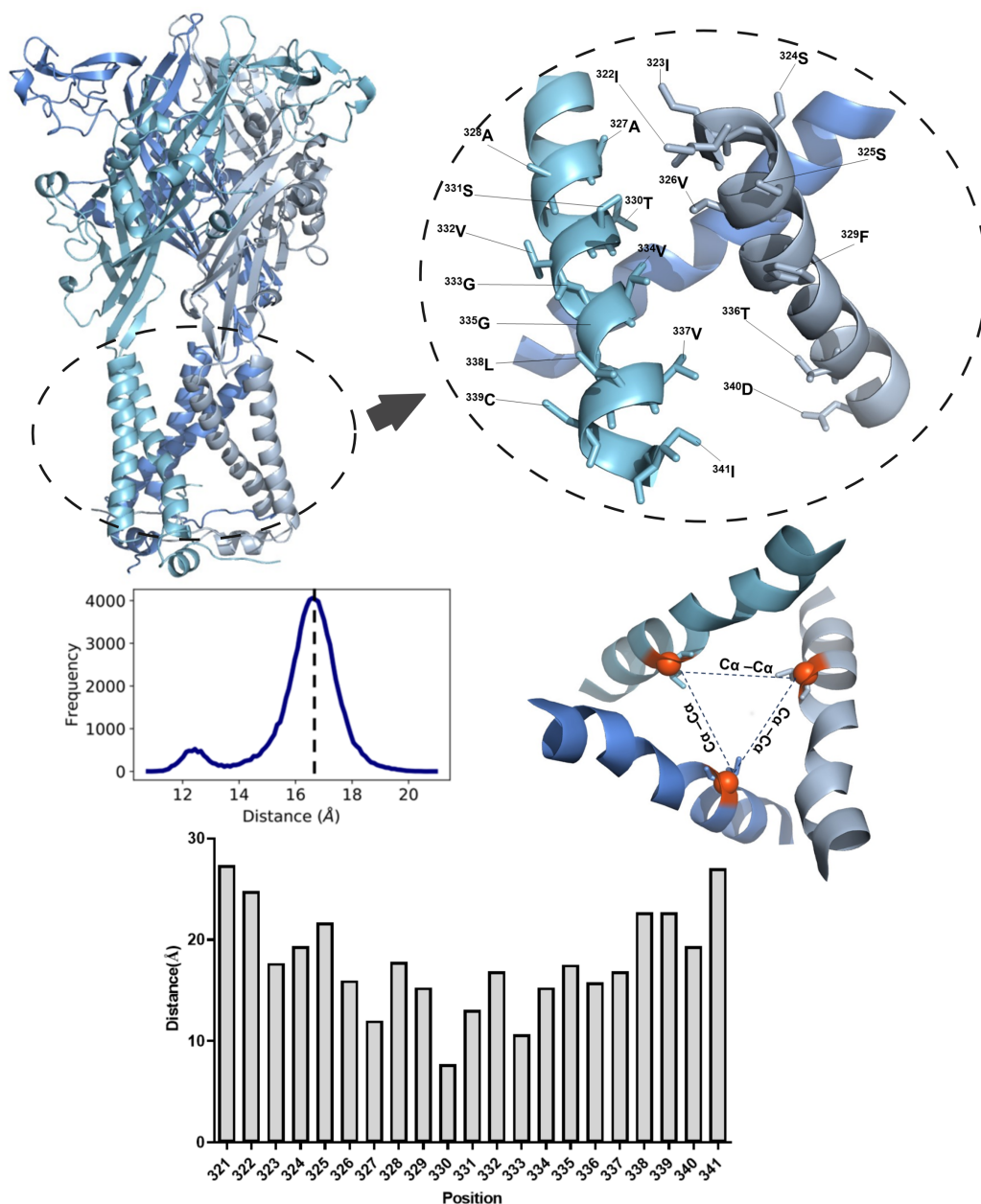


Figure 4.2.1: The upper panel shows the representation based on the crystal structure of the P2X3 in the open state (PDB: 5VSJ). The upper-left gives the full-length structure, with different monomers color-coded individually. The upper-right zooms in at the TM2 (TM1 was hidden for better viewing). Residues 321-341 are labeled with both positions and amino acids. Labeled ones are shown in sticks. The middle-right panel depicts how the Ca distances are determined, with vertical viewing of the TM2 from the extracellular side. Each monomer is color-coded differently. The exemplified residues are T330 with Cas shown in spheres colored in orange. The distance is described as the dashed line noted with "Ca-Ca". The middle-left panel gives an example of the Ca distances simulation results. The plot shows a distribution centered around a peak distance of approximately 16 Å, suggesting that this distance is the most common and likely conformation in the ATP-bound open state. The bottom graph shows columns representing the peak values from each simulation.

4.2.2 Cysteine-substitutions merely Alter Channel Function

The amino acids on TM2 were mutated into cysteines one by one based on the template of the C339S-triple mutant, which has previously been established in the **Results** as indistinguishable from the triple mutant. Here in the later study, these two were not specified differently unless needed. The mutagenesis was carried out from 321st to 341st residues, excluding residues 339th and 340th. The WT TM2 contains an endogenous cysteine at position 339, and 340C has been proven to be barely functioning in previous work. The rest of the cysteine mutations of the TM2 were all tested on TEVC.

RNA from all mutants, as well as the triple mutant serving as the control, was injected into oocytes with a dilution of 1:10. After 1-2 days of incubation at 19°C, the oocytes were tested on TEVC. The function tests provided with a single activation, as previously described, started with 10 seconds of washing with the Ori⁻, proceeded with activation from 10μM ATP for 20-30 seconds, and finished with further washing.

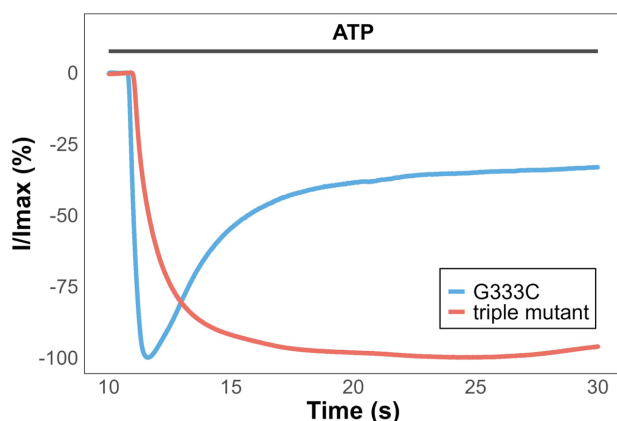


Figure 4.2.2: Overlapped recording traces from the triple mutant and G333C mutant. The traces are color-coded as indicated. The ATP application is shown. The responses were normalized to their maximal amplitudes.

The comparison of the amplitudes from each mutant, aligned with the control, is shown in Figure A.2.1 (Appendix). The triple mutant yielded an overall amplitude of $-8009 \pm 961.7\text{nA}$, while most cysteine insertions did not vary the channel functioning profile: T321C at $-7523 \pm 1038\text{nA}$, S324C at $9488 \pm 1256\text{nA}$, S325C at $-8265 \pm 839.1\text{nA}$, V326C at $-13618 \pm 1633\text{nA}$, A327C at $-7034 \pm 1072\text{nA}$, A328C at $-12875 \pm 2326\text{nA}$, F329C at $-12215 \pm 1203\text{nA}$, T330C at $-12331 \pm 1304\text{nA}$, 332C at $-6632 \pm 653.6\text{nA}$; G335C at $7996 \pm 1611\text{nA}$, T336C at $12188 \pm 1247\text{nA}$, V337C at -12574

$\pm 2253\text{nA}$, L338C at $-5096 \pm 1462\text{nA}$ and I341C at $-6288 \pm 682.2\text{nA}$. On the other hand, the mutation at I322, I323, S331, G333, and V334 raised significant differences. The replacements of isoleucines with cysteines at positions 322 and 323 dramatically decreased the responses, with current amplitudes of $-2488 \pm 359.3\text{nA}$ and $-297.4 \pm 29.6\text{nA}$, respectively. Similar effects were also observed on S331C, G333C, and V334C mutants, whose response to $10\mu\text{M}$ ATP dropped to $-3022 \pm 414.5\text{nA}$, $-2280 \pm 378.4\text{nA}$, and $1369 \pm 226.7\text{nA}$ respectively.

Although it was noticeable that some mutants exhibited a slightly accelerated desensitization, the relevant collective statistics were not performed, as most alterations were not as extensive, except for G333C, as shown in Figure 4.2.2. The cysteine at position 333 seems to not only decrease the amplitude but also able to accelerate the desensitization to an extent.

Cysteine substitutions can affect different functional attributes of the protein, including gating, trafficking, and conductance. The general function studies described here aimed to exclude the possible impact brought by the mutagenesis. Overall, the mutagenesis on the TM2 had minor impacts on most positions, although some residues were notably sensitive to the changes. These basic profiles of the mutants provide us with a solid foundation for a more detailed investigation.

4.2.3 Metal Bridge Characterizes Aligned Residues within TM2

As described in the **Materials and Methods**, Cd^{2+} selectively interacts with cysteine residues in the ion channel, forming metal bridges through non-covalent coordination with thiol groups (Linsdell, 2015b). Unlike disulfide bonds, which are covalent, Cd^{2+} bridges rely on coordination, where the metal ion binds to cysteine thiol groups without forming covalent bonds. During the recording, $10\mu\text{M}$ CdCl_2 dissolved in Ori^- was used to start the screening.

DTT could break metal bridges formed between two cysteines by competing with cysteine side chains to coordinate the metal ions (Alliegro, 2000). Besides, the application of DTT helps screen for spontaneous cross-linking between cysteine residues in the later experiments. 1 mM DTT was used to reverse possible Cd^{2+} -induced thiol group reactions.

The accessibility-testing protocol was modified based on the single activation one.

After 10-20 seconds of activation induced by ATP, the 10 μ M CdCl₂ + ATP solution was applied directly. A significant reduction in the currents represented the cysteine accessibility of the mutant. The Cd²⁺ blocking efficiency obtained from each construct was calculated and used for further comparison and statistical analysis.

G333C was not a suitable candidate for this part of the experiments and relevant calculations due to specific desensitization. Therefore, it was excluded from this analysis and will be discussed separately.

The representative recording traces from mutants identified as significantly different from the control, as well as the control, are shown in Figure A.2.4 (Appendix). The statistical results are shown in the left panel of Figure A.2.2 (Appendix). The triple mutant exhibits extreme insensitivity to 10 μ M Cd²⁺, with a blocking efficiency of 0.05 ± 0.02 , providing a solid negative control in the experiments. Compared to it, 10 out of 17 mutants showed no significant differences.

Viewing from the extracellular environment, T321C, and I322C, lining the pathway before the gating I323, were not significantly blocked by 10 μ M Cd²⁺, with a blocking efficiency of 0.13 ± 0.05 and 0.04 ± 0.03 in respect; the subsequent residues following the gate at I323, S324C, S325C, and C326C exhibited a blocking efficiency of 0.06 ± 0.03 , 0.15 ± 0.02 , and 0.16 ± 0.02 respectively, and A328C as well as F329C displayed blocking of 0.09 ± 0.05 and 0.09 ± 0.02 each. Surpassing the 330C, only three mutants did not show any significant cadmium-induced current decrease: S331C with a blocking of 0.08 ± 0.02 , G335C with 0.07 ± 0.03 , and I341C with 0.13 ± 0.03 .

I323C, which already showed a significantly altered response amplitude, displayed a significantly increasing current reduction upon applying Cd²⁺, with a blocking efficiency of 0.37 ± 0.07 . The blocking efficiency obtained from A327C was 0.51 ± 0.06 , while T330C showed a blocking efficiency of 0.85 ± 0.04 . The majority of residues located on the cytoplasmic end of TM2 seem to be sensitive to Cd²⁺: V332C with 0.70 ± 0.10 , V334C with 1.00, T336C with 0.62 ± 0.07 , and V337C with 0.57 ± 0.11 .

The V334C resulted in complete channel blocking, as the blocked efficiency exceeds 100% in most cases. As mentioned in the **Materials and Methods**, when certain mutants exhibited leak currents, the observed blocking sometimes exceeded

100%, indicating a more significant reduction than the initial ATP-induced response. To prevent negative values in the calculations, the blocking efficiency was set to 100% in these cases.

In comparison to the control group, the mutants sensitive to Cd^{2+} showed P values below 0.0001, whereas the rest groups resulted in P values exceeding 0.995, exhibiting a robust and clear binary response. The mean values of blocking efficiency plotted against the simulated C α -C α distances are shown in the right panel in Figure A.2.2 (Appendix) with P value = 0.0252, indicating a significant correlation.

To test the reversibility of the Cd^{2+} inhibitory effect, and thereby verify the very nature of the blockage, DTT-reducing experiments were introduced to the groups that showed apparent Cd^{2+} sensitivity. The resulting reactivity profiles were varied, recording trace examples of which are shown in Figure A.2.3 (Appendix).

After undergoing a current reduction after application of cadmium, I323C reacted dramatically to the DTT. The amplitude not only bounced back but also increased further compared to the original ATP-induced current. A327C, on the other hand, did not appear to be rescued by DTT. T330C exhibited a full recovery upon a prolonged administration of 1 mM DTT. As for mutants located after T330, as shown in the example trace of V334C, the inhibited current displays a rapid and full recovery. Overall, all inhibitory effects, except for what is shown on A327C, seemed to be reversible by the application of DTT, though the degrees and speeds might vary.

In summary, the Cd^{2+} sensitivities of the TM2 cysteine mutants were profiled. The screening showed that I323C, A327C, T330C, V332C, V334C, T336C, and V337C exhibited sensitivity towards Cd^{2+} , presenting as a decrease in ATP-induced current and varying in degree. Except for A327C, all current clamping from other mutants was able to be reversed by the application of DTT.

4.2.4 Cd^{2+} Experiments Raise Residue-specific Exploration

To build metal bridges, Cd^{2+} ion is coordinated by one to multiple amino acids with increased sensitivity (Linsdell, 2015b). In the experiments described above, different degrees of current blocking with position specificity throughout the ion

channel were observed. From the extracellular side, most residues that reacted to Cd^{2+} that are located above, including T330, reported a stable and DTT-reversible inhibitory effect of the Cd^{2+} , except for A327C. Cysteine mutations close to the cytoplasmic end were more likely to be more reactive, with, however, a less stable effect. Those results suggested that the Cd^{2+} effect observed here is most likely caused by the coordination covalent bond between Cd^{2+} ions and -SH groups from the cysteines (Giles et al., 2003).

Some residue-specific investigations were carried out to further explore the information provided by the metal bridge experiments.

V326C The first experiment took advantage of the distance sensitivity of the cadmium reaction and the particular location V326C, attempting to insert cadmium ions into the channel as an artificial selective filter, aiming to distinguish different ions passing through the channel by size.

The S-S distance in a disulfide bond is typically around 2 Å, with an associated $\text{C}\alpha$ - $\text{C}\alpha$ distance ranging from 3.8 to 6.8 Å. When a cadmium ion forms a metal bridge between two sulfide atoms, the total S-S distance increases, accounting for the sum of the cadmium ion's radius and its bond length to each sulfide atom. This extension results in an estimated $\text{C}\alpha$ - $\text{C}\alpha$ distance of approximately 7–11 Å. However, this calculation assumes an idealized structure and may not fully capture the flexibility and variations in real protein environments

As revealed by the crystal structure, four occlusions within the pore of the P2X3 channel were reported within the P2X3 channel: I323, V326, T330, and V334. Among all, cysteine substitution at V326 did not exhibit significant sensitivity to 10 μM CdCl_2 , unlike the other occlusion sites where such substitutions led to notable current inhibition. This unique response suggests that at position V326, the substituted cysteines are not positioned closely enough to coordinate CdCl_2 effectively through interactions involving multiple thiol groups.

However, the moderate distance between residues at V326 may still allow for partial coordination with CdCl_2 , potentially involving cysteines from two out of the three monomers in the trimeric structure. Meanwhile, less participation of the residues requires a higher CdCl_2 concentration. The stable complex of $\text{SH-Cd}^{2+}\text{-SH}$ potentially narrows the channel as an artificial filter that could differentiate

ions based on their size, providing valuable insights into the ion conduction mechanisms of the P2X3 receptor, particularly regarding its selectivity for hydrated versus dehydrated cations, which has not been systematically explored on hP2X3Rs pore.

Ion selectivity often depends significantly on ionic radius and hydration shell size (Degrève et al., 1996). Typically, smaller ions like Li^+ tend to be more strongly hydrated (thus effectively larger in aqueous solutions), while larger ions like K^+ have relatively weaker hydration shells, making their effective radius in solution comparatively smaller (Mahler & Persson, 2012). Although P2X channels generally permeate monovalent cations, Li^+ and K^+ were specifically chosen here due to their pronounced hydration differences compared to Na^+ . Thus, the metal-thiol barrier introduced by Cd^{2+} would affect these ions differently based on their hydration state and effective radii.

In these experiments, 90 mM LiCl and KCl were prepared to substitute the NaCl in the standard Ori⁻ solution individually. A concentration of 30 μM instead of 10 μM of CdCl_2 was used in these experiments, to facilitate more reactions between Cd^{2+} and cysteine residues. 10 μM ATP was applied as an agonist as before. During the TEVC experiments, the oocytes were washed with either Li^+ or K^+ -based Ori⁻, and activated for 20 seconds by ATP prepared in the corresponding buffer. The first activation was followed by 2 minutes of rinsing, then the ATP was applied again. After 20 seconds of activation, perfusion was switched to the 30 μM Cd^{2+} contained ATP solution. Both amplitudes before and after the co-application were recorded. The aim was to compare the blocking efficiency between the Li^+ Ori⁻ and K^+ Ori⁻ groups to determine if the Cd^{2+} -induced inhibitory effect was influenced by the permeating ions. The triple mutant was used as the control group.

The results are shown in Figure ?? (Appendix). The amplitudes for the triple mutant and V326C in both backgrounds were first compared to evaluate the conductance preliminarily. As is described in the left panel, both channels functioned in different Ori⁻ solutions. The triple mutant did not distinguish the two different permeating ions, with a current amplitude of -6553 ± 1280 nA in K^+ -based solution and -5638 ± 1080 nA in Li^+ . Conversely, V326C was sensitive to the switching between Li^+ and K^+ with current amplitudes of -3313 ± 295.4 nA and -6652 ± 740.2 nA, with a P value of 0.0003.

The addition of Cd^{2+} during the activation did not raise any observable changes

in the triple mutant, as indicated in the right panel, regardless of the permeating ions. In the K^+ solution, the blocking efficiency of $30\mu M$ Cd^{2+} was recorded as 0.06 ± 0.02 , and 0.04 ± 0.01 in Li^+ . By contrast, the same concentration of Cd^{2+} successfully induced a current decrease for V326C. In the K^+ background, the current was inhibited by 0.30 ± 0.05 , and this number was further increased to 0.54 ± 0.04 when the background buffer was switched to Li^+ -based ($P = 0.0005$).

In summary, this experiment aimed to induce Cd^{2+} -SH interactions at position 326 within the ion channel, to create a spatial obstacle serving as a potential filter for the ion channel. By evaluating the different degrees of impediments of ion conduction between Li^+ and K^+ caused by the same amount of Cd^{2+} , additional information on how the cations passed through the TM2 was provided.

A327C It was noticed in the crosslinking experiments that, although the blocking efficiency observed in A327C is not numerically special, this construct exhibits unique behavior in terms of reversibility. While A327C also exhibited current reduction upon Cd^{2+} application, it showed resistance to DTT treatment, and the current could not be effectively recovered, as shown in Figure A.2.3 (Appendix). Because of this, it was speculated that the Cd^{2+} blocked the A327C channel in a distinct pattern.

To start with, it was examined whether the differences in reversibility indeed significantly existed between A327C and the others. T330C was selected for comparison, for its steadily induced and reversed inhibitory. The TEVC experiments were conducted as previously described in the paragraph *Substituted Cysteine Accessibility Method* of the **Method** chapter. The recovery discussed here was defined as how much the reduced current recovered during the perfusion of solely ATP or ATP+DTT.

The results are shown in Figure A.2.5 (Appendix). In these experiments, A327C recovered 0.17 ± 0.07 in solely ATP perfusion after the current was reduced in $CdCl_2$, while T330C recovered 0.09 ± 0.02 . After adding DTT to the post- Cd^{2+} perfusion, the inhibited currents of A327C recovered by 0.17 ± 0.07 , meanwhile, a ratio of 1.06 ± 0.13 currents of T330C bounced back. T tests were conducted between A327C with/without DTT, T330C with/without DTT, A327C /T330C without DTT, and A327C /T330C with DTT groups. While no significant differences were detected within the A327C experiments, different conditions resulted

in different recoveries for T330C, with a P value = 0.0039. When DTT was not applied, A327C and T330C recovered in a similar pattern that showed no significant differences. The addition of DTT dramatically encouraged the recovery of T330C but not A327C, raising a P value = 0.0016.

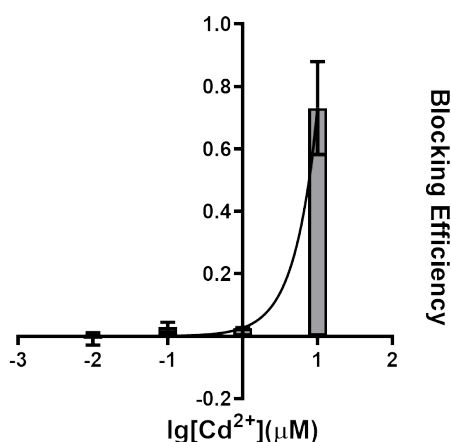


Figure 4.2.3: Concentration-dependent inhibitory effect of Cd^{2+} on F329C. The selected ranges of Cd^{2+} induced dramatic changes in current reduction on this mutant, from negligible to around 80% of inhibitory effect. Hence, a reliable IC_{50} value was not retrieved from the plotting.

Subsequently, the onset kinetics of Cd^{2+} in A327C was investigated and compared with other mutants. To assess onset kinetics, it was preferred to initiate with determining the individual concentration-response analysis IC_{50} s of Cd^{2+} for the mutants of interest, and then proceed to evaluate and compare the kinetics using different Cd^{2+} concentrations that correspond to a particular IC value for each. This method allowed concentrating on the differences in kinetic behavior while minimizing the impact of varying sensitivities among the mutants.

A wide range of Cd^{2+} from 0.01-1000μM was tested. Different ranges of concentrations were applied to different mutants, depending on the measurability: the chosen concentrations could comprehensively cover and define a reliable IC_{50} curve. The TEVC protocols remained similar. After 20 seconds of ATP activation, different concentrations Cd^{2+} were applied with further ATP perfusion for 20 seconds. The blocking efficiency data were collected as described. Besides, an exponential decay fitting was applied to the first few seconds of Cd^{2+} -induced current reduction, thereby calculating the onset constant τ .

Both MD simulations and previous experiments suggested that the segments below T330C, observed from the extracellular side, display high flexibility, reflected by relatively more unstable and unexpected results (a more detailed discussion of this flexibility will be provided in the **Discussion**).

Therefore, mutants S324C, S325C, V326C, F329C, and T330C were chosen for the comparative analyses. These extracellular end residues, positioned from 324 to 327 on a single α -helix, were selected due to their adjacent alignment and demonstrated higher robustness during preliminary experiments. By targeting these residues, the focus was on the similarities and differences in channel opening onset when cysteine side chains are oriented differently. Specifically, T330C served separately as a highly sensitive positive control, while the neighboring F329C mutant was included to provide additional insights into the structural dynamics of the channel.

The first observation from these experiments was that all mutants displayed a sure yet varied sensitivity to Cd^{2+} . Highly concentrated Cd^{2+} was able to block currents of 324-326 as well as F329C cysteine mutants to different degrees, as shown in Figure A.2.7 (Appendix). F329C was not included for further analysis, as the testing concentration appeared to fail to cover a reliable interval where IC_{50} can be predicted, see in Figure 4.2.3.

Results for S324C were obtained from Cd^{2+} concentration of 30/100/300/1000 μM , as inhibitory effects in lower concentrations were unable to be recorded. The onset τ values from tested concentration were 3.46 ± 0.35 , 2.62 ± 0.39 , 2.80 ± 0.50 , 3.30 ± 0.29 nA/s, with corresponding blocking efficiency of 0.33 ± 0.13 , 0.53 ± 0.05 , 0.74 ± 0.20 , and 0.82 ± 0.10 in respect. The dose-response curve fitting was achieved and reported an IC_{50} value of 110.5 μM . S325C was tested under $[\text{Cd}^{2+}] = 0.1/1/10/100/300/1000\mu\text{M}$, and only the last three concentrations induced sufficient inhibition to allow reliable exponential fitting of onset kinetics, of 3.74 ± 0.40 , 4.98 ± 0.79 , and 2.49 ± 0.34 nA/s. The blocking efficiencies for the ranging concentrations were 0.01 ± 0.03 , 0.02 ± 0.00 , 0.01 ± 0.01 , 0.36 ± 0.09 , 0.60 ± 0.02 , and 0.98 ± 0.01 . Dose-response curve fitting reveals an IC_{50} value of 430.8 μM . V326C was tested under Cd^{2+} concentration of 1/10/30/100/ 300/1000 μM , while τ values were collected from 30/100/300/1000 μM , as of 3.34 , 5.17 ± 0.83 , 4.00 ± 0.22 , and 2.48 ± 0.60 nA/s. The blocking efficiencies were 0.00 , 0.00 , 0.19 ± 0.01 , 0.68 ± 0.05 , 0.80 ± 0.08 , and 1 ± 0.08 , respectively. IC_{50} obtained from the fitted curve was 63.11 μM . - A327C as well as T330C were only tested under 0.01/0.1/1/10 μM as both were highly sensitive to Cd^{2+} . The τ values for A327C and T330C in a concentration-ascending order were 3.05 ± 0.58 , 3.09 ± 0.43 , 1.58 ± 0.07 , and 1.42 ± 0.18 nA/s and 2.71 ± 0.38 , 2.49 ± 0.23 , 2.36 ± 0.13 , and 1.62 ± 0.40 nA/s in respect. It was observed that during these experiments, neither of them showed a clear Cd^{2+} concentration-dependent blocking efficiency. This was confirmed by the one-way ANOVA test, as multi-

ple comparisons between every two values of blocking were compared for each of the mutant,s and no significant differences were detected. This indicated that even at the lowest concentration, 0.01 μ M, a maximal level of current inhibition was observed. In this case, the τ values were compared only between A327C and T330C, to investigate whether increasing inhibitor concentration could further encourage the binding speed of Cd²⁺ onto the channels when the blocking efficiency has reached the maximum.

The comparisons between the onset kinetics of A327C and T330C were done via one-way ANOVA multiple comparison tests, sorting the τ from each mutant by the same concentration. The first test was conducted within the same concentration, where no significant differences were detected. The second test performed was within the same mutant, where onset kinetics from both mutants at the same concentration were compared. The test revealed that all τ values of T330C, regardless of changes in Cd²⁺ concentration, showed no differences. In contrast to it, A327C exhibited acceleration of Cd²⁺ association along with increased [Cd²⁺]. 0.01 μ M and 0.1 μ M Cd²⁺ bind significantly slower than 1 or 10 μ M, with P values of 0.01-1 = 0.0369, 0.01-10 = 0.0486, 0.1-1 = 0.0211, and 0.1-10 = 0.032. The acceleration effect is indicated in Figure A.2.6 (Appendix).

Another intriguing effect observed was that, despite a lower sensitivity, other cysteine mutants were still able to be blocked by an increased amount of Cd²⁺ in the solution. It was interesting to find out whether the sensitivity is determined by the α -atom distances obtained from the simulation, or does the directions of the sidechains also play an indispensable role. Results are shown in Figure A.2.7 (Appendix). The IC₅₀ values of S324C, S325C, and V326C were plotted against the simulated distance of the carbon atom at these positions. As A327C and T330C appeared to be extremely sensitive to Cd²⁺, and their IC₅₀s could be considered nearly zero when in micromolar units, these two points were plotted on the x-axis. The plotting of available data strongly hinted at a positive correlation between Cd²⁺ IC₅₀ for each position and the α -c distances. A nonparametric Spearman correlation between the x and y coordinates of all data points was analyzed, resulting in P values = 0.0333, strongly suggesting a positive correlation. In Figure A.2.8 (Appendix), an exponential growth model was adopted for the fitting; the curve was plotted with $r^2 = 0.9842$.

During the preliminary function test of the cysteine mutants, it was observed that G333C exhibited a unique desensitization behavior compared to the other mutants

and the triple mutant. As a result, this mutant was analyzed separately.

To characterize G333C, minor modifications were made to the TEVC methods. Instead of reaching a plateau of ATP-evoked current before the perfusion of Cd^{2+} or DTT in the ATP solution, the G333C was activated directly by the ATP only or directly by the ATP + Cd^{2+} solution after baseline establishment. In some cases, the activation induced by Cd^{2+} +ATP was followed by the application of DTT + ATP to test for reversibility, as shown in the left panel of Figure A.2.9 (Appendix).

The stabilization of activation observed under the influence of ATP+DTT is presented in the left panel of Figure A.2.9 (Appendix). After the current activated by ATP in Cd^{2+} reached a plateau, the substitution of Cd^{2+} with DTT abolished the steady state and facilitated the current decrease. This effect is presented as DTT-specific, as a simple removal of Cd^{2+} did not lead to such a rapid and dramatic change in the channel behavior. In comparison, activation of channels with only ATP raised more dramatic desensitization, which appeared to be unaffected when DTT was added in addition. Unlike other mutants that were sensitive to Cd^{2+} , no amplitude-decreasing effect was observed. As shown in the middle panel of Figure A.2.9 (Appendix), the G333C yielded similar amplitudes in the ATP solution, with $(-3868 \pm 959.4 \text{ nA})$ or without $(-5414 \pm 1009 \text{ nA})$ $10 \mu\text{M}$ Cd^{2+} . Yet strikingly, Cd^{2+} seems to be far from insignificant here. The right panel shows that, under the perfusion of the combination of the ATP and Cd^{2+} , the channels are not desensitized to the full extent, remaining 0.68 ± 0.08 of the maximal current in 20 seconds, while the pure ATP-induced current decreased to 0.25 ± 0.05 in the same time.

4.2.5 Further Investigation of the Proposed Gating Residues

The crystal structure of P2X3R revealed four occlusions of its TM2 that potentially serve as gates: I323 as the extracellular gate, V326 that narrows the ion pathway, T330 that defines the narrowest part of the channel and potentially serves as a filter, V334 that gates during desensitization (Mansoor et al., 2016). The role of V326 and T330 has been described in the last section, and the I323 and V334 residues were further characterized and presented here, aiming to provide more information about these positions and the roles that the residues here might play.

I323 The earlier results proved that I323C was sensitive to $10\mu\text{M Cd}^{2+}$, most likely caused by the approaching of the cysteines' side chains at this position, which leads to effective Cd^{2+} coordination. Another feature noticed was that I323C was the only mutant that was potentiated after DTT rescuing the Cd^{2+} inhibitory effect, meaning the current amplitude not only recovered but also underwent additional growth. This phenomenon appeared to hint at a spontaneous crosslinking of the cysteines, which requires proximity of them to each other in addition to an oxidizing environment.

To verify this hypothesis, further experiments were conducted. The first experiment was to perform a DTT screening on all mutants (triple mutant as a control included), also to find out whether I323 was the only residue that approached extremely closely in the apo state. $10\mu\text{M ATP}$, 1mM DTT solution, and a solution containing both compositions were used. All mutants were tested for the DTT effect, by 20 seconds of DTT + ATP activation following 20 seconds of ATP application. Any DTT addition caused the amplitude to further increase and was recorded as the DTT potentiation. The screening did not include 333C, 338L, or 339C. The DTT potentiation calculation has been described in the **Method** chapter. To test whether the release of cysteines could be induced at an earlier stage, an additional DTT incubation of 30 seconds was applied to I323C before the ATP activation, immediately followed by DTT + ATP solution perfusion, to determine whether the DTT pre-incubation altered the DTT potentiation effect. Representative traces and results are shown in Figure A.2.10 (Appendix).

During the DTT scanning, it was observed that I323C was indeed the only cysteine mutant in the TM2 that exhibited sensitivity to 1 mM DTT (lower panel in Figure A.2.10 (Appendix)). As the upper right panel is shown in Figure A.2.10 (Appendix), the I323C current was dramatically increased upon the additional release of DTT into the ATP perfusion, and the current overall increased by 2.93 ± 0.27 . The triple mutant showed no reaction to DTT+ATP compared with ATP activation in the absence of DTT, with a current change of -0.01 ± 0.02 . Except for I322C with a slight yet not significant current increase of 0.18 ± 0.05 , the DTT application brought negligible changes in amplitudes for the others: I321C by 0.04 ± 0.06 , S324C by 0.01 ± 0.02 , S325C by 0.07 ± 0.07 , V326C by 0.06 ± 0.04 , A327C by 0.04 ± 0.02 , A328C by 0.01 ± 0.02 , F329C by 0.14 ± 0.07 , T330C by 0.06 ± 0.09 , S331C by 0.03 ± 0.01 , V332C by 0.01 ± 0.01 , V334C by 0.05 ± 0.04 , G335C by -0.01 ± 0.01 , T336C by 0.07 ± 0.09 , V337C by 0.05 ± 0.04 , I341C by 0.05 ± 0.02 .

The DTT incubation experiments provided extra information on the position. As the trace in Figure A.2.10 (Appendix) shows, the pre-activation DTT incubation decreases the post-activation DTT potentiation effect, as the current from the no pre-incubation group was enhanced by 2.93 ± 0.27 times, while the number of the DTT pre-incubation group was merely 0.40 ± 0.11 . Multiple comparisons found a significant difference between the potentiations with a P value < 0.0001 , suggesting that either encountering the receptor before or during its activation, the DTT facilitates the channel function similarly, likely involving the release of the channel from an SH-trapped state.

V334 During the previous experiments, cysteine scanning was done using the template of the non-desensitizing triple mutant. As no effects on desensitizing behavior were observed on the V334C-triple mutant, the cysteine mutation at this position was also generated on the fast-desensitizing P2X3 wild type to investigate further the potential role that V334 plays in desensitization, since this position was suggested to be the desensitization gate.

10 μ M ATP was applied twice on the same oocyte, each for 20 seconds, separated by 120 seconds of washing, and ending with a further wash step. The amplitudes yielded from both activations were recorded, and so were the remains of currents for 20 seconds. The ratio between the second and the first current was recorded as recovery. The WT was used as the control group. The results are in Figure A.2.11 (Appendix) (amplitudes comparisons in the scattered points graph were obtained only from the first activation). Both the hP2X3R-WT channel and the non-desensitizing triple mutant were selected as controls.

The V334C appeared not to significantly affect the current amplitude as compared to the WT receptors, as the results are shown in panel B of Figure A.2.11 (Appendix). The first activations recorded from both constructs presented to be not statistically different from each other, as the amplitude yielded from the WT was -2173 ± 431.8 nA, -4197 ± 1150 nA for the triple mutant, and -1236 ± 208.6 nA from the V334C-WT. The great variances found during recording for the V334-WT might account for no statistical differences.

However, the constructs behaved distinctively in terms of kinetics and recovery from desensitization. The P2X3R WT has been known for its fast desensitization and “rundown” effect, as shown in the trace in Figure A.2.11 (Appendix) upper left

panel, manifesting as a dramatically decreased current from the second activation, while the non-desensitizing triple mutant exhibits less level of rundown. On the other hand, the mutation at V334 slows down the desensitization to a moderate extent and almost lacks rundown. The fast desensitizing WT remained at 0.09 ± 0.02 by the end of 20 seconds of activation, the triple mutant remained at 0.93 ± 0.03 , and the V334C-WT remained at 0.32 ± 0.06 . After 120 seconds of washing, the second ATP application revoked an amplitude of 0.18 ± 0.10 on the WT compared to the first one, 0.54 ± 0.06 for the triple mutant, and 1.04 ± 0.14 for V334C-WT. Multiple comparisons between every two constructs found significant differences in both comparisons.

Overall, in these investigations, cysteine scanning on the TM2 region of the P2X3 receptor was conducted to examine the functional behavior of hP2X3 channels using TEVC. The mutant functions were characterized, and the reactive properties of cysteine side chains were exploited through the application of Cd^{2+} and DTT to investigate the structural details of the TM2 domain. In the **Discussion**, the TM2 structure will be further interpreted based on the general cysteine scanning data. Several mutants highlighted in this chapter will be analyzed in greater detail to clarify their structural roles within the pore and provide additional functional insights.

4.3 Profiling the ion pathway

The P2X family is usually described as non-selective cation channels, except for P2X5Rs with a remarkable permeability to chloride ions. The rapid desensitization of P2X3R wild types has limited the exploration of the ion permeability in these channels, besides knowing that they display fundamental properties like the rest of the family members. Taking advantage of the 13P, 15V, 16I-hP2X3 triple mutant, which exhibits a stable open state, the following investigation will focus on profiling the ion permeation properties of the P2X3Rs, mostly emphasizing the determinants of cation versus anion selectivity by comparing the differences between P2X3 and P2X5 receptors.

4.3.1 Aligning the Ion Pathways of P2X3 and P2X5 Channels

The ion pathway in P2XRs, as described in the introduction, can be divided into three sections: the extracellular fenestration, the pore (TM2), and the cytoplasmic fenestration. The ions can enter or exit the pore through these lateral fenestrations, which may also play a role in the regulation of the ion flux.

To identify the structural differences among hP2X3, rP2X5, and hP2X2, the amino acid sequences were aligned, as illustrated in Figure 4.3.1. Remarkably, rP2X5

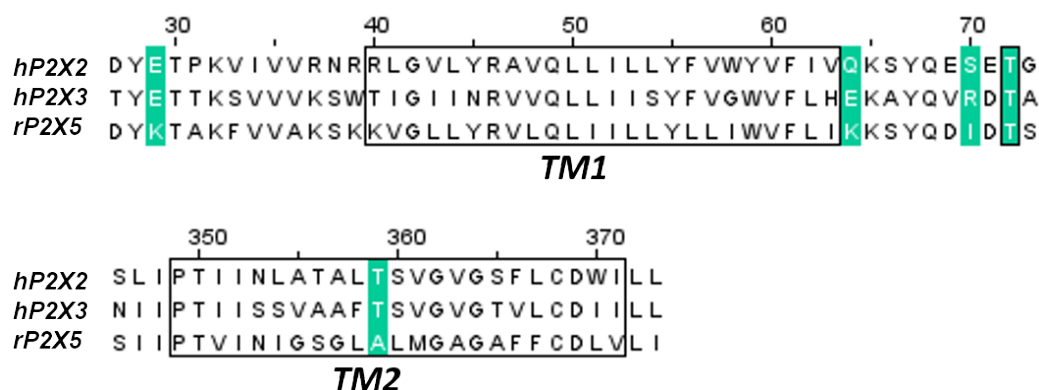


Figure 4.3.1: Sequence alignment of the lateral fenestration pathway among hP2X2, hP2X3 (WT), and rP2X5. TMs are noted. Residues of interest are colored in green. The numbering refers to hP2X2)

processes a unique lysine at position 17 within the ion pathway, in contrast with hP2X2 and hP2X3, which both carry a glutamic acid with the opposite charge. The variation between the K and the E has been shown to dramatically alter the chloride permeability in P2X2 by single-point mutation experiments (Tam et al., 2023). Beyond the TM1 and examining the extracellular fenestration ion pathway starting from residue E46 (numbered according to hP2X3 unless otherwise specified), where hP2X2 carries a glutamine (Q) and rP2X5 with a lysine (K), the residues' variance is proposed for further investigation. Another residue of interest is R52 in hP2X3 (S70 in hP2X2), as amino acids at this position exhibit quite a diversity, and the arginine is shared only between P2X3 and hP2X6. The T54 (T72 in hP2X2) is an additional feature only observed across P2X2/X3/X5, thus raising certain interest as well.

TM2, being the core structure of the pore, has been extensively studied in non-desensitizing P2X2. Specifically, threonine-to-lysine mutation at position 336 (T336K) in hP2X2 has been shown to enhance chloride permeability (Cao et al., 2009). This raises the question of whether a similar mutation at position T330 in hP2X3, which corresponds to this residue, would yield comparable effects. Besides, the sequence of rP2X5 TM2 shows a domain full of small and hydrophobic residues, which is different from both TM2s from hP2X2 and hP2X3 channels; whether the entire pore structure of rP2X5 contributes to its pronounced preference for chloride ions remains intriguing.

The experiments inspired by sequence comparison were designed to alter the part of the structure of hP2X3 accordingly, aiming to find out how modifications to the structure influence ion permeation. Details included single, partial, and complete mutation of the hP2X3 TM2 region and targeted single-point mutations at the residues of interest near the lateral portal.

In the following investigation, the triple mutant will still serve as the most important control. The P2X5, on the other hand, will not be included in most cases, due to its well-known biphasic current which gradually grows over time in the open state, making it hard to determine the current amplitude and will also cause other unwanted effects such as inaccurate identification of the reversal potential during the recording of the I/V curve due to the gradual current increase.

4.3.2 The P2X3 Channel Survived a Complete Swapping of rP2X5R's TM regions

As a key structural component transporting ion currents, the TM2 domain was the primary focus. The entire TM2 sequence, derived from rP2X5 using the megaprimer technique, was integrated into the triple mutant to substitute the native TM2, referred to as the P2X3/X5 TM2 chimera. To investigate whether the pairing of TM1 with its corresponding TM2 within the same channel is essential for maintaining biophysical properties, additional constructs were generated. These included a TM1 chimera (P2X3/X5 TM1 chimera) and a TM1/2 chimera (P2X3/X5 TM1/2 chimera) created by swapping domains between the two receptors. Furthermore, a reverse swapping experiment was conducted, where the TM2 region from P2X3R was transplanted into rP2X5R, resulting in the creation of P2X5/X3 TM2 chimeras.

Upon obtaining the constructs, the corresponding cRNA was injected into oocytes, with an undiluted concentration and a volume of 41nl. The triple mutant served as the control group, injected with 1:10 dilution and a volume of 41nL. After 1-2 days of incubation at 19°C, the oocytes were recorded on the TEVC.

The characterization of the mutants was initiated with examinations of the ion channel function, with ATP as the agonist. The background buffer was the Ori⁻ as in the routine, and the clamping voltage was -60 mV. ATP concentration was 10μM. The single-activation protocol was adopted, where recording started with a brief buffer perfusion, followed by 20-30 seconds of ATP application, and ended with further rinsing with Ori⁻.

The reversal mutations, specifically the P2X5-based chimera incorporating TMs from P2X3 (P2X5/X3 TM2 chimera), completely lost function when tested with 10 μM ATP perfusion. In contrast, all P2X3 channels remained functional after TM swapping, despite varying degrees of functional impairment. The current kinetics of all mutants closely resembled those of the triple mutant. Detailed functional results are presented in the upper left panel of Figure 4.3.1. Under identical activation conditions, the triple mutant displayed a peak amplitude of -7903 ± 1781 nA. The P2X3/X5 TM1 chimera experienced the most significant loss of function, with an average current amplitude of -96.37 ± 20.0 nA. The P2X3/X5 TM2 chimera was least affected, yielding an amplitude of -2139 ± 384.8 nA, though it

still showed decreased function compared to the triple mutant. Interestingly, the two domain-swapped P2X3/X5 TM1/2 chimeras exhibited only moderate impairment compared to the TM1 substitution alone, with an average amplitude of -1047 ± 176.6 nA.

As discussed in the **Method** chapter, the Goldman-Hodgkin-Katz (GHK) equation cannot be used to calculate relative permeabilities in this case. In standard applications, the GHK equation is simplified by eliminating intracellular ion terms, which is only valid when minor currents, such as those carried by Cl^- in typical cation channels like P2X3Rs, are negligible, as it is minor and not of interest. However, in this study, Cl^- permeability is the object of investigation. Since both Na^+ and Cl^- flow in opposite directions, and the intracellular Cl^- concentration is unknown, the key mathematical conditions required to apply the GHK equation are not met. Therefore, the equation cannot be used to reliably determine Cl^- permeability under these conditions.

A general solution is to examine the channels in the system where the internal solution is strictly controlled. Those constructs were therefore further transfected into the HEK293 cell lines and measured by the patch clamp. The external solution was dominated by 140mM NaCl and was used as the background solution. The initial function of the transfected constructs was tested under the NaCl external solution with 10 μ M ATP, the same as in the protocol described for the TEVC experiments. Besides the background of NaCl, a second external solution based on Choline-Cl with the same concentration and osmolarity was used. By substituting the Na^+ with impermeable organic cations, it was expected to observe the remaining current purely driven by Cl^- . The current amplitude measured in both solutions was compared; results are shown in panel B in Figure A.3.1 (Appendix).

Unlike the observed reduced current amplitudes of the chimeras in TEVC experiments, the amplitudes of the mutants obtained in patch clamp analysis displayed more similarity among different constructs, including the triple mutant. The triple mutant yields $-3.56 \times 10^{-9} \pm 2.59 \times 10^{-10}$ A in NaCl and $-6.35 \times 10^{-10} \pm 2.3 \times 10^{-10}$ A in Choline-Cl (Cl^-); the TM1 chimera yields $-5.43 \times 10^{-9} \pm 1.59 \times 10^{-9}$ A in NaCl and $-7.31 \times 10^{-10} \pm 3.2 \times 10^{-10}$ A in Cl^- ; the TM2 chimera yields $-4.40 \times 10^{-9} \pm 6.73 \times 10^{-10}$ A in NaCl and $-8.90 \times 10^{-10} \pm 1.78 \times 10^{-10}$ A in Cl^- ; the TM1/2 chimera yields $-6.95 \times 10^{-9} \pm 5.79 \times 10^{-10}$ A in NaCl and $-1.71 \times 10^{-10} \pm 9.75 \times 10^{-10}$ A in Cl^- . The One-way ANOVA did not detect any differences under both conditions.

After confirming the function of the channels in HEK cells compared to the ones in oocytes, stepped voltage protocols were utilized for the I/V measurement. After establishing the baseline of the cells clamped at -60 mV in the background buffer, the first baseline I/V curve was measured. Subsequently, the solution was switched to 10 μ M ATP. The activation was maintained briefly until the second I/V measurement took place. The differences between those two measurements are seen as the activation I/V curve of the channels. The differences between the two measurements on the same constructs also provide the possibility of calculating the relative permeation of the chloride ions.

The normalized I/V curves are shown in Figure A.3.1 (Appendix), with the middle panels describing the full measurement in the NaCl solution and the bottom panel of zoomed-in and comparable curves of the same channels in different extracellular solutions as indicated. A 2nd-order polynomial regression has always been fitted with each I/V curve to find out the reversal potential. The non-linear fitting yielded the following V_{rev} values for each construct under NaCl and Choline-Cl external solutions: triple mutant (NaCl = -9.42 mV, Choline-Cl = -37.12 mV), TM1 chimera (NaCl = -5.99 mV, Choline-Cl = -38.70 mV), TM2 chimera (NaCl = -3.95 mV, Choline-Cl = -36.67 mV), and TM1/2 chimera (NaCl = -5.18 mV, Choline-Cl = -27.61 mV). These V_{rev} shifts suggest a slight change in ion selectivity for the TM1/TM2 chimera, potentially indicating modifications in the pore region or selectivity filter.

Although V_{rev} values were collected and analyzed to evaluate P_{Cl}/P_{Na} using the GHK equation, no significant differences were observed brought by the chimeras. However, MD simulations suggest that permeant ions were concentrated at the pore entrance, creating local ion levels much higher than bulk values. Because the GHK equation relies on the actual local concentrations, these accumulations made the calculated permeability ratios unreliable, and the error increased with longer channel openings.

In view of this limitation, the plan for precise, concentration-controlled patch-clamp measurements was set aside. Instead, TEVC was chosen for qualitative assessment as it provides a simpler system of experiments. The TEVC protocol was adjusted with shorter activation steps and rapid solution exchange to minimise local ion build-up.

4.3.3 The T330K-hP2X3 Protomer Could Only be Expressed as Heteromers

Upon the general study of how the TM2 region affects the conductance of the ions, a more specific interest in a single residue arose. As mentioned above, the single mutation T336K of the P2X2 has been reported to dramatically change the ion selectivity of the channel to favor chloride. It was intriguing to see whether a similar effect could be reproduced on the corresponding T330 of the hP2X3Rs. The T330 residue, as has been discussed in detail in chapters discussing TM2 structure, was proposed to build the narrowest constriction within TM2 and potentially serves as an ion filter. The idea of lysine substituting was to insert a positive charge that attracts negatively charged ions without losing the ability to facilitate the hydration of the passing ions

The RNA of the mutated channel was injected into the oocytes with an undiluted concentration and volume of 41 nL. After 1-2 days of incubation, the oocytes were tested for function on the TEVC using 10 μ M ATP.

The TEVC results revealed that the T330K mutant was not functional, despite increasing the ATP concentration to 1 mM. Further biochemical experiments revealed that the T330K mutant abolished cell surface (plasma membrane) expression of the T330K-hP2X3R channel, explaining the lack of detectable current. Radio-labeling of the potential intermediate products of the protein synthesis, trying to locate which step was interfered with extensively, yielded no conclusive results. For comparison, a similar mutant that also carries a positive charge, the T330R, was generated as well, and the arginine appeared to be lethal to the channel at the expression level.

Eventually, it was suspected that the existence of positive charges in a very close proximity area might have interfered with the trimer assembly. The TM2 has been suggested to be the crucial area where subunit-subunit interaction takes place. When two lysines are forced to approach at an inappropriately close distance, the charges they carry compel each other, thus disrupting the interaction between two subunits, and further leading to the failure of the expression of an intact trimer. To confirm this assumption, T330K RNA was injected after mixing, either with hP2X2 or the previously discussed K63A-triple mutant, which eliminated channel function when more than one of them was incorporated into one trimer. Those

were attempts to artificially decrease the number of charges on lysine to eliminate the charge-collision-caused interference. The creation of P2X2/X3 heteromers was to test the final product with α,β -methylene-ATP, a specific form of ATP that can selectively activate P2X3 and P2X2/3 channels, but not P2X2 channels when used in concentrations up to 30 μ M. The co-injection with K63A (with a T330 as WT) was to see whether it was still possible to create a pure hP2X3 channel despite the problem of 330K.

Both assays confirmed that the P2X2/T330K-P2X3 heteromers and the K63A-T330K triple mutant channels are properly expressed and functional. For the work that follows, only the hP2X3-based construct (the K63A/T330K heteromer) was carried forward, as the P2X2/P2X3 combination differs more from the native P2X3 receptor and requires a distinct agonist.

4.3.4 Preliminary Examination of Mutants' effect on the TEVC

Function Study Besides the functional study of P2X3/X5 TM2 chimera and T330K described above, the remaining mutants, mostly focusing on single charge-reversal or polarity-altering variants (E11K, E46K, R52E, and VT54), were systematically investigated. MD simulations suggested that those mutants might play roles in ion permeation of the P2X3 channels. E11K was highlighted as a critical determinant of anion selectivity, while E46K was suggested to enhance extracellular cation attraction. R52E and VT54 were predicted to play essential roles in ion permeation, requiring further experimental validation.

After the RNA synthesis, the mutants were injected into oocytes with a 1:10 dilution of 41nL. The P2X3/X5 TM2 chimera and T330K were used in parallel for better comparison between different modifications on TM2, and so was the control of the triple mutant. The TM2 chimera and the T330K (heteromer) were injected without any dilution due to an expected reduced function, while the triple mutant was diluted by 1:20 as usual. The injection of hP2X2 (1:200 dilution) and K14C mutant (undiluted) was included in parts of the experiments. After 1-2 days of incubation, the oocytes were tested by TEVC, using 10 μ M ATP for activation. The protocols, as a single activation, were as described previously.

None of the constructs demonstrated altered kinetics compared to the non-desensitizing

triple mutant when tested in the Ori^- . The function test results are shown in the left panel of Figure ?? (Appendix).

Compared to the control, all mutants, with the exception of the E46K, showed a reduced function as compared to the triple mutant. While the triple mutant has an overall current amplitude of -5678 ± 530.9 nA, the TM2 chimera, as reported before, remained half of that of 2835 ± 305.6 nA. The R52E and the T54V functioned similarly, with currents of -1692 ± 232.2 nA and -1472 ± 396.9 nA in respectively. The T330K expressed in heteromers with the K63A-triple mutant was similar to the chimera, with a response of -2773 ± 411.4 nA. The E11K appeared to be affected the most, with currents of -558.2 ± 77.23 nA. Multiple comparisons detected significant differences between the control and every other construct ($P < 0.001$) except for the E46K.

Preliminary Establishment of TEVC Protocol After confirming a measurable current, I/V curve measurements were performed. The following specific improvements on the methods were assured:

First, the activation time of the channels was shortened to 2 seconds to minimize dramatic ion accumulation resulting from prolonged pore opening;

Secondly, measurements were conducted on the same cells under different solutions rather than relying on comparisons between separate groups of cells. This approach aimed to directly compare results within identical cellular environments, thereby reducing the variance caused by individual differences, such as variations in channel expression levels, intracellular ion concentrations, or membrane properties, which potentially buried changes induced by mutants. By using paired measurements on the same cells, it was possible to isolate the effects of external solutions with greater precision, enhancing the reliability of the data.

Those procedures aimed to decrease the disruption of the locally concentrated ions while establishing a robust and sensitive framework for evaluating changes in ion permeability. In particular, the ability to perform solution exchange within the same cell helps minimize variability caused by differences in intracellular environments or expression levels across oocytes. As a result, subsequent experiments using TEVC could be carried out more reliably.

The principle of these TEVC experiments was to replace the Na^+ or the Cl^- with impermeable organic molecules, either with Choline⁺ or gluconate⁻ respectively. The first attempt was to compare the reversal potentials measured from the constructs in normal Ori⁻ and modified extracellular solutions.

The measuring protocol was set up to test different ion environments within the same oocytes. The right panel in Figure ?? (Appendix) describes the protocol schematically. This approach involved measuring the same oocytes twice under controlled conditions to reduce variance and improve the reliability of the results. The detailed steps of the protocol are as follows:

Baseline Measurement (Baseline 1): The first I/V ramping measurement was performed before channel activation to establish the baseline voltage-current relationship.

Activation Measurement (Activation 1): The channel was activated for 2-3 seconds to ensure proper opening while minimizing ion accumulation around the pore. A second I/V measurement was then recorded in the ATP-induced open state.

Rinse and Reset: ATP was rapidly rinsed off for 2 minutes to recover channels that may have been affected by the rundown effect.

Switch to New Solution(Baseline 2 & Activation 2): The external solution was replaced with desired solutions, after which the protocol was repeated.

The I/V curves were obtained by fitting the current differences between activation and baseline (apo) measurements, from which the reversal potentials (V_{rev1} and V_{rev2}) were calculated. The change in reversal potential, ΔV_{rev} , defined as the difference between V_{rev2} and V_{rev1} , was documented for each construct and used to evaluate changes in ion permeability. This approach was for detecting subtle shifts in ion selectivity, which might otherwise be buried under variability between different oocytes.

Additionally, the ratio of current amplitudes between the first and second activations (I_2/I_1) was recorded. This provided a direct measure of the change in ion flow under different external environments.

As described in **Chapter 3, Section 3.3.1**, the assessment of I_2/I_1 and ΔV_{rev} is to evaluate the change in ion permeation from two perspectives: I_2/I_1 was used to

evaluate how sensitively the current responds to changes in external ion concentrations under the same voltage. A larger I_2/I_1 indicates that current decreases faster as the concentration gradient decreases, suggesting lower diffusion resistance between the ion and the pore. Conversely, a smaller I_2/I_1 indicates that the current is less responsive to concentration changes, reflecting a buildup of diffusion resistance. ΔV_{rev} was used to assess how stable the pore's ion preference remains when external ion concentrations are altered. A smaller ΔV_{rev} indicates that the pore maintains its preference for a particular ion even when its external gradient is reduced; a larger ΔV_{rev} suggests that the pore's selectivity is more easily shifted by concentration changes.

Accumulative Effect of E11K and TM2 Chimera Extreme experimental conditions were used initially with the modified TEVC protocol, with either Na^+ or Cl^- being completely swapped by choline or gluconate. The background buffer was 90mM Na-gluconate-based Ori^- (90 Na^+ /0 Cl^-), and the second buffer was 90mM Choline-Cl (0 Na^+ /90 Cl^-). I_2/I_1 and ΔV_{rev} values of each construct in these experiments were therefore referred to as how current or V_{rev} obtained in 90mM Choline-Cl Ori^- changes in relative to the values in 90mM Na-gluconate Ori^- ,

To test the sensitivity of the TEVC protocol, a double mutant combining the TM2 chimera and E11K was selected, as these two constructs would theoretically exhibit the most distinct ion permeation profiles compared to the triple mutant. Their combination was expected to produce a cumulative effect on ion selectivity and conductance properties. This preliminary screening aimed to determine whether such large differences could be detected despite the remaining limitations of the early TEVC protocol.

The double mutant, E11K-TM2 chimera-triple mutant, referred to as 11K-X3/X5 TM2 chimera, was prepared. After the RNA synthesis, it was injected undiluted with a volume of 41nL, alongside the triple mutant, as well as P2X3/X5 TM2 chimera and E11K as controls. The latter were injected as previously described. The oocytes were examined on TEVC after incubation.

Data points obtained from different Ori^- of all constructs were analyzed comprehensively via two-way ANOVA with a mixed effect model ($\alpha = 0.05$), instead of being compared one-to-one. The experiment results are presented in Figure ??,

and the detailed experimental results are listed in Tables A.3.1.

The statistical results regarding I₂/I₁ and the ΔV_{rev} values are shown in Table A.3.2, which summarizes the overall results. Analysis shows that significant differences among all constructs were detected both in I₂/I₁ and ΔV_{rev} values. Tables A.3.4 and A.3.5 are post-hoc Sidak's multiple comparisons test between every two constructs. Comparing the I₂/I₁ values, it was observed that significant differences were detected between the triple mutant and single mutant of E11K or another single (component) mutant, P2X3/X5 TM2 chimera. The E11K-TM2 chimera showed a similar current ratio in comparison with any other construct. When comparing the ΔV_{rev} values, the E11K-TM2 chimera was significantly different from both of the single-component constructs but not the triple mutant. Taken together, the E11K-TM2 chimera exhibits a compromised instead of an accumulative effect from E11K and P2X3/X5 TM2 chimera regarding the ion permeation profiles.

4.3.5 Characterizing Single Mutations on the Ion Permeation Pathway

In the above experiments, the TEVC method was established, and a signal of significant ion permeation behavior was detected as I₂/I₁ and ΔV_{rev} values. However, as previously noted in the **Chapter 3, Section 3.3.1**, when the original 90mM Na⁺/Cl⁻ was replaced by more than 50% by the impemeable organic molecules, unpredictable behaviors (i.e., baseline drift and increased noise) were consistently observed in the recordings, which were likely due to excessive amounts of impermeant ions in the solutions. baseline drift and increased noise. Besides, complete substitution of Cl⁻ caused a false signal caused by liquid junction potentials. Therefore, instead of complete substitution, a stepwise reduction of Na⁺ or Cl⁻ from 90 mM to 70 or 50 mM was used in the following experiments.

Besides, considering the rundown effect of P2X3Rs, which might affect the evaluation of I₂/I₁ signal, a control group was established for each construct to document potential intrinsic shifts in I₂/I₁ due to repetitive measurements. In this group, the second solution remained the standard 90 mM NaCl (Ori⁻), ensuring that ΔV_{rev} primarily reflected the effects of solution changes rather than measurement artifacts.

Varied $[Cl^-]$ Changes I2/I1 and ΔV_{rev}

The detailed results are presented in Tables 1-4 and Figure A.3.2; two-way ANOVA with a mixed effect model ($\alpha = 0.05$) was used to analyze the overall comparison between the triple mutant and every other construct.

Alterations of ion concentration and constructs were used as two independent parameters, and three fixed effects were tested: chloride concentration ($[Cl^-]$), construct type (*Construct*), and their interaction ($[Cl^-] \times \text{Construct}$). Effect $[Cl^-]$ suggested for constructs react to the change of extracellular chloride concentration; a significant difference suggests that, taking the constructs in comparison together, changes in I2/I1 or V_{rev} were observed in response to alterations of $[Cl^-]$. Significant differences of effect *Construct*, on the other hand, indicate a general difference was observed between constructs, which is stable across chloride levels; if a difference is detected for $[Cl^-] \times \text{Construct}$, different extracellular chloride concentrations might induce different construct-dependent effects. Each oocyte is treated as a random replicate to absorb oocyte-to-oocyte variability.

When comparing the change in V_{rev} , the hP2X2 and K14C were included explicitly. hP2X2 served as an additional control due to its well-characterized Cl^- permeability (Samways et al., 2014), allowing direct comparison with P2X3 constructs. In contrast, K14C, as the only functional K14 mutant, provided an opportunity to examine if that lysine residue, though not directly in the ion pathway, could influence Cl^- selectivity. However, these two constructs were excluded from the I2/I1 amplitude ratio comparison. Although I2/I1 was not used to indicate recovery here, it was still calculated from two consecutive activation responses, only under different conditions. Since hP2X2 shows no rundown and K14C displays extremely low recovery, as shown previously, both differ significantly from the other constructs with moderate recovery ratios resembling the triple mutants. To avoid introducing bias from their distinct rundown behaviors, they were excluded from this comparison.

Tables A.3.6, A.3.7 (Appendix), and Figure A.3.2 show I2/I1 values for each construct when Cl^- concentration was changed. Table A.3.7 (Appendix), shows that construct differences in diffusion resistances occur when Cl^- passes through the pores were detected between the triple mutant and every other tested constructs except for T54V, with P values of 0.0383 (vs. E46K), 0.0259 (vs. R52E), <0.0001 (Vs. P2X3/X5 TM2 chimera, T330K heteromers and E11K). This suggested that

all constructs but T54V changes have impacts on the resistance that Cl^- ions have to overcome when travelling through the pores, while P2X3/X5 TM2 chimera, T330K heteromers, and E11K might have more dramatic effects; however, comparing results from the triple mutant vs. E46K, R52E, and T54V, changes in Cl^- extracellular concentration are only significant for triple mutant vs. T54V group. In other words, while E46K and R52E change the Cl^- ion diffusion pattern, T54V introduces differences in the concentration dependency of the Cl^- permeation.

Tables A.3.8 and A.3.9 (Appendix) show the data and analysis of Vrev shifts of the constructs upon the changed Cl^- concentration. Example I/V curves could be seen in Figure A.3.2. Although E46K/R52E show different Cl^- ion diffusion patterns, statistical results suggest that they do not change the overall chloride preferences, which agrees with the results where the changes in Cl^- concentration are not significant for either of them when compared with the triple mutant. Similarly, T54V has no impact on Cl^- preference either. P2X3/X5 TM2 chimera, though with a different Cl^- diffusion pattern, does not show any changed Cl^- selectivity. hP2X2Rs shows a difference in Cl^- selectivity, with P value = 0.0111, while $P < 0.0001$ was detected when comparisons between T330K heteromers and E11K, suggesting an increase in Cl^- selectivity of those constructs: Vrevs of them shifted less during the changing of extracellular chloride concentration, seen in Figure A.3.2.

Varied $[\text{Na}^+]$ Changes I₂/I₁ and ΔV_{rev}

Unlike highly varied intracellular Cl^- concentration, intracellular Na^+ is negligible. Therefore, the decrease in extracellular sodium concentration constantly resulted in decreased concentration gradients.

Table A.3.10 and A.3.11 (Appendix) show I₂/I₁ values of the constructs under the changed Na^+ concentrations. Similar to the I₂/I₁ values during the chloride experiments, the I₂/I₁ of Na^+ current is an analog of how fast the resistance that sodium ions encounter during permeation changes, along with a decreased driving force of the concentration gradient. The Two-way ANOVA detected significant differences except for comparisons between the triple mutant versus E46K, E11K, R52E, and T330K heteromers both show dramatic sodium permeation, with P values < 0.0001 , 0.0005 , and < 0.0001 , respectively, revealing that those constructs differ from the triple mutants regarding sodium ions permeating resistance within the channel. T54V and P2X3/X5 TM2 chimera showed not only different diffusion

resistance, with P values in factor *construct* of 0.0002 and <0.0001, but also differences in *factor [Na⁺] x construct*, with P value of 0.008 and 0.0259. Those results together suggest that the diffusion of Na⁺ in T54V or P2X3/X5 TM2 chimera is significantly different from the triple mutant.

Table A.3.12 and A.3.13 (Appendix) show V_{rev} shifts from the constructs under different concentrations of Na⁺, with examples of the shift shown in A.3.3. Two-way ANOVA analysis detected no differences in absolute Na⁺ preference between the triple mutant versus R52E or P2X3/X5 TM2 chimera; however, P2X3/X5 TM2 chimera still presents overall changes in sodium permeation compared to the triple mutant, with a P value of 0.0039. E46K and T54V both showed differences in factor *construct*, but not the *[Na⁺] x construct*, suggesting that those two constructs have altered sodium selectivity, but not differ from the triple mutant in how to respond to changed Na⁺ concentration. T330K heteromers and E11K, on the other hand, differ from the triple mutant in the factor *construct*, both P < 0.0001, as well as *[Na⁺] x construct*, with P = 0.0002 and 0.0011, respectively. exhibiting significant behavior of those two constructs from the triple mutant.

5. Discussion

5.1 The Cytoplasmic Cap Network

The first characterization of the cap was to determine how the intracellular termini plays various roles in the construction of the cap.

5.1.1 Participation of N-/C- Termini

The Role of N- or C- Terminus To investigate the distinct roles of the N- and C-termini in the assembly and functionality of the cytoplasmic cap, a series of RNA co-expression experiments were conducted, using varying ratios of the WT, triple mutant, and mutants based on these two constructs.

In the first part of the experiments, mixed RNA from wild-type hP2X3 and the triple mutant were co-expressed in varying ratios (2:1, 1:1, 1:2). The enormous amplitudes from the activation of triple mutant homomers could mask the response of the $N^{WT}/N^M/N^M$ and $N^{WT}/N^M N^M/N^M$ receptors. To minimize this masking effect, the amount of triple mutant RNA injected was reduced. The wild-type homomers, due to their fast onset but slow recovery of desensitization and thus relatively small amplitudes did not significantly interfere with the overall channel opening kinetics.

The 2:1 group (WT to triple mutant ratio) showed the most noticeable reduction in this masking effect, as the overall current was large enough to cover the contribution from the WT component. Compared to the triple mutant, a change in

kinetics was observed, indicating that the contribution from heteromers could not be ignored. This suggests that despite the remaining interference from the triple mutant, the heteromers could still be successfully detected. Overall, the results from each group show a clear trend of decrease in desensitization rate with increasing proportions of the triple mutant RNA. These observations suggest that as the proportion of triple mutant RNA increases, leading to more mutant subunits in heteromeric channels, the desensitization becomes slower. This indicates that the incorporation of the mutated N-terminus into heteromers alters the desensitization kinetics.

For precisely determining the changes in the desensitization phenotype caused by different termini assemblies during trimerization, it was preferable to rely on signals from specific heteromeric channels. The introduction of the K63A mutation effectively removes functional responses of homomeric hP2X3 triple mutant channels from subsequent experiments. Meanwhile, truncation of either the N- or C-terminus of the WT protomers resulted in non-functional homomeric WT channels, further eliminating any possible interferences from the signal of WT homomers. These attempts of interference elimination ensured that only heteromers produced measurable currents in the following experiments.

The initial experiments involved co-injecting oocytes with RNA combinations of ΔN -WT and N^M -K63A. None of these oocytes responded to 10 μ M ATP, raising questions about the underlying cause. A key consideration was whether these mutations directly interfered with trimer assembly. However, it is unlikely that either ΔN -WT or N^M -K63A disrupts trimerization, as neither mutation has been shown to engage in the trimerization process of P2X3 channels extensively. previous studies indicate that K63A does not affect subunit assembly (Hausmann et al., 2012), and there is no direct evidence linking the cytoplasmic N-terminal domain to trimerization (Murrell-Lagnado & Qureshi, 2008). Thus, the lack of ATP response is likely due to other mechanisms.

If trimerization had occurred and heteromeric receptors were present, two possible configurations would be $\Delta N/\Delta N/K63A$ or $\Delta N/K63A/K63A$. As described in **Section 4.1**, receptors containing two K63A-mutated ATP-binding pockets (N^M -K63A protomers) were reported to be non-functional (Stelmashenko et al., 2012), explaining why the $\Delta N/K63A/K63A$ heteromer could not generate a response. This leaves the $\Delta N/\Delta N/K63A$ configuration, which also appeared non-functional, leading to the conclusion that the absence of two N-termini within a trimer si-

lences the receptors under TEVC conditions. In other words, the loss of two or three N-termini severely disrupts the formation of the cytoplasmic cap, preventing receptor function. This conclusion aligns with MD simulation results, which predict a closed pore when the N-terminus is absent.

In the experiments where WT and K63A-triple mutant RNA were co-injected, different channel functions were observed. The possible receptor assemblies in this case include WT homomers, WT/WT/K63A, WT/K63A/K63A, and K63A homomers. As previously discussed, the last two configurations were non-functional despite their possible existence. Knowing this, the remaining functional assemblies can be classified based on their N-terminus composition as $N^{WT}/N^{WT}/N^{WT}$ and $N^{WT}/N^{WT}/N^M$, which N^M represents the mutated N-terminus introduced by the K63A mutation.

The co-injection groups, with WT: N^M - K63A ratios of 4:1 and 1:1, exhibited significantly higher signal amplitudes compared to the WT-only group, where only $N^{WT}/N^{WT}/N^{WT}$ existed. These observations suggest that $N^{WT}/N^{WT}/N^M$ heteromers may enhance channel function primarily by stabilizing the open state through the mutated N-termini, a conclusion supported by the altered kinetics. Since K63A substantially decreases ATP affinity, it is unlikely to account for this improved performance. Notably, the K63A mutation does not impede the assembly and surface expression of the receptor subunits (Bodnar et al., 2011). Additionally, a recent study suggested that mutations in the N-terminal region do not appear to interfere with subunit assembly or membrane trafficking (Lin et al., 2024). Therefore, while other possibilities cannot be entirely excluded, the stabilizing effect of N^M is the most likely explanation for the increased current. Although no significant differences were observed between WT and WT: K63A = 1:4 group, the latter still responded to ATP with bigger amplitudes, indicating a minor enhancement in channel activity.

Repeated activations led to "rundown" effects, manifesting as decreased amplitudes of the responses in the WT groups due to the accumulation of desensitized receptors. The same phenomena were also observed in WT: K63A = 4:1 and 1:1 groups, as the amplitudes during the 2nd and 3rd activations were decreasing in both groups. Noticeably, the 4:1 group showed a more severe response suppression compared to the 1:1 group. The 1:4 group, conversely, resembled the triple mutant rather than the WT, maintaining a consistently similar response magnitude regardless of repetitive ATP applications.

Throughout three activations, both the sole WT and the WT: K63A=4:1 groups exhibited progressive desensitization, indicating that a higher fraction of WT subunits drives stronger desensitization. Meanwhile, mixtures with fewer WT subunits (such as 1:4) displayed weaker desensitization, consistent with lower level of rundown. As an intermediate, the 1:1 group initially showed moderate desensitization (between 1:4 and 4:1) but gradually shifted toward the 1:4 behavior upon repeated activation. This shift may reflect preferential suppression of WT subunits by rundown, reducing their influence and thereby making the channel behave less like the WT channels. Specifically, the 1:4 group maintained a smaller yet stable response, likely driven by two opposing effects: a more stabilized cap-enhancing function vs. the reduced ATP-binding pockets diminishing channel conductivity. Together, these forces produce the 1:4 group's intermediate phenotype.

1 μ M ATP was also tested on the WT+K63A groups. Although statistical analysis was not possible due to the small sample size, the recording traces suggested that the differences in desensitization kinetics, which were previously observed among groups with different RNA ratio mixtures, were no longer present. This suggests that either the $N^{WT}/N^{WT}/N^M$ and WT homomers behave similarly under 1 μ M ATP or one of them was less responsive at this concentration.

Given that only two bound ATP molecules are required to open the channel (Chataigneau et al., 2013), it is expected that the $N^{WT}/N^{WT}/N^M$ did not lose the high sensitivity to ATP. However, due to the cooperative nature of ATP binding (Ding & Sachs, 1999), the absence of the third binding site may result in a receptor that, while still responsive, exhibits reduced maximal current amplitude.

The similar rundown effect observed across all groups challenges the notion that both receptor types behave identically at 1 μ M ATP, as previous studies have shown that WT receptor desensitization is highly sensitive to nanomolar ATP levels (Pratt et al., 2005). Therefore, it's plausible that at this lower ATP concentration, the $N^{WT}/N^{WT}/N^M$ heteromer phenotype becomes more pronounced, as WT homomers may respond with even smaller magnitudes. The heteromers exhibit slower desensitization compared to WT receptors, providing evidence that incorporating a single mutated N-terminus can significantly stabilize the cytoplasmic cap.

The Δ C-WT + N^M -K63A groups revealed additional insights and provided further support. Neither Δ C-WT nor K63A solely injection groups could be activated by 10 μ M ATP, suggesting that functional heteromers are assembled by two con-

structs that cannot form functional homomers by themselves, which compensate for each other's deficiencies when expressed together. Furthermore, heteromers containing two K63A-incorporated subunits are supposedly not activated by 10 μ M

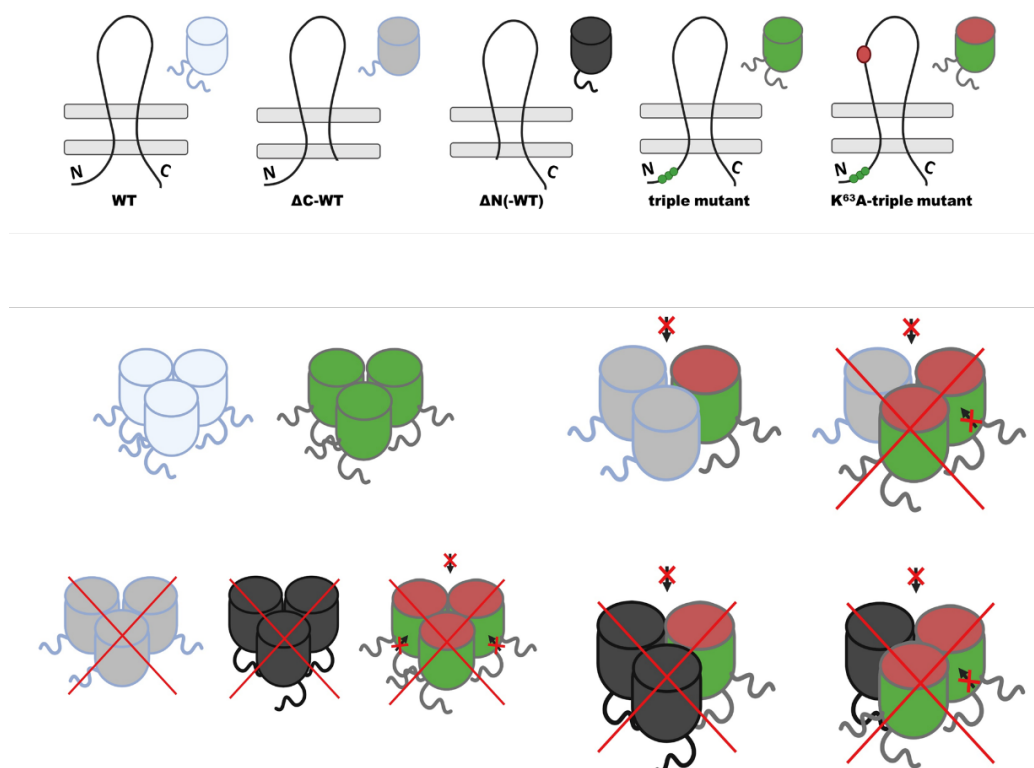


Figure 5.1.1: Schematic representation on incorporation of Δ N- / Δ C-WT and K63A-triple mutant in trimeric assemblies. The details of each construct (resulting in the indicated protomer) are specified in the upper panel. Based on the WT, Δ C-WT lacks the C-terminus, and Δ N-WT is deficient of the N-terminus (notice that without the N-terminus, WT and triple mutant are identical and thus are not distinguished, here it's referred to as Δ N-WT, as it was generated from the wild type). The three mutations of the triple mutant (T13P, S15V, V16I) on the N-terminus are colored in green, and the ATP-binding deficient K63A substitution is colored in red. In the lower panels, all possible assemblies are listed. The ones that are crossed are non-functional, according to literature and /or from TEVC observation; detailed discussion is in the text. The homomers of WT and triple mutant (lower left panel) are functional, but when lacking three N or C-termini or having no non-mutated ATP binding pockets, the function is disrupted. Heteromers are described in the lower-right panel. Any heteromers bearing more than one K63A protomer should not be activated by 10 μ M ATP. The K63A with two Δ N protomers appeared to be non-functional as seen in TEVC. Δ C/ Δ C/K63A should be retained as the functional heteromers here, which contributed to the signal also observed in TEVC(Created in <https://BioRender.com>)

ATP, as mentioned above, the observed TEVC signals must originate from the $\Delta C/\Delta C/K63A$ heteromer. This indicates that a single C-terminus is sufficient to support the formation of the cytoplasmic cap and enable the activation. This finding aligns with simulation results showing that the pore remains open under conditions where both termini are intact, as well as when one or two C-termini are removed.

Additionally, no significant changes in desensitization were observed in the 4:1 and 1:1 ΔC -WT + K63A groups compared to the WT + K63A groups, where excessive WT participation led to pronounced desensitizing and rundown effects. Meanwhile, the 1:4 ΔC -WT + K63A group, where the K63A-triple mutant was in excess, demonstrates poor performance, which may be caused by the decreased expression level of C-termini truncated WT subunits, thus also a reduced amount of $\Delta C/K63A/K63A$ heteromers, leading to a decreased formation of functional $\Delta C/K63A/K63A$ heteromers. When K63A was the less abundant component, this issue was largely resolved. Both observations aligned with what has been previously reported that the C-terminus facilitates the expression (Chaumont et al., 2004). Consequently, the K63A protomers with an intact C-terminus were expressed more proficiently, ensuring that, even with a reduced RNA injection ratio, sufficient K63A-expressed proteins were available to form heteromers with ΔC -WT, ensuring functional receptor assembly.

Figure 5.1.1 summarizes the subunit assembly in the above experiments, and functional/dysfunctional trimers are specified. In conclusion, these experiments provided evidence that 1). deficiency of the N-terminus prohibits the construction of the cytoplasmic cap, thus preventing the channel from normal function; 2). On the contrary, even one C-terminus is sufficient to support functional receptors; 3). The incorporation of only one cap-stabilizing mutated N-terminus into a trimer is sufficient to enhance the stability of the cap and significantly decelerate the overall desensitization kinetics of the P2X3Rs.

5.1.2 Cap Structure Supported by Residues and Residue-interaction

Later, the specific residues on the cytoplasmic termini were studied, aiming to outline the details of how the cap is constructed and to determine the role of specific

residues on cap stability.

Ionic Interaction The salt bridge study began by examining the relationship between residues D340 and K357. The two residues are conserved in human isoforms of the P2XRs, and a potential salt bridge between D340 and K357 has been reported earlier (Oken et al., 2022). To investigate their functional significance, the aspartic acid at 340 was mutated to both alanine (D340A) and lysine (D340K). Both mutants yielded poor functioning performance with responses reduced to barely 1% of the triple mutant.

The dramatic impairment of function lays out the crucial importance of the native aspartic acid. MD simulations have highlighted the importance of D340 and its interaction with K357 in maintaining the integrity of the cytoplasmic cap. Additionally, D340 is located within the cytoplasmic fenestrations (Mansoor et al., 2016) suggesting its potential interaction with ion flow due to its charged side chain. Experiment data here showed that substituting the negatively charged D340 with a neutral alanine or a positively charged lysine disrupts the facilitation of cation passage through the channel, manifesting as a current reduction. This disruption could be due to destabilization of the open state or altered electrostatic interactions within the pore, leading to decreased current. Thus, the presence of a negatively charged residue at this position appears crucial for both channel stability and efficient ion conduction.

Substitution of its supposed counterpart K357, which is also located on ion fenestration (Mansoor et al., 2016), with aspartic acid (K357D) resulted in a dramatic decrease in function while neutralizing K357 (K357A) caused less extensive functional damage, yet the currents were still significantly smaller than the ones of the K357 channels. This variability indicates that the positive charge at residue 357 is crucial for function. This is further confirmed by mutating K357 to arginine (K357R) and glutamic acid (K357E). The charge-reversed K357 nearly abolished channel function, while a replacement by an arginine, where the side chain is changed structurally but the positive charge remains, maintains the current amplitudes comparable to the triple mutant. These results indicate that the positive charge at position 357 enhances channel conductivity primarily by stabilizing the cytoplasmic cap through ionic interactions, as suggested by MD simulations. Given the channel's strong cation selectivity, it is unlikely that this lysine residue directly facilitates ion permeation—an alternative mechanism by which charged

residues might typically enhance ion flow. Instead, the observed functional impairment upon mutation highlights its structural role in maintaining protein integrity.

Consistent with these observations, the charge-neutralized mutation K357A results in less severe functional impairment compared to the charge-reversed mutation K357D. This clear trend—where function progressively declines from positive (K, R) to neutral (A), and finally negative (D, E)—further supports that electrostatic interactions strongly contribute to the structural integrity provided by K357.

Interestingly, despite severe functional impairment, the K357D mutation exhibited desensitization kinetics similar to the triple mutant, whereas K357A showed notably faster desensitization and a more unstable open state. Given that both lysine and aspartic acid possess relatively large side chains, this observation suggests that beyond charge, side-chain size and flexibility at position 357 might also contribute to structural stabilization. However, the distinct kinetics observed with K357A indicate that structural features alone cannot fully compensate for the absence of appropriate electrostatic interactions. Thus, the residue's electrostatic properties appear dominant, but its non-electrostatic structural attributes may also influence the receptor's open-state stability, as has been suggested (Schwehm et al., 2003).

The double mutant D340K, K357D which was expected to restore the ionic residue interaction by compensating for the charge reversal by charge swapping, exhibited an even greater loss of function. Despite the functional loss in terms of current amplitudes, the desensitization kinetics of the double mutant were re-established to resemble the triple mutant. This suggests that the ionic interaction between D340 and K357 is crucial for the slow desensitization kinetics observed in the triple mutant and thus stability of the cytoplasmic cap, but it is not solely responsible for the observed functional impairments, as interactions between local protein structural and individual residues might play a crucial role in protein overall function, as discussed above. In support of this, positive results of interaction suggestion from the double-mutant cycle further evidence the interaction between D340 and K357, which significantly contributes to the stability of the open state of the receptor.

Similar investigations were done on E359 and its suggested interaction partners of K14, K356, and K357. The mutation K14E showed severely damaged channel function, suggesting that K14 is highly involved in the gating processes. Despite the loss of function, the glutamic acid at 14 does not impair the desensitization ki-

netics significantly. This site appears to be highly mutation-sensitive, where K14 is structurally and electrostatically optimized for gating, and even minor modifications (such as K to Q or E) disrupt function. In a previous study, it was suggested that the aligned residues TXK (X representing uncertain residue), from position 12 to 14, form a critical motif in P2X3 receptors, a most likely target for phosphorylation, in which, neither T12E nor K14Q resulted in measurable current (Paukert et al., 2001). Similarly, the T12A mutation also led to poor functioning, which will be discussed in a subsequent study.

Interestingly, a cysteine substitution at K14 does not significantly affect function, as current amplitudes remain largely unchanged. TEVC recordings of K14C revealed extensive rundown between the first and second activations, with a small second current contrasting the large, fast-desensitizing initial signal. Since Position 14 is located in the cytoplasm, where the reductive environment does not favor disulfide bond formation, the effect of cysteine substitution is likely due to its smaller side chain rather than specific chemical interactions. Although K14C reduces the current to a lower level, its rapid desensitization and pronounced rundown, resembling WT behavior, indicate that it significantly disrupts open-state stability. This reinforces the idea that K14 is crucial for maintaining sustained activation.

Size reduction may introduce compensatory flexibility that minor modifications, such as K to Q, do not provide. The suggestion would align with previous conclusions that small amino acids such as alanine or cysteine were found to be considerably less disruptive, indicating that smaller or flexible side chains can sometimes be better tolerated than bulkier changes, as they introduce adaptability that helps accommodate local conformational changes (Gray et al., 2017). While K14 is essential, the surrounding region appears highly sensitive to mutations yet retains a degree of flexibility, permitting certain small substitutions, like K14C, to preserve gating function despite the loss of charge. The balance between mutation sensitivity and structural adaptability highlights the dynamics of this gating region.

The mutations on 356K surprisingly didn't impair the function, regardless of substitution with a bulkier side chain (356R) or reversed charge (356E). The results reveal that the 356K might not directly interfere with the pore opening, gating or the fenestration pathway with its charged side chain. When mutated to arginine (356R), the receptor remained slow desensitization, whereas reversing the charge with 356E affected desensitization, indicating that the positive charge at this posi-

tion may be important for cap stability.

In contrast, substituting lysine with glutamic acid at residue E359 (E359K) significantly impaired both receptor function and cap stability. As there is a lack of comparisons among different substitutions, it is challenging to determine precisely how E359 facilitates receptor function. When combining E359K with other mutants located in positions suggested to be interacting with E359, more information is obtained.

The simultaneous charge-reversing and thus charge swapping between K14 and E359 suppresses receptor function further, without restoring desensitization kinetics, providing weak evidence for their direct ionic interaction. The combination of K356 and E359K resembles both of the single mutants, suggesting that their interaction does not exhibit additive effects. However, the 357E,359K double mutant successfully restored desensitization kinetics through charge swapping, although receptor function remained impaired. The restoring of the desensitizing behaviors suggests that interactions between these residues contribute to cap stability. Double-mutant cycle analysis is further evident that the salt bridge formations between K356-E359 and K357-E359 are critical for receptor stability, whereas the K14-E359 interaction does not appear to be significant.

Single and double mutants were also generated to probe the role of the hydrophobic network in maintaining the structure of the cytoplasmic cap. This network is especially important around the area with hydrophobic residues oriented towards the lipid environment and hydrophilic residues lining the pore (Nicke et al., 1998a). The hydrophobic interactions in this area are critical for the receptors to maintain the structures and function normally.

The phenylalanine at position 8 and tryptophan at 21 within the N-terminus was suggested by MD simulations to form direct hydrophobic interactions crucial to the intracellular protein domains and the cytoplasmic cap. Single and double mutants, all to alanine (F8A and W21A) were generated accordingly. It was observed by TEVC analysis that the alanine substitution of the alanine substitution on the W21 seems to be similar to the WT, with neither function nor desensitization kinetics affected by substituting W with A. This outcome was unexpected, given that the tryptophan possesses a large and complex aromatic side chain capable of extensive hydrophobic interactions with surrounding residues. The fact that the smaller, less hydrophobic alanine can maintain the structural stability of the

cytoplasmic cap suggests that even modest hydrophobic side chains are sufficient to stabilize the local environment.

In contrast, F8A resulted in an increased function and accelerated desensitization. The phenylalanine resembles tryptophane, possessing a large aromatic side chain that contributes to hydrophobic interactions. The observation of the faster desensitization after alanine substitution indicates that the side chain of F8, being larger and more hydrophobic than alanine, plays a more critical role in maintaining the structural stability of the cytoplasmic cap. However, the flexibility introduced by alanine could allow a high level of freedom for local conformation adoption, which potentially leads to a more conducive open pore, which might explain why F8A increased the current amplitudes.

Furthermore, the double mutant 8A,21A exhibited enhanced performance with desensitization accelerating beyond that of 8A alone. Double-mutant cycle analysis between F8A and W21A confirms that the two single alanine substitutions do not add up to the double mutant, leading to a conclusion that both residues are functionally coupled and a hydrophobic interaction exists between residues F8 and W21. This interaction is essential for the stabilization of the cytoplasmic cap, as disrupting both hydrophobic interactions simultaneously results in distinct functional outcomes.

To further explore the hydrophobic network, the single mutations were performed on T12 and V17 located in the N-terminus, and F358 of the C-terminus. As mentioned above, the T12 is highly conserved and was suspected to be involved in constructing a critical motif on the N-termini. Substituting the T12 with an A was observed to indeed significantly weaken the function while accelerating the desensitization. Similarly, aspartic acid mutation at position 17 (V17D) resulted in comparable functional impairments and accelerated desensitization. It suggests that the negatively charged D can be a great disturbance to a local hydrophobic network, thereby disrupting receptor stability, especially near the membrane.

The F358A resembles the F8A on the accelerated desensitization kinetics, without providing any functional improvement. This similarity suggests that replacing phenylalanine with alanine at both positions reduces structural stability due to the smaller, less hydrophobic side chain of alanine. However, unlike F8A, the F358A mutation does not enhance channel current amplitudes. This difference indicates the distinct local environments of the F8 and F358. At position 358, the same

alanine substitution might not have induced significant conformational changes that affect receptor function, compared to the F8A which enhances the flexibility and increases channel conductance. Therefore, F8A is slightly favored in terms of functional enhancement, while F358A appears to have a negligible impact on function. This outcome highlights the importance of the local structural context in determining how hydrophobic residue substitutions influence receptor activity and stability.

5.1.3 Movements of the Intracellular Termini during Channel Gating

It was one of the key interests in understanding how the termini moved during the transition from the apo state to the open state of the channel. MD simulations indicated that certain residues on the termini experience significant changes in their relative distances to each other during activation. Residue pairs E11-V15, V15-F358, and V18-V361 were selected as reporter sites for conformational changes during the activation. These residues were mutated into cysteines, and with a crosslinker with appropriate length, it was expected to be able to capture and crosslink the approaching of the residues.

All single cysteine substitutions show similar levels of current amplitudes compared to the triple mutant on TEVC, with only slight reductions on E11C, V15C, and V18C. Regarding desensitization kinetics, mutants V18C and F358C showed mildly accelerated desensitization, while desensitization kinetics of the E11C, V15C, and V361C mutants remained unaffected.

E11 is both polar and charged, yet substituting it with cysteine does not affect desensitization, suggesting that it does not significantly contribute to local electrostatic interactions or structural constraints that maintain the cytoplasmic cap's integrity. Accordingly, the unchanged desensitization of E11C indicates that E11 is unlikely to play an active role in stabilizing the cytoplasmic cap. However, the mild reduction in current amplitude suggests that E11 contributes to ion conductance. As E11 has been reported to participate in cytoplasmic fenestration of the channel (Tam et al., 2023), its substitution may slightly alter ion flow without affecting gating dynamics.

Valine at position 15 is critical for stabilizing the cytoplasmic cap, as proven by the previously reported S15V-hP2X3R and the triple mutant (13P,15V,16I-hP2X3R)'s performance (Hausmann et al., 2014). Since valine stabilizes the cytoplasmic cap, its substitution with cysteine is expected to destabilize, leading to faster desensitization which may also manifest as a reduction in maximal current, as was seen during the TEVC measurement.

Molecular dynamics simulations have suggested that V18 plays a significant role in constructing hydrophobic interactions essential for channel function. Consequently, mutating V18 to cysteine disrupts these interactions, leading to loss of channel function, which is consistent with the observations. Similarly, V361 was relatively more sensitive to the cysteine substitution, manifesting as moderate acceleration of the desensitization, suggesting that its hydrophobicity is crucial for maintaining structural stability as well.

As mentioned in the **Results** chapter, crosslinking experiments could not be successfully conducted with readouts of the functions, using current TEVC protocols. Therefore, biochemical experiments were performed instead, to analyze whether the proximity of specific residue pairs changes during channel activation. These experiments aimed to detect crosslinking by covalently linked dimers and trimers on SDS-PAGE gels. Given that the triple mutant exhibits a relatively stable open state, the prolonged exposure of residues in close proximity should increase the likelihood of successful crosslinking. A successful capture of these conformational states is reflected in the formation of SDS-resistant dimers and trimers, confirming that the cysteine residues were trapped upon approaching each other during channel activation.

In these experiments, the E11-V15 was ruled out in the early stage, because their proposed interactions are intrasubunit (within the same subunit) and thus would not be detectable by SDS-PAGE because crosslinking does not concatenate adjacent subunits.

For the residue pairs V15C-F358C and V18C-V361C, SDS-PAGE analysis showed that dimers formed regardless of ATP presence, while trimers appeared almost exclusively with ATP. The appearance of trimers in the ATP condition confirms that these double mutants successfully report the movement of the termini during channel activation. This supports the idea that in the open state, these residues move into proximity, enabling trimer formation.

Meanwhile, the fact that the dimer signal was always present suggests that it does not provide a usable readout. However, its exclusive appearance in double mutants, and not in single mutants, confirmed that spontaneous, unspecific dimerization does not occur. Given that crosslinkers react within a tolerance of 3 Å beyond their theoretical distance (Merkley et al., 2014), it is possible that dynamic movement of the termini allows crosslinking to occur even before full activation. The consistently strong dimer signal as well as the weaker and open-state-exclusive trimer signal suggests that trimer formation requires a more precise subunit arrangement and is inherently less favorable. This aligns with previous observations that higher-order oligomers form less frequently in biological systems, as their assembly is constrained by both energetic costs and structural requirements (Danielli et al., 2020). While dynamic movement of the termini and the crosslinker's tolerance range may allow dimers to form even outside the fully open state, trimerization likely depends on a much stricter conformational alignment. This makes the trimer signal naturally weaker.

A key factor limiting trimer formation could be the structural effects of the cysteine mutations. Although the triple mutant stabilizes the open state, the additional double cysteine mutations accelerate the desensitization as shown by the TEVC recording traces, prevent the perfect alignment needed for efficient crosslinking. Since trimer formation requires a precise relative distance between all three monomers, even small deviations from this ideal conformation could reduce crosslinking efficiency. This suggests that, while the experiment successfully reports the movement of the termini, the extent of trimer formation might have been lower than expected due to these structural constraints.

Overall, in these experiments, the purpose was to establish the critical role of the intracellular termini in the structural integrity and functional dynamics of the cytoplasmic cap in hP2X3 receptors. It was found that the absence of N-termini disrupted receptor function, whereas a single remaining C-terminus was sufficient for activation. The cap was stabilized by both ionic interactions and hydrophobic networks, with disruptions in these interactions leading to impaired receptor function and altered desensitization kinetics. Structural rearrangements of key residues during channel gating were observed, indicating that precise spatial organization of the termini is necessary for maintaining receptor activity. These findings demonstrate that the cytoplasmic cap is indispensable for receptor stability and function, governed by specific residue interactions that regulate gating and desensitization dynamics.

5.2 Structural-function Study on the TM2

The following section describes the structural implications of the SCAM results for the TM2 structure and its dynamic changes during gating.

5.2.1 SCAM Study Potentially Outlines TM2 Structural Dynamics

To investigate the ion channel pore formed by TM2 of the P2X3 receptor, cysteine mutations were introduced across the entire TM2 region. TEVC measurements on the cysteine mutants were performed to establish the background of the following study. TEVC confirmed the functionality of the mutations and provided direct observation of any alterations in channel biophysics. These basic assessments ensured that subsequent studies on channel dynamics and access pathways were built upon reliable and functional mutants.

The functional test results demonstrated that the substitutions of cysteine at most positions were not destructive to the channel function. Compared to the control group of the triple mutant, mutants I322C, I323C, S331C, G333C, and V334C exhibited a decreased channel function (reduced current amplitude), while the others behaved similarly to the control (with the exception of D340C). The introduction of cysteine at certain positions leads to a loss of function, suggesting that the corresponding residues play an irreplaceable role in ion conduction and that even a subtle structural change can result in notable effects on the ion channel's functionality, for example, S331C. The change in desensitization behavior of the G333C also raised interest. This will be discussed in a later section where G333 is profiled.

Cadmium was chosen as the cysteine-evaluating reagent. Compared to MTS reagents that are usually used, metal ions such as cadmium or silver have a similar size as biological cations passing through ion channels such as sodium or potassium, which allows the ion to access the entire channel, and the restrictions in channels that limit the biological ions should also apply to the cysteine-reactive ones (Linsdell, 2015b). Another feature that distinguishes the binding profile of metal ions from MTS reagents is that the reaction between cysteines and the ions is expected to have moderate stability. The coordination bond between metal ions and the cys-

teine side chain is more easily disrupted compared to the covalent bond between MTS reagents and the side chain. Previous studies recorded changes in single-channel conductance over channel exposure to Cd^{2+} , suggesting an occasional release of the metal ions from the cysteines that eased the blockage brought by the ion coordination in the pore (Choi et al., 2013). While adding redox reagents such as DTT can break both kinds of interaction, simply removing the metal ions by washing or chelation also disrupts their binding to the cysteine residues (Peris-Díaz et al., 2020).

The binding affinity variants are another key to the interpretation of the result. Although favoring coordination via four cysteines with extremely high affinity, it is reported that Cd^{2+} can still be attached to one cysteine, which requires a much higher concentration of Cd^{2+} . Each additional cysteine coordinating with Cd^{2+} can increase binding affinity by approximately 1000-fold (Linsdell, 2015b). As Tsushima et al. reported, a 50-fold of Cd^{2+} -sensitivity alteration for cysteines at different positions was observed (Tsushima et al., 1997). Besides the accessibility and local environmental features, the spatial relationship between cysteines and side-chain orientation plays a role, which means a transient conformational change and protein flexibility potentially decide on the formation and breaking of effective Cd^{2+} coordination. An approach between cysteines during conformational change can result in metal bridge formation, while a protein geometry-suboptimal Cd^{2+} coordination that is beyond the adapting ability of the receptors will be ruptured from the tension or lead to distortion of protein secondary structure (Gao & Hwang, 2016).

The selected concentration for the Cd^{2+} examination was $10\mu\text{M}$. This concentration is sufficient to facilitate coordination with three cysteine residues when they are in close proximity, allowing for the detection of such specific interactions. However, it is not so high that Cd^{2+} would coordinate with cysteines that are not as closely spaced, which could diminish the assay's sensitivity to spatial differences between cysteine residues. The results of the preliminary Cd^{2+} screening suggested that compared to the non-reactive C339S-triple mutant, 323C, 327C, 330C, 332C, 334C, 336C, and 337C have shown a significantly reduced current amplitude induced by $10\mu\text{M}$ CdCl_2 .

Cytoplasmic End of TM2 is Highly Flexible

The hP2X3R crystal structure reports that I323, V326, T330, and V334 are pore-lining residues, while I323, T330, and V334 were observed to be gating the channel in the apo, open, and desensitized state in respect (Mansoor et al., 2016). These structural observations align closely with the results of the current study. The high Cd^{2+} sensitivity observed in cysteine mutants at these positions strongly suggests structural features associated with spatial proximity and side-chain orientation toward the pore, creating ideal conditions for Cd^{2+} coordination. Meanwhile, the sensitivity of residues 332C, 336C, and 337C to low concentrations of Cd^{2+} implies distinctive characteristics in the TM2 segment near the cytoplasmic end.

Considering the proposed hourglass-shaped ion pathway and simulation results regarding the distances between $\text{C}\alpha$ atoms, the narrowest part of the channel is around residue T330. As the pathway moves deeper toward the cytoplasmic side, it was expected that the residues on neighboring protomers would become more separated. However, the results indicate the opposite, showing a closer spatial proximity between residues in this region. This finding suggests that the structural dynamics of the channel are more complex than previously thought.

MD simulation suggested a highly flexible region of the TM2 cytoplasmic end, where precise estimation of the distance relationship was harder to achieve. The presence of small side chains, notably two glycines, likely contributes to this flexibility. TEVC recordings revealed that cysteines at these flexible positions exhibit high reactivity and efficiency when inhibited by Cd^{2+} , however, these inhibitory effects are less stable compared to those at other positions. A comparison of the Cd^{2+} washing profiles between residues at the extracellular end (323C, 330C) and the cytoplasmic end (332C, 334C, 336C, and 337C) showed that removing Cd^{2+} from ATP perfusion resulted in a loss of current blocking at the cytoplasmic end, but not at the extracellular end. The instability of the reaction between the residues and the metal ion might result from the flexible nature of the protein, which might disfavor the temporary trapping of the cysteines, resulting in the interruption of the coordination from the dynamic movements.

Extracellular End Is Not Completely Rigid

When plotting the blocking efficiency of each cysteine mutant against the simulation result according to C α -C α distance, a negatively correlated relationship was observed, as seen in Figure A.2.8. This negative correlation indicates that as the distance between cysteine residues decreases, their ability to coordinate with Cd²⁺ increases due to closer spatial proximity. Thus, IC50 values can serve as an indirect measure of residue distances, providing a dynamic and functional approach to evaluating pore structure in real time.

However, this interpretation is primarily based on measurements taken near the extracellular end, where the structure is more rigid and residue positions remain relatively stable. In contrast, the lower part of TM2 is more flexible, which could affect how reliably IC50 reflects static distances. While structural models suggest that residues in this region may be farther apart, their flexibility could allow transient conformations where they still frequently come into close proximity. As a result, IC50 measurements in this area might still provide meaningful insights, even if their interpretation might be less straightforward.

Mutants S324C, S325C, V326C, A327C, A328C and T330C were tested under concentration range of Cd²⁺. The results first reported a Cd²⁺-induced current blocking at the positions where C α -C α is supposed to be far apart. Specifically, for positions 324, 325, and 326, C α -C α distances were suggested to exceed 15 Å by simulations in respect, and almost fully blocked currents were observed when Cd²⁺ was highly concentrated. Cysteines that are sufficiently far apart tend to bind Cd²⁺ independently, influenced by the distance between them, their side chain orientations, and the local environment, all dictating the reactivity between the residues and the metal ions. Higher concentrations of Cd²⁺ lead to increased current blocking, raising questions about the role of excess Cd²⁺, given that multiple Cd²⁺ ion accumulation within the same channel is unlikely. This observation is supported by previous research, which found no evidence of a second ion binding (Choi et al., 2013), and by control experiments of the triple mutant, which dismissed the possibility of unspecific binding at high concentrations.

It is proposed that, despite the region's rigid structure, the flexible nature of the α -helix permits conformational changes that affect the positioning of amino acid side chains. At high Cd²⁺ concentrations, these rare but favorable conformations are more likely to be captured, leading to stable metal bridge formation. Once

these bridges form, they persist and effectively block ion flow. The strong correlation between IC50 values and residue distance suggests that blocking is mainly caused by metal bridge formation rather than multiple Cd^{2+} ions binding separately to different cysteines. This aligns with observations from the cytoplasmic end, where the structure is more flexible, allowing Cd^{2+} to bind frequently but not remain trapped. In contrast, at the extracellular end, occasional but stable Cd^{2+} coordination leads to stronger and longer-lasting channel inhibition, especially at high concentrations.

The curve-fitting results suggested an unambiguous correlation between the simulated C α -C α distance and IC50 values, as in Figure A.2.7. Particularly for residues T330C and A327C, where IC50 values are less than 10nM, these values effectively appear as zero on the micromolar-to-millimolar concentration scale, resulting in an exponential decrease in Cd^{2+} sensitivity with increasing distances between the residues. This trend implies that spatial distance is a dominating factor in Cd^{2+} reactivity, possibly because the side chain orientation can be readily adjusted by the protein's flexibility. This adjustment allows for effective Cd^{2+} coordination even when initial alignments are not ideal. Although this correlation appears to weaken towards the cytoplasmic end of the residues, where reactivity is predictably higher, the potential to accurately define the shape of TM2 by extrapolating the Cd reactivity measurements of individual residues remains intriguing.

In this section, Cd^{2+} -induced current blocking was observed during the SCAM experiments, from which an overall structure analysis of the TM2 was conducted. A dynamically transforming TM2 during channel opening was seen, with a relatively rigid extracellular end and a highly flexible cytoplasmic end. Moreover, the concentration dependency of the Cd^{2+} inhibitory effect provided valuable information on the details of the structure.

5.2.2 Crucial Residues Regulate The Ion Pore

Although X-ray crystallography has provided detailed insights into the TM2 structure of the hP2X3R, exploring how these structural details manifest during channel function is crucial, especially to determine if the interpretations from crystal structures accurately represent the receptor's state in a living system. Focusing on the functional study of the key residues, a more profound understanding of the de-

tailed changes and roles of the TM2 in dynamic receptor activity was expected to be obtained.

I323 Controls the Opening and Closing of the Ion Channel

The cysteine mutagenesis performed at position 323 initially impaired the channel function significantly compared to all cysteine mutants. The amplitude comparison between the control and the I323C showed approximately 20-fold differences. In the 10 μ M Cd²⁺ screening experiments, I323C exhibited a sensitivity towards Cd²⁺ binding. While taking a look at the recording trace shown in Figure A.2.3 and A.2.10, a fast onset of the Cd²⁺ effect was noticed, which resulted in an average blocked current of 37%. A prolonged exposure to Cd²⁺ seemed to raise further current decrease, with a significantly slower binding. Despite this, among all mutants that were significantly 10 μ M Cd²⁺-reactive, I323C current was inhibited to the least extent. This suggests that while the position of I323C may permit the formation of metal bridges, such a reaction may not be structurally favored, judging by the relatively low level of inhibition observed.

After applying DTT to the Cd²⁺-bound channel in the open state, a dramatic increase in the ATP-induced current, approximately threefold, was observed. This potentiation was specific to the I323C mutant, as determined from DTT screenings conducted across all cysteine mutants. However, if the channels were pre-incubated with DTT before activation, the subsequent potentiation effect of DTT was markedly reduced. In these cases, a second application of DTT led to less than a 1-fold increase in current potentiation compared to those not treated with DTT beforehand.

The hP2X3R crystal structure describes the I323 as the extracellular boundary in the apo state, with pore radius = 0.29 Å, rejecting dehydrated Na⁺ when the channel is closed (Mansoor et al., 2016). The captured open-state structure displays an upward and outward rotation of the I323, seen in Figure 5.2.1, resulting in the opening of the pore. While artificially introduced cysteines at this position resulted in reduced ion conductance, this effect could be partially reversed by DTT. A reasonable hypothesis is that a spontaneous disulfide bond forms among the 323C residues during the apo closed state. This assumption was further established by a decreased potentiation during activation caused by a DTT treatment performed during the apo closed state. When the entrance of the ion pathway is “locked”

by the disulfide bond and restricted by the bond length to $\sim 2\text{\AA}$, the pore can no longer adapt to a completely open conformation, whose naturally opened entrance is estimated to be around $6\text{\AA}$ (Mansoor et al., 2016). Treating the receptors with DTT, either before or during activation, breaks the disulfide bond and enables activation of the “locked” receptors. A DTT pre-incubation preponed frees most of the channels that are closed and decreases the number of channels that could be released during ATP+DTT application, which explains the different potentiation efficiencies observed between the two treatment protocols.

The fact that no other mutants responded similarly confirmed that position 323 is the strictest restriction in the apo closed state, as cysteines at other positions do not approach into enough to form a disulfide bond. Therefore, it could be concluded that I323 defines the entrance of the pore and determines whether the channels are closed or open, thus gating the pore during the apo closed state.

Although DTT treatment recovers part of the channel function, it was still noticeable that the rescued current was smaller than the WT channel. The disulfide

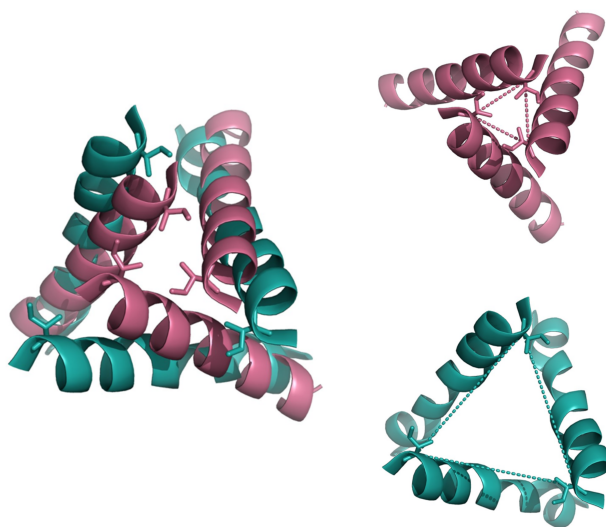


Figure 5.2.1: Viewing the trimer TM2 from a top-down perspective on the external side (PDB: open-state 5SVK, apo-state 5SVJ). The apo closed state is colored in red, and the ATP-bound open in green. The α -helices are shown in the ribbon representation, with I323 noted in sticks. The left panel shows overlapped apo- and open-state. The two right panels show the distances among three I323 in dash lines in apo- and open-state in respect.

bond trapping the ion entrance appears not to be fully responsible for the function impairment. As mentioned above, the pore radius at position 323 was estimated to be small enough to expel any conductive ions, and the cysteines inserted were expected to be positioned in extreme spatial proximity as well. However, it's less common for reactive cysteine residues to come so close (side chain distance of less than 0.5 Å as the pore radius) in the natural environment. When the cysteines are placed like this via specific guidance, as in this case, the formation of a disulfide bond distorts the local conformation of the protein. When the distortion surpasses the adaptive ability of the protein, the structure could face permanent damage, leading to a decrease in the channel function (Pu et al., 2018). Besides the disulfide bond causing the function loss, the different properties between isoleucine and cysteine, such as polarity and hydrophobicity, should also be taken into account.

Although, in theory, one out of three I323Cs may remain uncrosslinked and retain some movability, relocation of this single residue might not be sufficient for a single channel opening. Single ATP binding is not sufficient for pore opening in P2X receptors (Ding & Sachs, 1999), and intersubunit crosslinking has been shown to abolish channel function (Stephan et al., 2016), suggesting that the mobility of only one protomer is not enough to support P2X3 channel opening. Therefore, despite their dramatically reduced currents, the partial functionality of the I323C channels indicates that spontaneous crosslinking occurs only in a portion of the expressed channels in the apo state, otherwise, no current should have been observed during ATP application. This indicates that, despite the distance favoring disulfide formation, there is significant dynamic movement in the local structure. This movement likely rotates the 323C residues away from each other, allowing the channels to remain partially open despite rapid disulfide bond formation. Spontaneous opening in the absence of ATP was not observed, suggesting the flexibility reaches certain limits and does not allow a dramatic translocation of the gating residues. The conformational changes likely occur at the level of side chains rather than involving significant backbone rearrangements. Even though the 323C residues are far apart during activation, the formation of metal bridges still occurs, which partially blocks the currents. While there is indeed a blockage of a small magnitude, it not only indicates a movement of the I323Cs in the open state that results in a temporary metal bridge trapping among the three cysteines but also suggests that the structure strongly favors keeping the I323Cs separated during channel opening.

In summary, the examination of I323C suggested that position 323 takes responsibility for controlling the opening and closing of the pore. While comparing the data

from the crystal structure, additional information was provided about the flexible local environments that allow gentle relocation at this position. Yet these dynamic changes are still limited, suggesting that the consistency of such structure is vital for the initiation of the channels' functional cycle.

T330 Defines the Narrowest Part of the Channel

The TEVC experiments performed on T330C suggest first a certain level of tolerance toward cysteine substitution at this position. As no significant differences in function in terms of current amplitudes were observed between the T330C mutant and the non-desensitizing triple mutant control, a slight desensitization of the T330C mutant was seen, suggesting a more unstable open pore. Contrary to this, it was reported that a cysteine substitution at the equivalent 339T in P2X2Rs destabilizes the apo closed state of the P2X2 channels (Cao et al., 2009). The fact that no change in function or spontaneous crosslinking was observed in DTT screening indicates that in neither of the states, the 330C side chains approach into the distance range within 2Å, and the substitution with cysteines shows no evidence of altering the pore dramatically. The hP2X3R crystal structure suggests a pore radius of 0.7Å at T330, implying a smaller pore observed in crystallography compared to the ones in living cells.

In the Cd²⁺ screening experiments, a rapid inhibition was observed when Cd²⁺ was introduced during channel activation of the T330C channels, which could be profoundly reversed at a moderate rate by DTT perfusion. Different concentrations of Cd²⁺ were tested on this mutant as well, leading to the surprising conclusion that effective current blocking was achieved with a concentration of Cd²⁺ under 10nM.

T330 was observed as the cytoplasmic boundary of the pore, which defines the second restriction site. During the activation, T330 undergoes rotation and results in an up and outward relocation, leaving the pore open to approximately 4Å (Mansoor et al., 2016). The proximity, even in the open state where residues at position 330 are separating, allows the effective formation of the metal bridge when Cd²⁺ is added. The sensitivity to low concentrations of Cd²⁺, a stable inhibitory observed when Cd²⁺ was removed from the solution and a moderate reversing process under DTT treatment, see in **Results** Figure A.2.3, collectively suggest a strong interaction between 330C and Cd²⁺ ions. Both the optimal distance and

the orientation of the 330C side chains contribute significantly to this interaction. Despite an outward relocation, the side chains at position 330 still point into the pore for effective multi-side chain coordination; this conclusion agrees with previous results showing that 339T interacts with passing ions in P2X2 channels and potentially serves as a selectivity filter (Migita et al., 2001).

The strong interaction between the 330C side chains and the Cd^{2+} , however, did not achieve the maximal inhibitory effect when compared with mutants, which showed complete pore blocking, such as V334, with 85% current blocked on average, regardless of the Cd^{2+} concentration. The maximal effect without achieving a full blocking suggests that the position of Cd^{2+} in the center of the pore does not result in a full occlusion. The geometry of the pore changes during activation, such as the coiling of the helix, causing the rearrangement of the residues on the helix. Despite being constrained by the metal bridge, subtle movements at position 330 might still occur, potentially creating small gaps that allow limited amounts of ions to pass.

A327 Movements Reveal the Rotation of the Local Helix

The A327C mutant behaved in a specific way that distinguished itself from the other mutants. First, the substitution of cysteine did not alter the channel function regarding both current amplitudes and desensitization kinetics. Comparing the cysteine to the alanine, a more polar and slightly more bulky side chain was observed. The lack of significant functional alterations indicates that the local environment tolerates such a kind of subtle modification of the residues, and the cysteine contributes to the helix stability in a comparable way as the alanine. Moreover, the reactive side chain of the cysteines, when the channel is activated normally, does not manifest itself, which means no extra critical polar contact or spontaneously formed disulfide bond was introduced.

After $10\mu\text{M}$ Cd^{2+} was applied to the channel, the current of A327C was significantly blocked. Yet, noticeably, this current decrease was not reversed by further perfusion with DTT. Manipulating the concentration of the Cd^{2+} , from 0.01 to $30\mu\text{M}$, resulted in only the differences in the onset kinetics of the current block but not the blocking efficiency. This suggests two possibilities: either the 327C residues are positioned so closely that rapid formation of metal bridges occurs, similar to what is observed with T330C, or a different mechanism underlies the

Cd²⁺-induced current reduction

A close positioning of 327 residues contradicts the crystal structure data, which shows up-and-outwards movements at position 327, and therefore, theoretically, will not increase the approaching possibility, as shown in Figure 5.2.2, and a single cysteine-Cd²⁺ reaction cannot explain such a high reactivity. Additionally, if coordination between A327Cs and Cd²⁺ is responsible for the pore blocking, it should be expected to see an effective to almost full blocking, as what was seen on 330C, as such a high reactivity would require all 327Cs to engage and inevitably constrict the pore and reduce the current to a great extent. This was not observed during the experiments. Besides, the current reduction at A327C showed minimal reversability to DTT treatment. This suggests that the Cd²⁺-SH complex at this position may be either inaccessible to the reducing agent or stabilized in a way that prevents its reversal.

Taken together, these inconsistencies suggest that a different mechanism may underlie the Cd²⁺-induced current reduction at A327C. A commonly proposed alternative for ion channel current inhibition by metal ions involves their function as channel blockers. The A327C mutation may introduce or expose a binding cavity for Cd²⁺, thereby leading to inhibition through a mechanism distinct from direct

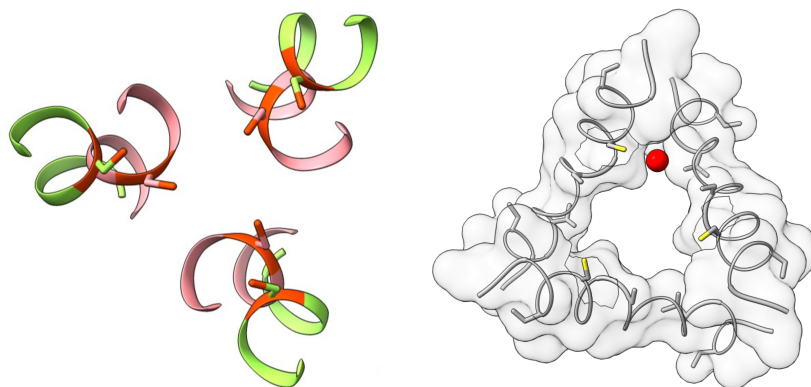


Figure 5.2.2: The left panel views the trimer TM2 from a top-down perspective on the external side (PDB: open-state 5SVK, apo-state 5SVJ). Apo-state is colored in red and open-state in green. The α -helixes are shown in the cartoon, with I323 noted in sticks. The left panel shows overlapped apo- and open-state. The two right panels show the distances among three I323 in dash lines in apo- and open-state in respect. Panel B proposes the Cd²⁺ docking within the pore during the open state. Cd²⁺ is represented by the red sphere. S atoms from the 327Cs are marked in yellow.

thiol coordination.

To do a preliminary confirmation of this theory, an onset kinetics assessment was performed. When comparing both T330C and A327C under a series of Cd^{2+} concentrations — all producing maximal blocking effects for both constructs—a consistent acceleration of Cd^{2+} onset was observed only in A327C and not T330C. These saturated concentrations ensured that differences in onset kinetics were not due to insufficient Cd^{2+} binding, thus validating the comparison between mutants. If Cd^{2+} functions as a channel blocker on the A327C channel, the results can thereby be explained: at low concentrations, Cd^{2+} may saturate this site, resulting in maximal blocking. At higher concentrations, the occupancy of this binding site may be accelerated, thereby hastening the onset of current reduction without altering the degree of block. When cysteines are incorporated in this region, Cd^{2+} may be positioned near the pore, where it exerts steric and electrostatic hindrance. Since the number of available binding pockets for Cd^{2+} is limited, those that are occupied may exclude further Cd^{2+} binding, leading to a rapid yet incomplete current block.

As the current blocking of the 327C channel was not a consequence of multiple sulfide groups coordinating a Cd^{2+} ion, it might be concluded that the side chains of the cysteines moved away from each other during the opening, especially when the estimation of the distance among residue at this position does not deny the possibility of metal bridge formation. As described in Figure 5.2.2, the movement of the cysteines promotes more exposure of their backbone and probably buries the side chain. The more reactive side chain of the cysteine might allow for spatial approaching(s) between them. Although the interactions are minor and do not impact the function, they could be sufficient to alter the orientation or position of the backbone. The subtle conformation adjustment can be enough to cluster a binding pocket for the Cd^{2+} .

Although 327A was not explicitly discussed in the crystallography study from the study of Mansoor. et al, the corresponding T336 in P2X2Rs were studied in previous research (Li et al., 2008, 2010). It was concluded that the 336T was lining the pore of P2X2 channels and was accessible to large MTS reagents in both the apo and open states, which indicates a relatively fixed position of T336 and, thus, a fixed TM2 at this position in the P2X2 channel during channel opening. In the case of P2X3R, the A327C was seen to be away from the pore, especially in the open state. The movement of 327C depicts a counter-clockwise rotation of the

right-hand helix in detail. The formation of the cavity from the backbone of the 327C and the surrounding environments allows the binding of the Cd^{2+} , further elucidating the overall rigid but still dynamic local helix.

G333 Serves as the Essential Hinge in the Open-state

As introduced in the **Introduction**, the G-V-G motif from positions 333 to 335 in P2X3R is conserved through the P2X family. Therefore, the research conducted on other subtypes is interesting to note for the sake of comparisons. The G333C mutant stood out for its unique kinetics during the function test. As a construct built on the non-desensitizing background of the triple-mutant, G333C exhibited fast desensitization, with only 20% of remaining current left after 20 seconds of exposure to ATP. The maximal current obtained from the mutant was also significantly smaller than the one from the control, with a comparable ratio of approximately 1:4. This function-impairing mutation was reported as functionally well-tolerated in P2X2 (G342C) (Cao et al., 2009) and does not alter kinetics in P2X7 (G345C) (Browne et al., 2013)

Activation by ATP or ATP+10 μM Cd^{2+} solution raised similar maximal responses on G333C, as shown in Figure A.2.9. However, ATP+ Cd^{2+} activated channels sustained a larger current amplitude after 20 seconds of agonist exposure, about three times more than the ones with ATP alone, which showed continued desensitization. As the example in **Section 3.2**, Figure 4.2.2 indicates, applying DTT eliminated the “current stabilization” effect from the Cd^{2+} . After a transient current rebound, the response to ATP with DTT quickly fell to levels similar to those with ATP alone. In other recordings where mutated channels were only activated by ATP+DTT, similar kinetics to sole ATP activation were observed (trace not shown). The facilitation of channel opening from Cd^{2+} is explicit on G333C, and the reversibility under DTT implicates that this effect is SH- Cd^{2+} coordination-specific, as a simple removal of Cd^{2+} out of ATP perfusion did not cause the same effect. DTT + ATP application without any previous Cd^{2+} involvement did not cause any difference, suggesting that spontaneous disulfide bond formation among 333C cannot explain the phenomenon.

As suggested by the hP2X3R crystal structure, the G333 advantage of its small size enhances the flexibility of the helix. Mutating equivalent 342G in P2X2 into proline or alanine either helps or damages the flexibility feature individually, resulting

in increasing or decreasing current flows, respectively (Habermacher et al., 2016). Figure 5.2.3 describes the differences in conformation between 3_{10} helix and α -helix, where the involvement of G333 is essential for the former. In previous research, it was observed on P2X2 channels that MTSEA led to the potentiation of the G342C mutant, but not MTSES or MTSET (Rassendren et al., 1997). Additional research showed that the MTSEA-caused increase in channel function was only observed when 20 mM intracellular cysteine was present (Egan et al., 1998). Those results together proposed that the positively charged thiol group modification reagent MTSEA, with a proper size, potentiates the P2X2 channels with G342C mutations.

The crystal structure of hP2X3Rs in the open state shows that pore opening involves an outward repositioning of TM2, accompanied by a structural transition from an α -helix into a 3_{10} helix at the G-V-G hinge. Based on this observation, it has been proposed that the cytoplasmic cap, which links directly to the TM2 region, stabilizes this 3_{10} helix conformation. The cap is thought to anchor TM2 to the membrane, thereby maintaining the expanded conformation required for sustained pore opening (Mansoor et al., 2016). Mansoor et al. thereby offered an insight into the energetic aspects of this structural coupling: the 3_{10} helix conformation is known to be less stable compared to an α -helix (Vieira-Pires & Morais-Cabral, 2010), suggesting that the stable cytoplasmic cap (as observed in the triple mutant) helps offset the energy cost associated with this structural transition.

In the case of G333C, compared to glycine, cysteine is larger and less flexible, making the TM2 transition into 3_{10} helix more complex; in other words, it requires more energy. This resembles a stretched spring, which is energetically strained and prone to snapping back into the more stable α -helix. In the triple mutant channel (with WT TM2), this strain is balanced by the cytoplasmic cap, which connects the TM2 and anchors to the membrane, thereby stabilizing the open conformation. However, the G333C mutation increases this internal potential energy, leading to a even more energetically unfavored 3_{10} helix: the likelihood of 3_{10} helix forming is dramatically reduced, explaining current reduced by 80% in G333C channel; and when the 3_{10} helix is indeed formed, this spring is tighter, composing higher level of potential energy and causing the cap to be "pulled out", in terms of spring recoiling, or simply collapse, manifesting as fast-desensitizing kinetics during activation, resulting from the dissembling of the cytoplasmic cap.

The insertion of Cd^{2+} ions provides additional structural support through the cys-

teine side chains. Although Cd^{2+} coordination may partially decrease ion flow due to its charge and size, this is counterbalanced by its stabilizing effect on the open state. As a result, the maximal current amplitude remains largely unchanged. This assumption might explain why DTT application resulted in a current renouance followed by fast desensitization of the channel: removing the Cd^{2+} temporarily clears ion pathway before the structural support collapses. This reversibility confirms that the effect is cysteine-specific and supports the interpretation of Cd^{2+} as a conformational support.

In these experiments, it was further confirmed that G333 acts as a necessary hinge, enhancing the open state. Substitution with cysteine at this position revealed functional consequences of the conformational change during gating. The G333C mutant showed significant current reduction and rapid desensitization, indicating that cysteine at this position interferes with the formation or stabilization of the 3_{10} helix required for channel opening. Coordination with Cd^{2+} at this site compensates for the energetic instability, acting as a structural brace that helps preserve the 3_{10} helix and thereby prolongs the open state without increasing maximal conductance.

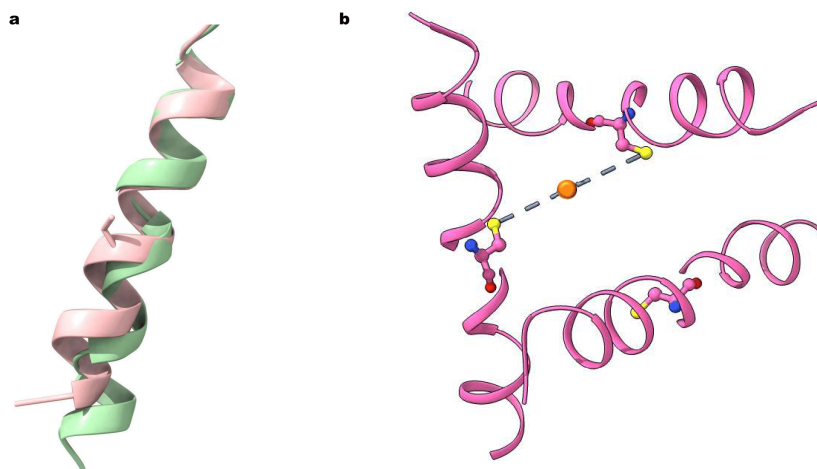


Figure 5.2.3: Panel A, Overlapped the TM2 in 3_{10} -helix and α -helix in the open state (5SVJ). 3_{10} -helix (green) contains a G333 (cannot be seen here), α -helix (pink) contains a 333C, which shows in sticks; Panel B, illustration of possible metal bridge formation in G333C when the pore is open and the 3_{10} -helix is formed (5SVJ). The cysteines are shown in ball-sticks, and -SH groups are colored in yellow. Cadmium ion is indicated by the orange ball, and the metal bridge by the dashed line.

While structural evidence suggests that the transition into 3_{10} helix couples TM2 and the cytoplasmic cap during the gating process, the current functional experiment provides more evidence: Disruptions in TM2 stability can reflect on the distalized cap structure, compromising its anchoring role and ultimately leading to pore closure. Together, these findings reveal that the gating process relies on a finely tuned structural coordination between the TM2 helix and the cytoplasmic cap to maintain a stable open state.

V334 Determines Desensitization Actively

When moving down to the cytoplasmic end of the TM2, V334 was reported as the last constrictive site. The V334C, built on the background of the triple mutant, exhibited a significantly smaller response when compared to the control, with almost unchanged desensitization behaviors. Yet when taking a close look at the recording trace, what stood out was that these channels appeared to have larger leak currents sometimes, which manifested as a more negative baseline. The amplitude was defined as the differences between the maximal current and the baseline, and a leaky baseline could be taken into account for the smaller amplitude.

During the Cd^{2+} modification, V334C was blocked by $10\mu\text{M}$ Cd^{2+} rapidly and extensively. For those samples with large leak currents, the inhibitory effect always surpassed 100%. As discussed earlier, the fast-blocking effect suggests a high solvent accessibility of 334C. In earlier research, similar results were reported on the equivalent V343C in rP2X2 channels (Li et al., 2010). The MD simulation results did not support the possibility of a metal bridge establishment due to the unfavored distance among the cysteines. Other simulations raised the same question, as they found out that 351Ls of zfP2X4 channels (corresponding to V334 in P2X3) were far apart as well (Habermacher et al., 2016).

Here, these disagreements were suggested to be explained by a rearrangement of the TM2 and an electrostatic change brought by the Cd^{2+} . This assumption also aligns with the flexible structure addressed earlier. The fast and effective Cd^{2+} coordination suggested a frequent approach of the 334Cs during the opening state. On the other hand, a less stable binding between the Cd^{2+} and sulfur group, as the current blocking effect was gradually erased by simply removing Cd^{2+} from the ATP application, describes that this kind of approach is not rigid. On the contrary, it is not as easy to trap the V334C within the same position, as the dissociation also

showed a high tendency. Together with the similar results observed on other cysteine mutants from positions 332 to 337, an extremely dynamic structure during channel opening in this area was observed. As no function decrease was observed, the dynamic movement of the TM2 must be still limited within a certain range, so an extreme approach among the 334C shall only happen during desensitization which is unlikely to take place on the non-desensitizing channels, otherwise spontaneous crosslinking caused gradual function decreasing at this position should have been observed.

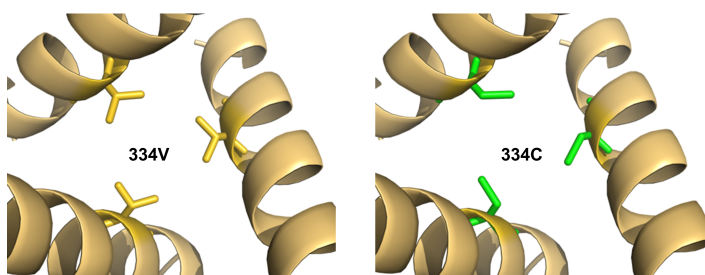


Figure 5.2.4: Viewing part of the trimer in the desensitization state from the extracellular side. The left panel shows the WT (wild type) channel, with V334 displayed in sticks and colored in yellow. The right panel shows the V334C channel, with 334C shown in sticks and colored in green.

To find out how the V334 affects desensitization, as it was determined as the gating residue during desensitization, the V334C was constructed on the fast-desensitizing WT receptor as well. Surprisingly, an indispensable impact on desensitization behavior was indeed observed.

As shown in Figure A.2.11, it was noticed that V334C-WT exhibited significantly slower desensitization compared to the typical fast-desensitizing WT, with larger remaining currents during continuous exposure to ATP perfusion. Compared to the triple mutant, the stability of the open state introduced by V334C within WT was moderate. However, when comparing the “run-down” effect, V334C even surpasses the triple mutant, showing almost 100% recovery during repetitive activations.

The moderate level of desensitization observed for V334C-WT strongly suggests that residue V334 actively stabilizes the desensitized state in hP2X3Rs. The conformational change between opening and desensitization was suggested based on the crystal structure. The suggested disassembly of the cytoplasmic cap releases the TM2, leading to its subsequent movement. As described above, the 3_{10} helix on the TM2 resembles a stretched spring that falls back to a more relaxed α -helix after the absence of the fixation at the end from the anchoring of the cap. The recoil of the helix causes an upward rotation (from the extracellular side) of the residues,

during which valines at position 334 approach and form the new restriction gates in the desensitization. become a new constriction site (Mansoor et al., 2016), as shown in Figure 5.2.4.

In V334C-WT, even though cytoplasmic cap disassembly and TM2 recoil still occur, the cysteine substitution at position 334 reduces the residue's ability to stabilize the desensitized conformation. Unlike valine, which forms an effective gate through hydrophobic interactions and steric bulk, cysteine has a smaller and more flexible side chain. No disulfide bond formation was observed at position 334, likely due to its position near the cytoplasmic end of TM2, where the reducing environment disfavors crosslinking. Additionally, local conformational changes introduced by the cysteine substitution may subtly alter the alignment of side chains, further reducing the likelihood of effective gating interactions. As a result, these cysteines do not form a stable gate and fail to provide sufficient structural resistance to support complete pore closure. This weakened gating mechanism directly explains the slower desensitization kinetics observed for V334C-WT channels. Together, these findings support that V334 actively contributes to the transition into the desensitization state.

What is more interesting is that, degree of desensitization kinetics observed during channel activation and the level of recovery do not correlate straightforwardly. While the WT channels were characterized by a low level of recovery, or "run-down", the V334C-WT channels displayed a notably high recovery ratio, even higher compared to the triple mutant, which desensitizes minimally yet exhibits only partial recovery.

Earlier research found that the rate-limiting step for recovery is the dissociation of ATP from the receptor; however, this process *per se* is restricted by the desensitized conformation, which traps the agonist within the binding pocket (Giniatullin & Nistri, 2013; Li et al., 2019; Pratt et al., 2005; Sokolova et al., 2004). As a result, the channel remains locked in the desensitized state until ATP unbinding allows the conformational reset back to the apo state.

Interestingly, the degree of desensitization does not directly correlate with recovery levels in the V334C experiments. In the triple mutant, stabilization of the cytoplasmic cap was expected to prevent the channels from entering the desensitization state during opening, but the recovery level is restricted. The previously proposed "High Affinity Desensitization" (HAD) suggested that P2X3 channels

can be desensitized by a nanomolar level of ATP without eliciting macroscopic current (Sokolova et al., 2006), and a direct transition between desensitization and apo states of the triple mutant can explain the moderate recovery here. This is supported by the fact that, despite a higher open probability, the peak current in the triple mutant was not significantly larger than that of WT under 10 μ M ATP: If almost all channels completely avoided desensitization, a much larger current increase would be expected. In other words, a portion of channels were never activated, both in WT and the triple mutant receptors. The cap-dissmeble-independent desensitization happens repeatedly when the triple mutant channel encounters ATP; hence, the accumulation of desensitized channels is reflected by the incomplete recovery.

In contrast, the V334C mutation impairs the formation of the desensitization gate itself. Without this gate, channels are far less likely to enter any desensitized state, whether from open or apo, and therefore exhibit nearly complete recovery. This comparison strongly supports the suggested kinetics model, that, unlike P2X1Rs, which must enter the open state before desensitizing (Rettinger & Schmalzing, 2003), P2X3Rs can transit from apo directly into the desensitization state.

Although no structural evidence has yet shown the direct transition from apo to desensitized state, the strong sensitivity to the V334C mutation suggests that TM2 movement, especially at position 334, still plays a key role in forming and holding the desensitization gate during the alternate desensitization. In support of this, V334C channels often show a higher level of leak currents, which can be partially elevated during the Cd²⁺ treatment, where current clocking efficiency exceeds 100%, suggesting that spontaneously opened channels might be responsible. This raises the possibility that a small amount of naturally existing ATP might trigger partial TM2 transitions in some channels, or, considering the previously proposed view that I323 can spontaneously rotate away from the pore axis, such a rotation itself might transiently expose the pore entrance, requiring a rapid protective closure at V334. In that case, separation around I323 might drive the opening, while position 334 works to simultaneously seal the pore from the cytoplasmic end, which is weakened or missing in the case of V334C.

In summary, changing a key gating residue in TM2 strongly affects the desensitization process, not by modifying the cap directly, but by disrupting how TM2 closes the channel. This disruption weakens desensitization, makes ATP unbinding easier, and allows for more recovery. These findings highlight how movements at

the lower end of TM2 can influence not just pore closure but also upstream domains like the agonist binding site, showing that gating and desensitization involve dynamic, tightly linked steps across the entire receptor.

5.2.3 V326C Provides Evidence of Ion Passing From

When the channels are in the apo state, the ions and water molecules diffuse freely over the extracellular domain. Once the channels are opened, the ions are gathered at the entrance of the lateral portals and pore, where the inward flows start. Thereby, it was suggested that the ions are hydrated at least while entering the channel (Samways et al., 2014). Whether the ions are going through full/half-dehydration while travelling in the pore is intriguing.

As discussed, the V326 is located at one of the constriction sites within the TM2. The insensitivity of V326C to 10 μ M Cd²⁺ suggests a relatively large spatial distance relationship among the cysteine at position 326 and a relatively rigid local structure during channel opening. In the later experiments, a higher Cd²⁺ concentration was used, aiming to increase the attachment of Cd²⁺ to the 326C, thereby artificially inserting a barricade to the passing ions. Cd²⁺ not only acts as a steric barrier but also brings an extra electrostatic factor interfering with the ion flow. The P2X3R is described as “non-selective” to monovalent cations, thus, the aim here was to compare how different alkali metal ions are discriminated by the Cd²⁺ filter.

The K⁺ and Li⁺ current amplitudes comparisons reveals that, unlike the nonselective triple mutant, V326C conducted more K⁺ flow than the Li⁺ one, while no differences were observed between the 326C and the triple mutant in currents obtained from K⁺-Ori⁻. The attachment of the Cd²⁺ onto the cysteine further restricts the channel. It was observed that the Li⁺ flow in the V326C was more sensitive to the Cd²⁺ filter, manifesting in a higher percentage of current blocking.

K⁺, with its larger ionic radius, forms a thinner and less compact hydration shell than Li⁺, whose small size and high charge density result in a compact and multi-layered water shell. The immediate increase in the preference for K⁺ in V326C channel, and the different blocking efficiency of Cd²⁺ to Li⁺ and K⁺ currents, suggests the half-dehydration process of the different ions when passing the pores.

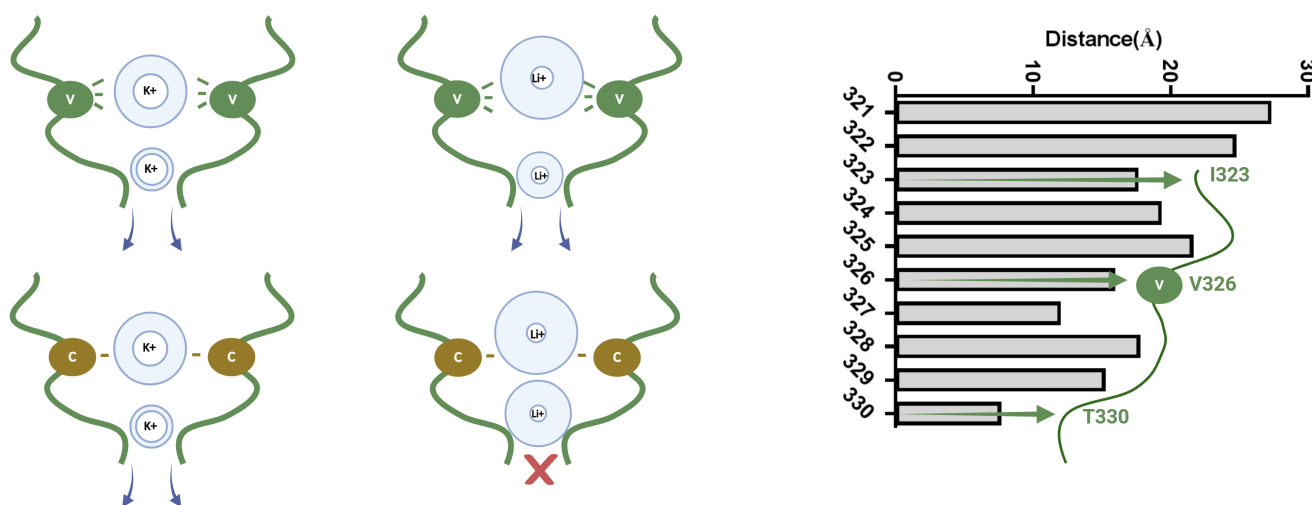


Figure 5.2.5: Left panels schematize ion permeation through the upper part of the pore, whose outline is based on estimation of simulated Ca–Ca distances for residues 321–330 (horizontal gray bars) in the right panel. I323, V326, and T330 are noted, with V326 specified in green ball. The green trace reflects the relative spatial constriction along the pore, but does not represent the actual pore radius as it considers no side chain volume. V326C mutations are specified in brown. Residue-induced dehydration effects are specified in the colored lines, with density representing dehydration efficiency. The upper describes that in the V326 pore, both K⁺ and Li⁺ (as named circles, water shells are the outer light-blue rings) undergo partial dehydration at 326. Both ions shed the outer layer of water and pass through the narrowest part of the channel; the lower describes the V326C with weakened dehydration drive, from which K⁺ still partially dehydrates and passes, while Li⁺ faces more difficulty passing the T330 with its more intact hydration shell, as it is more compact.

Flanking from I323 to T330, the hydrophobic environment of the TM2 channels, from I323 to 329F, disfavours the stabilization of water molecules and thereby promotes dehydration as ions enter the pore. Along the path, V326 is suggested to be the first occlusion in the pore beyond the gating V326, whose hydrophobic side chain is oriented towards the pore and contributes significantly to ion dehydration. Compared to valine, V326C slightly raises the polarity and reduces the ions' dehydration ability. As shown in Figure 5.2.5, this shift might be sufficient for discriminating between K⁺ and Li⁺: tightly bound Li⁺ carries more water and fail to pass the subsequent narrowest gate at T330, whereas K⁺ still manages to loose part of the water shell and permeates further.

The insertion of Cd²⁺ into the V326C background further differentiates ions. Cd²⁺ introduces both spatial obstruction and electrostatic changes: besides a direct re-

pulsion effect, which is similar for monovalent cations like Li^+ and K^+ , the charge promotes local water polarization and thereby the hydration shell of nearby ions. Carrying ions, it not only potentially changes the local energetic environment, but also further reduces the dehydration of passing ions by facilitating the polarization state of the water molecules. This stabilization reduces the efficiency of dehydration during permeation. As a result, the more pronounced current reduction observed for Li^+ likely reflects both its larger hydrated size and its increased difficulty in shedding water, which collectively limit its ability to pass through the Cd^{2+} -narrowed pore.

Half-hydration state, where ions in the pore reduce in size by shedding only outer layers of water molecules, is usually favored by ions when passing a non-selective pore, as full hydration requires an unrealistic large pore while full dehydration is highly energy-consuming. In the P2X3 pore, V326 contributes significantly to ion dehydration, helping ions to reach a size compatible with the filter at T330. T330 restricts the pore and may interact with the left hydration shell of ions and stabilize this intermediate state. The presence of this polar residue is further evidence that ions are not fully dehydrated: complete dehydration would lead to excessively strong interactions with T330 and a risk of getting stuck in the pore.

A similar role of polar residues within the TM2 segment of P2X2 was also described, where threonines at positions 336 and 339 were suggested to aid ion permeation through the pore (Migita *et al.*, 2001). T339 in P2X2 is positionally equivalent to T330 in hP2X3, indicating a conserved role in stabilizing partially dehydrated ions at the narrowest region of the pore. Although the residue equivalent to V326 in P2X3 is A335 in P2X2, experimental data suggest that T336, which faces the pore interior, is likely the key contributor to ion interaction and size restriction at this location (Li *et al.*, 2008). Compared to P2X2, P2X3 TM2 possesses not only fewer polar residues, but also filters ions more strictly due to a smaller pore (Mansoor *et al.*, 2016; Rokic & Stojilkovic, 2013). Together, these might suggest different dehydration strategies between P2X2 and P2X3 pores. The bigger P2X2 pore can accommodate more hydrated ions, thereby a threonine at 336 is tolerated, as it assists in stabilizing the hydration shell during permeation without reducing ion passing. This could explain why Migita *et al.* observed a clear preference for Li^+ in the T336 but not T336V pore, as the strongly binding hydration shell of Li^+ making it greatly benefits on polar interactions provided by threonine, also suffer the most from hydrophobic mutations.

In summary, V326 represents a critical point along the P2X3 permeation pathway where dehydration likely initiates. Its hydrophobic nature facilitates the removal of loosely bound hydration water, preparing ions for passage through the narrower gate at T330. Mutation to cysteine, while only modestly increasing local polarity, significantly alters dehydration efficiency—preferentially affecting Li^+ due to its tighter hydration shell and greater dependence on a permissive dehydration environment.

In this part of the study, SCAM was applied to systematically evaluate the functional contribution of individual residues within TM2 of the hP2X3 receptor. Several positions, including I323, T330, and V334, were identified as functionally critical, supporting their proposed roles as gating determinants. The residue V326 was found to influence ion access, offering insight into its potential involvement in pore occlusion. Furthermore, the analysis of A327 and G333 clarified how TM2 may act not only as the pore-forming segment but also as a functional interface linking the extracellular gate to the cytoplasmic cap. Together, these findings contribute to a broader understanding of how TM2 coordinates with surrounding domains to regulate receptor activation.

5.3 The structure-permeation relationship study

The following discussion will outline the keys of ion permeation regulated by the P2X3 structure, which were derived from the experimental data.

5.3.1 Extracellular Ion Pathway

The sequence alignment between the hP2X3 and rP2X5, together with MD simulation, highlighted E46, R52, and T54 as the residues of interest. Later, charge-reversal mutants of E46 and R52 were tested in parallel with the triple mutant, while a hydrophobic valine substituted for hydrophilic threonine at position 54.

Data from TEVC functional experiments revealed that the current amplitude of E46K was not reduced compared to the triple mutant, while those of both R52E and T54V were reduced to a certain level. It appeared that the substitution of the negatively charged glutamic acid with a positively charged lysine did not affect the ion flow significantly, even though it was suggested by the MD simulation that this glutamic acid might help with forming a local electrostatic environment, attracting cations at the entrance of the pore. None of the mutants exhibited a kinetic that dramatically differed from the triple mutant.

To find out how the mutations affect the ion flow, by altering ion selectivity or conductance, components in the Ori⁻ solution were substituted by organic ions stepwise, concerning those described: choline⁺ replacing Na⁺ or gluconate⁻ replacing Cl⁻. The study assessed changes in ion flow from two perspectives: first, by comparing the ratios of current amplitudes of the same construct between two solutions to evaluate changes in conductance; and second, by analyzing shifts in the reversal potential (ΔV_{rev}) as the extracellular ion environment varied, which provides insights into the ion selectivity of the channels. Two-way ANOVA was conducted to increase the sensitivity of detecting differences among groups. Additionally, due to a rundown effect, repetitive measurements at the same concentration within individual channels were required to capture the varying degrees of rundown observed across different constructs. Incorporating this control data into the analysis enabled a more accurate interpretation of the changes associated with each construct. As a result, two-way ANOVA allowed for simultaneous com-

parison across multiple groups within the dataset, enhancing both sensitivity and interpretability. This approach also permitted the isolation of construct-specific effects by statistically controlling for other sources of variability.

Choline ions were introduced into the background buffer, substituting for sodium, during which a significant current decrease was expected to be observed. The change in current amplitude observed in the triple mutant served as a control, to which the levels of current reduction in the mutants were compared. During the reduction of sodium concentration, the concentration gradient across the membrane decreases, resulting in a smaller sodium current. The extent of current reduction reflects how efficiently sodium ions contribute to conduction in each channel variant. Similarly, where gluconate molecules gradually replace extracellular Cl^- ions, the extent of current decrease is also positively correlated to chloride conductance.

In parallel, changes in the V_{rev} reflect the electrochemical equilibrium established by key permeable ions, which, in these cases, involve intracellular K^+ , extracellular Na^+ , and Cl^- , and are directly related to the channel's ion selectivity.

When comparing different constructs, a more pronounced negative shift in V_{rev} during decreasing extracellular Na^+ concentration suggests a higher relative permeability to Na^+ . This larger shift occurs because, for such constructs, Na^+ permeation plays a more dominant role in determining the electrochemical equilibrium potential. Similarly, higher sensitivity of V_{rev} shifts during Cl^- removal suggests that the construct has a higher chloride preference. Together, these two approaches—current amplitude changes and V_{rev} shifts—independently capture diffusion efficiency and selectivity, respectively.

Compared to the triple mutant, the E46K displayed a higher level of rundown effect, as the level of current decrease was significantly larger when measured twice in the same NaCl -based Ori^- . Although not statistically significant, the E46K mutant consistently showed a slightly greater sensitivity, compared to the triple mutant, to decreasing extracellular sodium concentrations during the stepwise Na^+ reduction protocol, where P values from the ANOVA tests were 0.06 and 0.08 for factors of $[\text{Na}^+]$ and *construction* variable, respectively. Those results suggest that E46K shows no significant change in Na^+ conductance relative to the triple mutant. However, the stronger rundown observed in E46K may have masked subtle effects caused by the E-to-K substitution at the extracellular fenestration, making it harder to detect actual differences in sodium conductance.

The 2-way ANOVA test of the ΔV_{rev} values between the E46K and the triple mutant indicated a significant difference with a P value of 0.0269 when evaluating the importance of changes in constructs (factor "*construct*"), suggesting that the substitution of E46 into K resulted in a change in Na^+ selectivity. Specifically, the E46K mutant exhibited lower sodium preference than the triple mutant, as reflected by smaller ΔV_{rev} shifts under varying sodium concentrations. When both sodium concentration and construct identity were considered together (factor $[\text{Na}^+] \times \text{construct}$), no significant interaction was detected. This suggests that while the E46K mutation does affect sodium selectivity, the effect is modest and may not strongly depend on sodium concentration.

R52 is an intrinsically positively-charged residue, and its precise role at the fenestration site was difficult to predict. To investigate its function, R52 was mutated to glutamic acid (R52E). Compared to the triple mutant, the R52E mutation significantly altered the current response to changes in sodium concentration ($P = 0.0007$). Further analysis showed that while the triple mutant's current dramatically decreased upon Na^+ removal, the R52E mutant was significantly less sensitive to this change. This result suggests a significantly smaller Na^+ conductance of R52E. Similar findings were observed for the T54V mutant. Two-way ANOVA detected a significant difference in the current reduction in response to sodium reduction between constructs ($P = 0.009$ when comparing T54V to the triple mutant's response to decreasing ion flow). This result suggests that the T54V mutation also significantly reduced the channel's Na^+ conductance. Furthermore, a significant interaction effect between " $[\text{Na}^+] \times \text{Construct}$ " was observed for the T54V mutant ($P = 0.0149$). This significant interaction ($P = 0.0149$) means that the difference in Na^+ conductance between the T54V mutant and the triple mutant changes depending on the extracellular Na^+ concentration.

The R52E exhibited a negligible impact on the sodium ion selectivity of the channels. Comparing the shifting of the ΔV_{rev} when sodium decreased, the mutant and the control were indistinguishable from each other, with a P value of 0.9123. This result suggests that charges of R52 do not affect the cation selectivity. T54V, on the other hand, displays a lower sensitivity to the ion concentration alterations, with a P value of 0.0308, indicating a decreased selectivity for the Na^+ .

To assess chloride permeability, the external Cl^- concentration was reduced while other solution components were kept constant. Such alteration reduces the driving force for Cl^- efflux, leading to a change in outward Cl^- current, and the resulting

I2/I1 ratio indicates the change in chloride conductance

The relationship between V_{rev} shifts and Cl^- selectivity was then considered. For a channel with high Cl^- selectivity, its V_{rev} is expected to shift significantly. Conversely, for a channel with lower Cl^- selectivity, the V_{rev} shift will be smaller. In this scenario, the V_{rev} is more influenced by the electrochemical gradients of other significantly permeable ions (Na^+ and K^+) within the system.

When comparing the amplitudes of the chloride current, the two-way ANOVA detected a P value of 0.0353 between E46K and the triple mutant, with a significantly lower amplitude decrease for E46K compared to the triple mutant. Notably, under standard 90mM NaCl conditions, the current amplitudes of E46K's are comparable to those of the triple mutants, implying that its overall channel function, including general conductance and gating, remains intact. Therefore, the altered current observed under chloride-specific conditions is more likely attributable to changes in chloride permeability rather than a general loss of channel function. However, the chloride selectivity of the E46K remained largely unchanged, with a P value of 0.7414. Together, the E46K might conduct the chloride current less effectively, but it does not show any statistically significant changes in chloride selectivity.

Similarly, the R52E mutant exhibited altered Cl^- conductance but not selectivity. The reversal potential changed to a similar degree when comparing the two constructs, with a P value of 0.4525, with multiple comparisons showing a small but insignificant bigger shifting of the R52E. The conductance comparisons, on the other hand, resulted in a higher sensitivity to the chloride change of the mutant, with a P value of 0.011.

Although the T54V had shown a significant impact on the sodium flow, no evidence indicated a change in chloride permeation induced by the mutation. The test results of the amplitude ratio approached but did not reach significant differences, with a P value of 0.0501. The multiple comparisons between the control and the mutant suggested that the current flows of the control changed to a greater extent compared to the mutant. The shifts of the reversal potential were similar between the two constructs, with a P value of 0.345.

In Figure 5.3.1, the residues studied here are labeled on a trimer. The region shown in ribbons is residue 46-54, which was suggested to be where the lateral fenestration is located (Samways et al., 2014). Previous calculations of the electrostatic

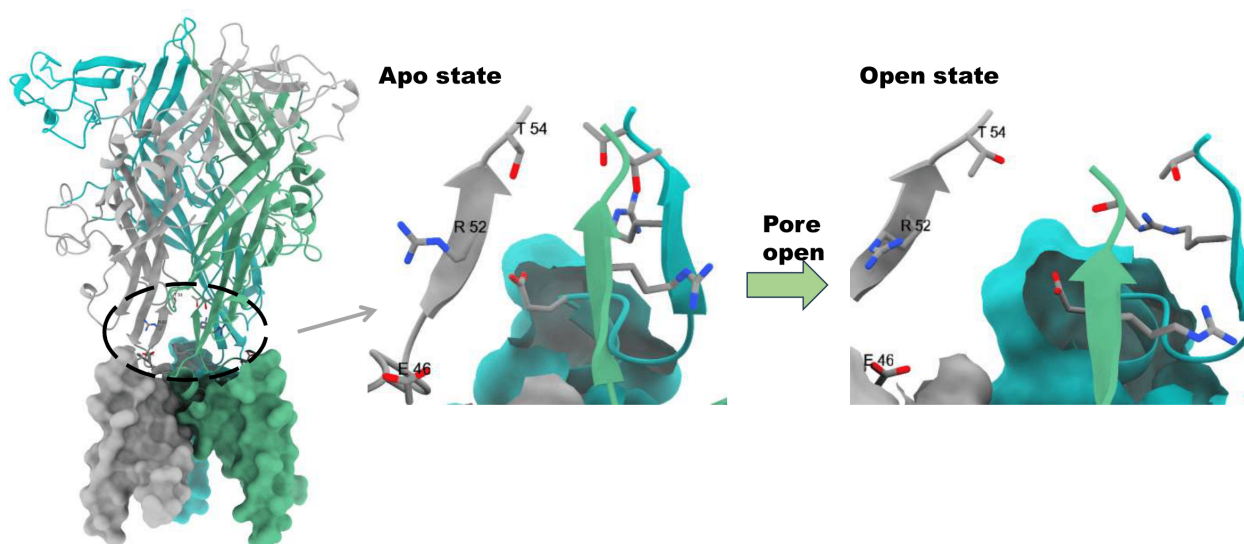


Figure 5.3.1: Residues 46E, 52R, and 54T on a trimer. The left structure shows a trimer of the hP2X3 channel in the closed, apo state (5SVJ), with the segments and intracellular termini shown in surface representation, while the rest are in ribbons. 46E, 52R, and 54T are specified in sticks. Different monomers are color-coded. The middle part zoomed in on the residues 46-54 with partial TM2s. 46E, 52R, and 54T are labeled and shown in sticks. The right part shows the same region in the open state. (5SVL).

accessibility of the lateral fenestration on the zfP2X4 and rP2X2 channels have suggested that the sodium ions are energetically favored on this pathway (Kawate et al., 2011b). A negative electrostatic surface might play a role in the ion selection. Thereby, the negatively charged E46, which is located around the entrance of the pore, caught interest. Cysteine and alanine scanning, including equivalent residues in other P2X channels, have been carried out. Cysteine substitutions at this position, either in rP2X4 or hP2X1 channels, did not result in a change of function. However, when the equivalent E51 in rP2X4 was replaced by alanine, the current decreased (Allsopp et al., 2011; Rokic & Stojilkovic, 2013). It appeared that, compared to the charge, losing the polar (and slightly bulkier) side chain seems to matter more for the conductance of Na^+ -influx: in this part of the experiments, the E46K channel behaved similarly in the function tests. However, results suggested glutamic acid might still play a role in cation selectivity, as part of the electrostatic negative pathway. A decreased selectivity of sodium was found when the E was replaced by the K, suggesting the positively charged lysine tampered with the sodium ions' preference. These findings somewhat match the previous conclusions that the equivalent E on the P2X1 channels regulates the calcium flow (Samways & Egan, 2007). The Cl^- selectivity of the E46K reflected by the ΔV_{rev} remained unchanged compared to the control, though the current conductance was less effective.

Overall, the maintenance of polarity at E46 is crucial for sodium conductance, probably because the hydration of sodium at the entrance is key for the ion diffusion through the pore with relatively low resistance. However, although a mutation into lysine retains the polarity contact, thus keeping the diffusion difficulty unchanged, the positively charged lysine still disturbs the passing Na^+ electrostatically. In other words, the E46 is the most optimal amino acid at this position for P2X3 channels for proper cation permeation: the polar, negatively charged side chain holds a stable shell of water around incoming Na^+ and, at the same time, is electrostatically favored. At this position, a substitution with lysine might have been too late to be decisive on chloride preference, as the main energy barriers are presented upstream, but the positive charge does reduce the Cl^- diffusion resistance by flipping the original repulsion from the negatively-charged E, allowing a moderate Cl^- current increase. Interestingly, rP2X5 possesses exactly a lysine at this position, suggesting the E/K is likely indeed engaged in the ion selectivity strategies of the P2XRs.

The R52 stands out as a positive charge located in a generally negatively charged

area. Only human P2X6 carries an arginine at the same position alongside the P2X3Rs compared across the family. A study of the equivalent threonine on the rP2X4 suggested that while the threonine is structurally involved in the extracellular vestibule, it does not play a crucial functional role in receptor activation (Rokic & Stojilkovic, 2013; Rokic et al., 2014). Here, the substitution of arginine for glutamic acid not only decreased the function, regardless of the sodium or chloride current the channels conduct, but also had no impact on ion selection. This result suggested that the arginine at this position might be involved in the lower body gating process instead of participating in the lateral fenestrations. It implies a unique local conformation that the hP2X3 undergoes during activation.

The T54 is highly conserved in P2XRs. The substitution of the T54 with valine caused a decrease in current during function tests in hP2X3Rs function, also suggesting a crucial role that it plays in maintaining the normal channel function. The threonine is located beyond the pore's entrance, especially in the apo state, where the clustered T54s, as shown in Figure 5.3.1, are embedded as part of a polar environment likely favouring transient Na^+ gathering. While having no impact on chloride permeation, the T54V had a distinct sodium permeation profile compared to the triple mutant: both conductance and preference for sodium changed, and the conductance decreasing is sodium concentration depend, as the factor $[\text{Na}^+] \times \text{construction}$ was detected as significant during these comparisons between T54V and the triple mutant.

As discussed above, the polarity environments near the pore entrance seem overwhelmingly important, so it is seen on T54V as well. During the mutation from T to V, the local polarity that attracts Na^+ and helps to keep them hydration is greatly disturbed. While comparing T54 to the E46, the gathering of ions at T54 is purely dependent on polarity interaction, while, besides the polarity, a more active electrostatic attraction is observed at E46, which could explain why ion accumulation at T54V shows changes that are ion-concentration-dependent; meanwhile, E46K creates a fixed electrostatic barrier that is difficult to be overcome by increasing in ion concentration. The moderate shifts of V_{rev} suggested a mild loss of Na^+ selectivity of T54V as well. As the sodium influx is highly dependent on the polarity in this area, the removal of T54 directly causes a decrease in sodium current, which might increase the favoring of outward K^+ current during the I/V voltage ramping.

Overall, R52 by the entrance of the pore might be directly engaged in the gating, as

its replacement reduces current without altering selectivity, implying a structural or kinetic effect rather than direct ion interaction; E46K and T54V show how a highly polar local environment is critical for inward cation flow. The addition negative charge of E46 further enriches Na^+ near the pore, whose electrostatic effect might even barricade the outward Cl^- current as a small energy barrier, helping explain the low chloride conductance of the typical P2X3 conductance.

5.3.2 The Transmembrane Domain

The transmembrane domains dominate the ion conduction of the channel to a great extent, as the pathway guides ions to travel through the cell membrane along their electrochemical gradient. In these experiments, the TMs were to be swapped with the ones from the rP2X5 channels, to investigate whether the TMs of the rP2X5 channel carry any structural features that contribute to the favoring of chloride.

The P2X3/X5 TM chimeras were first tested after expression in *Xenopus* oocytes. The TEVC results showed that all mutants generated on hP2X3 channels survived the domain swappings, while the rP2X5R with the TM2 region obtained from the hP2X3R did not. The TM1 domain, together with the TM2 domain of the hP2X3 channels, was later replaced by the ones obtained from the rP2X5. Function-damaged channels were seen on both the TEVC and patch clamp. Previous research also successfully created P2X1 channels that survived entire TMs swapping from P2X2Rs (Werner et al., 1996). This different tolerance level suggested that P2X5 might be composed of a unique architecture or undergo special conformational change during activation, which restricts its normal function within its TMs. From the perspective of biosynthesis, the more hydrophobic TM2 domain of the rP2X5 potentially requires an explicit terminus to provide a minor level of stabilizing effect during the transmembrane anchoring compared to the rest of the isoforms in the P2XR family (Öjemalm et al., 2013). Therefore, substituting rP2X5R TM2 with the TM2 domain from other isoforms might not collaborate with the rest of the rP2X5R structure. Performing further experiments to determine the expression level should help identify the reason for the silence of this construct.

The I/V curve of single activations obtained from the chimeras on the patch clamp revealed that no significant shifting of the reversal potential was observed for all

function TM chimeras. When comparing the I/V curves measured in NaCl-based or choline-Cl-based external solutions within each construct, the negative shifting of the V_{rev} varied, but without reaching significant differences. Similar results were seen when comparing the current amplitudes in different solutions of different constructs. The transmembrane domains appeared not to be the key to the high conductance and selectivity for chloride of the rP2X5 channels.

Subsequent experiments switched to TEVC, as the method improvement allowed such a kind of measurement. Compared to patch clamp, TEVC is also more straightforward, so it became the preferred approach. When comparing the shifts in V_{rev} observed in both sodium and chloride experiments, the ANOVA test did not identify construct differences as a statistically significant factor, suggesting neither a decrease in Na^+ nor an increase in Cl^- preference is detected during the TM2 from rP2X5 into hP2X3 channel.

However, in the sodium-based experiments, the differences in constructs, while not directly influencing the ΔV_{rev} , were still relevant. The two-way ANOVA revealed that although the '*construct*' factor itself was not significant, the interaction of '*construct* $\times$ $[Na^+]$ ' was significant with a P value of 0.0048. This indicates that, although the differences between selectivities in the two constructs do not reach a significant level, the two constructs react differently as external Na^+ concentration changes. The triple mutant exhibited a more pronounced response to changes in $[Na^+]$, indicating distinct permeation characteristics of the different channels. Such differences were not observed in the permeation of the chloride, as the factor of " *$[Cl^-] \times construct$* " had a P value of 0.9168. Overall, a better accommodation of accumulated Na^+ but not Cl^- ions of the P2X3 TM2 is seen, as in hP2X3 TM2, an increasing selectivity accompanies the increasing concentration of extracellular Na^+ .

Despite less impact on selectivity changes, the P2X3/X5 TM2 chimera is distinct from the triple mutant while focusing on ion conductance. Compared to the triple mutant, current amplitudes of the TM2 chimera dropped more slowly in response to decreased extracellular concentration; *vice versa*, a more rapid amplitude change was observed in the chimera when extracellular Cl^- changed. $P < 0.0001$ was detected in both experiments concerning the factor "*construct*". Those data revealed distinctive ion diffusion patterns in different TM2, suggesting that different pore structures in hP2X3 and rP2X5 contribute to their specific ion permeation: compared to the P2X5 TM2, the TM2 from rP2X5 is intrinsically smoother

for Cl^- ions traveling while creating a higher level of barricades for Na^+ .

Together, the results suggest that the TM2 of rP2X5 alters the conducting properties of the channel, likely by reducing the energy barrier for chloride while increasing it for sodium. This shift leads to a relative increase in chloride current and decrease in sodium current, without changing the intrinsic preference of the hP2X3 channel for conducting cations over anions.

The structural comparison of the different TM2 is shown in Figure 5.3.3, with hydrophobicity/hydrophilicity specified in the surfaces. Compared to the TM2 of hP2X3, the TM2 segment of rP2X5 enriches more small and hydrophobic residues, especially leucine, glycines, and alanines. These residues not only contribute to the local hydrophobicity, but the small size of glycines and alanines also potentially allows greater structural flexibility. Altogether, the rP2X5 channel possibly possesses a wider and more dynamic pore structure.

As discussed in the previous chapter, when cations such as Na^+ travel through the pore, they most likely go through mild dehydration before arriving at the T330, due to the restricted pore size and hydrophobicity of the pore lining V326 (relevant

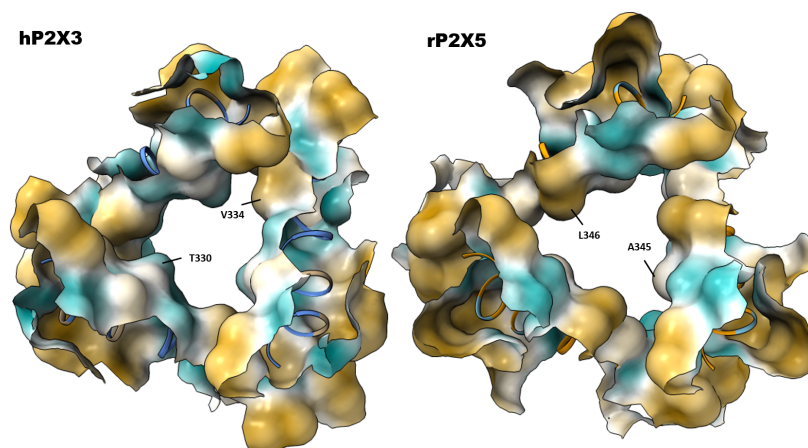


Figure 5.3.2: Top-down views of the TM2 helices of hP2X3 (PDB: 5SVJ) and rP2X5 (homology model generated by Swiss-Model) from the extracellular side, showing as surfaces with hydrophobicity color-coded from hydrophobic (yellow) to hydrophilic (blue). The narrowest points of the pore—residues T330 and V334 in hP2X3, and A345 and L346 in rP2X5, are labeled. Note that the hP2X3 pore is more constricted from the pore-lining residues' sidechain and more hydrophilic, while the rP2X5 pore is wider and more hydrophobic.

discussion see in **Chapter 5.2.3**). Subsequently, the T330 facilitated the partially hydrated ions passing the narrowest part of the pore. The partial dehydration is a compromise of low dehydration energy cost and size reduction of the passing ions. In the case of rP2X5 TM2, the small side chains of pore lining residues before the corresponding restriction side of A345/L346 do not encourage partial dehydration as V326 in P2X3 TM2; therefore, more Na⁺ ions still carry intact hydration shells when reaching the restriction site. Attempts to generate rP2X5-A345K homomers were unsuccessful (similar to the expression failure of T330K in the hP2X3 triple mutant context, which will be interpreted below), suggesting that this pore constriction cannot accommodate bulky, charged residues and is thus unlikely to permit passage of a fully hydrated Na⁺ (radius around 5 Å (Degreé et al., 1996)). Therefore, Na⁺ ions, having undergone less efficient dehydration yet still needing to significantly dehydrate to pass the constriction site, encounter A345/L346, which both lack polarity to effectively coordinate and stabilize these necessarily dehydrated Na⁺ ions. This combination of inefficient initial dehydration followed by an unassisted passage through a very restricted site lined by Alanine likely accounts for the observed decrease in Na⁺ conductance in rP2X5.

As for the chloride outflux, the hydrated chloride ions are easy to go from the entrance to the restriction of both TM2s of hP2X3 and rP2X5, due to the remarkably flexible area, as previously discussed: a highly flexible area is seen in hP2X3 TM2 (see **Chapter 5.2.1**). When considering the segment extending from the pore constriction site towards the intracellular side (e.g., corresponding to residues 331-341 in hP2X3), rP2X5 shares strong sequence similarity with hP2X3 in this specific region, suggesting that their flexibility in this particular segment is likely comparable. Although the restriction positions might differ in size, T330 in P2X3 might act as the first barrier for chloride ions. After the efflux passes the restriction site, the chloride ions in rP2X5 are surrounded by another highly flexible area, given the appearance of glycines and alanine, while in p2x3, as discussed, the corresponding area is significantly more rigid. As a consequence, the rP2X5 TM2, compared to the hP2X3 one, exhibits increased Cl⁻ conductance and decreased Na⁺ conductance.

Besides the complete TM2 swapping, the T330 residue in hP2X3, which was previously proposed as part of the ion filter, was also mutated to lysine. In contrast to the equivalent T339K in P2X2, which expressed normally, the T330K mutant in P2X3 failed to express as a homomer, as did the T330R and the corresponding A345K in rP2X5. This suggests that lysine at this position is less tolerated in P2X3

and P2X5, possibly due to structural constraints within the pore. A similar effect has been observed in AMPA receptors, where four arginines at adjacent positions within a tetramer disrupted proper assembly (Greger et al., 2003). Compared to the tolerance of three lysines at the same position in P2X2 pore, the incapability of P2X3 and P2X5 pores to accommodate may reflect their more restricted trimer architecture.

In theory, the successfully generated heteromers of T330K and T330-based K63A construct contain two lysines at the position of 330 (T330-T330K-T330K as in one heteromer), as only one K63A monomer was allowed to be incorporated in a functional trimer, to ensure at least two out of three binding pockets are available for ATP docking, as activation of the channel requires a minimum of two bound ATP molecules. This also implies that, while the T330K mutant cannot be assembled as homomers, the pore can still tolerate up to two lysine substitutions at this position.

The 2-way ANOVA detected significant differences in all tests and revealed a distinctive ion permeation pattern induced by the lysine insertion. During the decrease of extracellular sodium concentration, significant differences between the triple mutant and the T330K homomers were not only detected in the factor *construct* (I₂/I₁ and ΔV_{rev} values) but also factor *construct* $\times$ $[Na^+]$ (ΔV_{rev} values). Those results indicated that the T330K exhibits less conductance and a preference for sodium ions, while the two constructs also differ in their sodium concentration dependency. Similarly, the significance in the tests from I₂/I₁ and ΔV_{rev} values comparisons in Cl⁻-decreasing experiments suggests more pronounced Cl⁻ ion selectivity and conductance of the T330K heteromers compared to the triple mutant. Taken together, the insertion of lysine in the narrowest part of the pore has a great impact on both Na⁺ and Cl⁻ flows, and from the perspectives of both ion diffusion resistance and active selectivity.

The insertion of lysines within the pore creates electrostatic barricades for sodium influx, while compensating energy cost that the chloride eflux requires. Moreover, the charged site directly repels cations and attracts anions, amplifying the selectivity between them. This demonstrates that although conductance and selectivity are deliberately distinguished as diffusion resistance versus ion preference, strongly charged residues at key pore positions can influence both properties in parallel. Due to the intrinsic expression problem, it cannot be concluded whether a homomeric T330K-hP2X3 channel could further increase the chloride preference as

observed on rP2X2Rs by Browne et al (Browne et al., 2011). However, it indeed suggests that the clustered positive charge in the pore could serve as an ion filter.

In summary, while a positively charged residue at position 330 clearly alters both conductance and selectivity, swapping the entire TM2 from P2X5 into hP2X3 leads to increased chloride conductance without altering selectivity. This implies that although TM2 can, in principle, contribute to ion selectivity, as shown by the T-to-K substitution, it does not function as a selectivity filter on its own in the P2X5 background. This indicates that while TM2 does not determine chloride selectivity in P2X5, it facilitates chloride conductance.

5.3.3 Intracellular Cytoplasmic Fenestration

The cytoplasmic fenestration is supposedly the ion pathway connecting the transmembrane pore and the intracellular side. Like the extracellular fenestration, the pathway is hypothesized to be tilted away from the central axis, allowing the ions to exit and enter the pore via the sides of the cytoplasmic cap instead of the central pore (Mansoor et al., 2016).

Sequence alignment among P2X channels showed that a positively charged lysine is inhabitant on the N-termini of P2X5 and P2X6 channels, where the rest of the family possesses a negative charge. Replacing the original negative charge in the P2X2 channel caused an increased anion permeation (Tam et al., 2023). The equivalent glutamic acid at position 11 of the hP2X3R was mutated into K in this part of the experiments, to test whether the charge reversal affects the ion preference. Besides, the previously discussed K14C was added in parallel in part of the experiments as well, to find out whether the conserved cytoplasmic lysine also contributes to the intrinsic anion permeation of the channel, though it is located away from the proposed cytoplasmic fenestration.

The replacement of E11 with lysine, E11K, reduced the current amplitudes extensively. up to 90% compared to the triple mutant. In **Chapters 4.1** and **5.1**, where the cytoplasmic cap was characterized structurally, the same E was also mutated into cysteine (E11C), from where no such dramatic functional interferences was observed. This result was in accordance with what was reported for the equivalent E17C in rP2X2 channels by Tam et al. (Tam et al., 2023). These results

together report that rather than the loss of the negative charge, the addition of a positive charge is more likely responsible for the functional damage. Besides, no change in kinetics during activation was observed in either mutant, which, adding up to similar results observed from the E11C, implied that the contribution of the glutamic acid to the stability of the cytoplasmic cap might be minor as well.

Like the T330K channels, the E11K mutant demonstrated a dramatically altered ion permeation. A significant increase in the chloride current and a decrease in the sodium current together proved that the E11K channel favored chloride current. In the I₂/I₁ comparisons, E11K showed significant differences from the triple mutant in both Na⁺ and Cl⁻ experiments, with p-values below 0.0001. ΔV_{rev} comparisons also indicated that the E11K mutant preferred Cl⁻ over Na⁺ ions. Interestingly, the factor “[Na⁺] × Construct” was found to be significant only in the ΔV_{rev} comparison under Na⁺ conditions, similar to what was seen with T330K, suggesting that the concentration-dependence of sodium selectivity of the E11K changes compared to the triple mutant.

Therefore, the insertion of lysine, either within the transmembrane channel (T330K) or in the cytoplasmic fenestration (E11K), functioned as a Cl⁻ selector by reducing the sodium current and enhancing the Cl⁻ current. Because of the similarity, the comparison between the two lysine mutants was performed. Results demonstrated that no differences in effect were seen as the factor *construct* was insignificant; meanwhile, the factor *[ion] × construct* was significantly different, indicating that their overall impact on chloride permeability is comparable, but the Cl⁻ concentration dependency is different.

The 14K is highly conserved within the P2X family, substitutions of which are often (functionally) lethal. In previous experiments, the K14C channel was found to be damaged to a minor level and was thereby tested in this part of the experiments. This mutant was only tested for the ΔV_{rev} values in varied Cl⁻ concentrations, as its I₂/I₁ values might be distorted by its dramatically larger rundown effect. No significances were found during the comparison between the mutant and the control (0.0737), however, the value approaching the threshold made it intriguing to evaluate the trend of the comparisons. Multiple tests showed that K14C was not as sensitive to the change in [Cl⁻] as the triple mutant, indicating a tendency towards lower Cl⁻ selectivity. In other words, the hP2X3 channels possess an intrinsic and weak chloride selectivity.

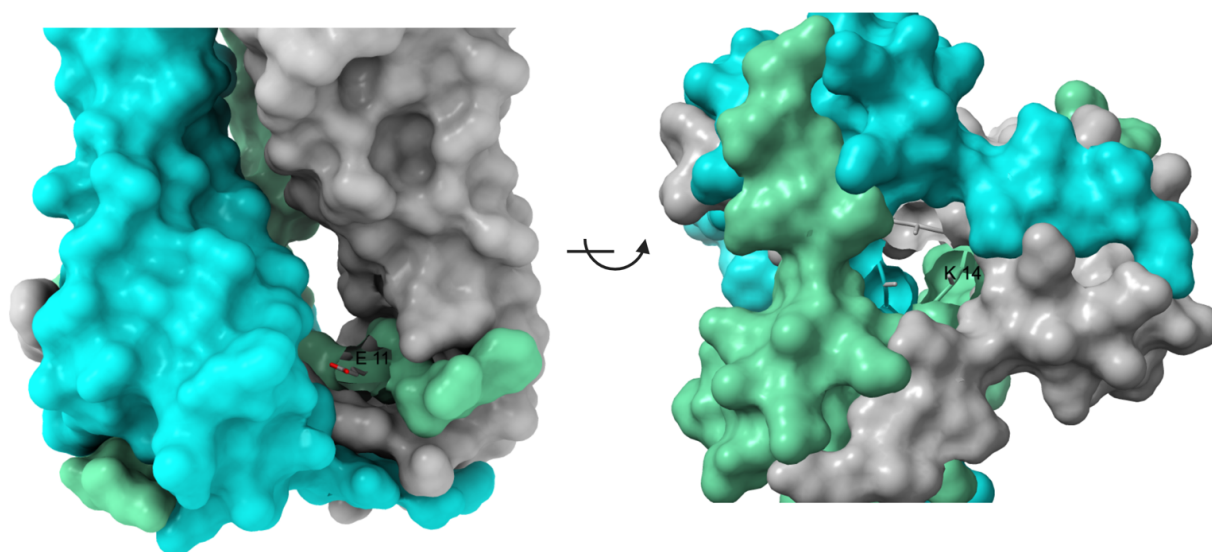


Figure 5.3.3: TMs and cytoplasmic cap of the hP2X3 trimer in the open state (5SVJ). The main structure is meshed in surface with monomers in different colors. The left image provides a horizontal view of the structure (with the central axis perpendicular to the cell membrane), where E11 is shown in sticks. It can be observed that E11 is located at the lateral entrance of the cap. The right image shows the structure after being rotated 90° along the horizontal axis, viewed from the direction of cytoplasm. It reveals that the cap center, formed by the three monomers, has created a pore, with each contributing a lysine (K14) approaching at this location.

The two residues studied here are indicated in Figure 5.3.3. The E11 is located adjacent to the pore from the intracellular side, while the K14 is at the central pore constructed by the cap. The enormous changes brought by the E11K but not the K14C, proved the importance of the lateral fenestration for ion selectivity.

Overall, E11K demonstrates a clear gain in Cl^- and loss in Na^+ selectivity, whereas the rP2X5 TM2 provides a more ideal ion pathway for Cl^- without adding further selectivity. Together, E11K sets the preference, and the TM2 swap improves conductance, providing two distinct yet complementary mechanisms to enhance chloride flow.

After establishing a robust method and clearly validating the effects of E11K and the TM2 chimera individually, the earlier experimental approach, which was initially discarded due to higher noise and a weaker signal-to-noise ratio and brought extra error (details described in **Results**), was revisited. By directly comparing these previously obtained data with the current validated results, the reliability of the initial experiments for detecting sufficiently strong mutant effects could be assessed. If so, the data of the double mutant, E11K-TM2 chimera-triple mutant, collected under the original experimental conditions, are also valid for analysis and comparison to an extent.

The double mutant, E11K-TM2 chimera, was initially tested alongside the triple mutant and single mutants (E11K and TM2 chimera) under the following experimental design: channels were activated first in standard Ori^- solution and subsequently reactivated after washout in solutions lacking either sodium or chloride ions. Current ratios reflecting changes in conductance and shifts in ΔV_{rev} , indicating ion selectivity differences, were recorded. This step aimed to verify whether comparisons made under the initial conditions between the triple mutant and single mutants (E11K and chimera) yielded results comparable to those obtained later under more robust experimental conditions.

The solution factor used for these two-way ANOVA analyses was switched from the concentration gradient for each ion to a comprehensive inclusion of standard NaCl, sodium-only, or chloride-only conditions, while other aspects of the analysis remained unchanged. When comparing values obtained in both solutions, the TM2 chimera showed a distinct conductance profile compared to the triple mutant, with a P value of construct differences less than 0.0001. Meanwhile, its ion selectivity, represented by the ΔV_{rev} , was similar to the triple mutant ($P = 0.1569$). In the

case of E11K, both results were significantly different from the triple mutant, with P values of 0.0015 and less than 0.0001 in respectively, demonstrating changes in both conductance and selectivity. These findings were consistent with the previously established results, confirming that the initial experimental approach was at least trustworthy at the qualitative analysis level.

After validating the initial experimental conditions through the adjusted two-way ANOVA, the analysis was then extended to the double mutant, E11K-TM2 chimera. When comparing the I2/I1 values obtained in both solutions, the differences between the triple mutant and the E11K-TM2 chimera were identified, with a P value of 0.0007. Further multiple comparisons indicated that the significant difference primarily arose from comparisons between the two tested conditions: the E11K-TM2 chimera exhibited higher I2/I1 ratios when solutions were switched from standard NaCl to chloride-only conditions than when switched to sodium-only conditions. Given that there is currently no evidence suggesting that changing between sodium-only and chloride-only solutions would differently affect the rundown effect, the higher I2/I1 ratio in chloride-only conditions indicates that the double mutant exhibits improved conduction in the Cl⁻ solution, implying enhanced chloride permeability. However, when comparing the double mutant with the single mutant, no significant differences were detected, with P values of 0.4988 (vs. TM2 chimera) and 0.4932 (vs. E11K).

A similar effect was found during the test of ΔV_{rev} values. The E11K-TM2 chimera still showed an altered selectivity, with a significant difference of 0.005 when compared to the triple mutant. However, the differences between the control and were smaller than the one with E11K.

Overall, the double mutant of E11K and the TM2 chimera did not show any additivity to the ion permeation modifications. On the contrary, the resulting channels, while still exhibiting similarity to the single mutants, behaved not as dramatically differently from the triple mutant. Instead of inheriting from both mutants, the double mutant was a more moderate construct, from the perspectives of both conductance and selectivity. This result implies an interaction or coupling between the pore and the cytoplasmic fenestration.

In summary, the ion permeation properties of the hP2X3 channel were examined through three structural domains: the extracellular fenestration, the transmembrane pore, and the cytoplasmic fenestration. Charge clusters near the lateral fen-

estration appear to assist hydrated cations in reaching the pore entrance, though single-residue mutations showed only minor individual effects. The TM2 pore of hP2X3 exhibits intrinsic chloride conductance, and the incorporation of the rP2X5 TM2 further increased conductance without enhancing Cl⁻ selectivity, suggesting that structural differences in the pore region influence general ion flow rather than strict ion preference.

In contrast, mutating residues at the cytoplasmic fenestration had a strong and selective impact, shifting the channel's overall cation/anion selectivity and thereby altering net ion permeation. While permeation was assessed in terms of both conductance and selectivity, changes in selectivity were found to have a greater influence on global ion flow. This also indicates that conductance differences between hP2X3 and rP2X5 are relatively modest.

6. Summary

In this study, the human P2X3 receptor was investigated from a biophysical perspective to reveal how specific structural domains regulate receptor gating and ion permeation under functional conditions. Guided by available crystal structures and electrophysiological data, the research focused sequentially on the cytoplasmic cap, the adjacent TM2 helix, and the receptor's ion permeation pathway.

The cytoplasmic cap was shown to be essential for receptor activation and functional stability, with structural integrity maintained primarily by ionic and hydrophobic interactions. Experiments revealed that while removal of the N-terminus disrupted receptor function, the presence of a single C-terminus was sufficient for receptor gating. These findings indicate a critical role for precise spatial organization within the cap region for proper receptor function.

The TM2 domain was systematically investigated by cysteine scanning mutagenesis, identifying residues I323, T330, and V334 as critical determinants of channel gating. Additionally, residue V326 appeared to influence ion accessibility to the pore region, suggesting involvement in gating and pore occlusion. The results further clarified TM2's dual role: it forms the primary pore structure and serves as a functional interface linking extracellular and intracellular domains.

Lastly, the study assessed ion permeation through extracellular fenestrations, the TM2 pore, and cytoplasmic fenestrations. Although extracellular fenestration residues individually contributed minimally, charge clusters near these regions facilitated cation entry. Intrinsic chloride conductance of the TM2 pore was sensitive to structural variations, as introducing the rP2X5 TM2 to the hP2X3 channel resulted in increased ion conductance without affecting selectivity. In contrast, mutations within the cytoplasmic fenestrations profoundly altered channel selectivity, sig-

nificantly impacting overall permeation characteristics. These findings identify key structural determinants governing the ion permeation profile of the hP2X3 receptor.

Together, this study provides insights into the structural and functional interplay among key receptor domains, contributing to a more comprehensive understanding of P2X3 receptor function under physiological conditions.

Bibliography

- Addgene. (2023). *Plasmids 101: A guide to understanding plasmids* (Fourth Edition) [Accessed: October 21, 2024].
- Aguero, T., Newman, K., & King, M. L. (2018). Microinjection of xenopus oocytes. *Cold Spring Harbor Protocols*, 2018(2). <https://doi.org/10.1101/pdb.prot096974>
- Alliegro, M. C. (2000). Effects of dithiothreitol on protein activity unrelated to thiol–disulfide exchange: For consideration in the analysis of protein function with cleland’s reagent. *Analytical Biochemistry*, 282(1), 102–106. <https://doi.org/10.1006/abio.2000.4557>
- Allsopp, R. C., El Ajouz, S., Schmid, R., & Evans, R. J. (2011). Cysteine scanning mutagenesis (residues glu52–gly96) of the human P2X1 receptor for ATP: Mapping agonist binding and channel gating. *The Journal of Biological Chemistry*, 286(33), 29207–29217. <https://doi.org/10.1074/jbc.M111.260364>
- Axon Guide. (2021). The axon guide for electrophysiology and biophysics laboratory techniques.
- Barish, M. E. (1983). A transient calcium-dependent chloride current in the immature xenopus oocyte. *Journal of Physiology*, 342, 309–325. <https://doi.org/10.1113/jphysiol.1983.sp014852>
- Bennetts, F. M., Mobbs, J. I., Ventura, S., & Thal, D. M. (2022). The P2X1 receptor as a therapeutic target. *Purinergic Signalling*, 18(4), 421–433. <https://doi.org/10.1007/s11302-022-09880-4>
- Bhargava, Y., Rettinger, J., & Mourot, A. (2012). Allosteric nature of P2X receptor activation probed by photoaffinity labelling. *British Journal of Pharmacology*, 167(6), 1301–1310. <https://doi.org/10.1111/j.1476-5381.2012.02083.x>

- Bodnar, M., Wang, H., Riedel, T., Hintze, S., Kato, E., Fallah, G., Gröger-Arndt, H., Giniatullin, R., Grohmann, M., Hausmann, R., Schmalzing, G., Illes, P., & Rubini, P. (2011). Amino acid residues constituting the agonist binding site of the human P2X3 receptor. *Journal of Biological Chemistry*, 286(4), 2739–2749. <https://doi.org/10.1074/jbc.M110.167437>
- Boué-Grabot, E., Archambault, V., & Séguéla, P. (2000). A protein kinase c site highly conserved in P2X subunits controls the desensitization kinetics of P2X(2) ATP-gated channels. *The Journal of Biological Chemistry*, 275(14), 10190–10195. <https://doi.org/10.1074/jbc.275.14.10190>
- Browne, L. E., Cao, L., Broomhead, H. E., Bragg, L., Wilkinson, W. J., & North, R. A. (2011). P2X receptor channels show threefold symmetry in ionic charge selectivity and unitary conductance. *Nature Neuroscience*, 14(1), 17–18. <https://doi.org/10.1038/nn.2705>
- Browne, L. E., Compan, V., Bragg, L., & North, R. A. (2013). P2X7 receptor channels allow direct permeation of nanometer-sized dyes. *The Journal of Neuroscience*, 33(8), 3557–3566. <https://doi.org/10.1523/JNEUROSCI.2235-12.2013>
- Burnstock, G. (1972). Purinergic nerves. *Pharmacological Reviews*, 24(3), 509–581.
- Burnstock, G. (1976). Do some nerve cells release more than one transmitter? *Neuroscience*, 1(4), 239–248. [https://doi.org/10.1016/0306-4522\(76\)90054-3](https://doi.org/10.1016/0306-4522(76)90054-3)
- Burnstock, G. (1990). Noradrenaline and ATP as cotransmitters in sympathetic nerves. *Neurochemistry International*, 17(2), 357–368. [https://doi.org/10.1016/0197-0186\(90\)90158-p](https://doi.org/10.1016/0197-0186(90)90158-p)
- Burnstock, G. (2000). P2X receptors in sensory neurones. *British Journal of Anaesthesia*, 84(4), 476–488. <https://doi.org/10.1093/oxfordjournals.bja.a013473>
- Burnstock, G. (2018). Purine and purinergic receptors. *Brain and neuroscience advances*, 2, 239821281881749. <https://doi.org/10.1177/2398212818817494>
- Cao, L., Broomhead, H. E., Young, M. T., & North, R. A. (2009). Polar residues in the second transmembrane domain of the rat P2X2 receptor that affect spontaneous gating, unitary conductance, and rectification. *The Journal of Neuroscience*, 29(45), 14257–14264. <https://doi.org/10.1523/JNEUROSCI.4403-09.2009>
- Chataigneau, T., Lemoine, D., & Grutter, T. (2013). Exploring the ATP-binding site of P2X receptors. *Frontiers in Cellular Neuroscience*, 7, 273. <https://doi.org/10.3389/fncel.2013.00273>

- Chaumont, S., Jiang, L.-H., Penna, A., North, R. A., & Rassendren, F. (2004). Identification of a trafficking motif involved in the stabilization and polarization of P2X receptors. *The Journal of Biological Chemistry*, 279(28), 29628–29638. <https://doi.org/10.1074/jbc.M403940200>
- Choi, L.-S., Mach, T., & Bayley, H. (2013). Rates and stoichiometries of metal ion probes of cysteine residues within ion channels. *Biophysical Journal*, 105(2), 356–364. <https://doi.org/10.1016/j.bpj.2013.04.046>
- Coddou, C., Stojilkovic, S. S., & Huidobro-Toro, J. P. (2011). Allosteric modulation of ATP-gated P2X receptor channels. *Reviews in the neurosciences*, 22(3), 335–354. <https://doi.org/10.1515/{RNS}.2011.014>
- Conant, A. R., Fisher, M. J., McLennan, A. G., & Simpson, A. W. (2000). Diadenosine polyphosphates are largely ineffective as agonists at natively expressed P2Y(1) and P2Y(2) receptors on cultured human saphenous vein endothelial cells. *Journal of Vascular Research*, 37(6), 548–555. <https://doi.org/10.1159/000054088>
- Danielli, L., Li, X., Tuller, T., & Daniel, R. (2020). Quantifying the distribution of protein oligomerization degree reflects cellular information capacity. *Scientific Reports*, 10(1), 17689. <https://doi.org/10.1038/s41598-020-74811-5>
- Degrève, L., Vecchi, S. M., & Junior, C. Q. (1996). The hydration structure of the Na^+ and K^+ ions and the selectivity of their ionic channels. *Biochimica et Biophysica Acta*, 1274, 149–156.
- Dhuna, K., Felgate, M., Bidula, S. M., Walpole, S., Bibic, L., Cromer, B. A., Angulo, J., Sanderson, J., Stebbing, M. J., & Stokes, L. (2019). Ginsenosides act as positive modulators of P2X4 receptors. *Molecular Pharmacology*, 95(2), 210–221. <https://doi.org/10.1124/mol.118.113696>
- Ding, S., & Sachs, F. (1999). Single channel properties of P2X2 purinoceptors. *The Journal of General Physiology*, 113(5), 695–720. <https://doi.org/10.1085/jgp.113.5.695>
- Egan, T. M., Haines, W. R., & Voigt, M. M. (1998). A domain contributing to the ion channel of ATP-gated P2X2 receptors identified by the substituted cysteine accessibility method. *The Journal of Neuroscience*, 18(7), 2350–2359. <https://doi.org/10.1523/{JNEUROSCI}.18-07-02350.1998>
- Egan, T. M., & Khakh, B. S. (2004). Contribution of calcium ions to p2x channel responses. *The Journal of Neuroscience*, 24(13), 3413–3420. <https://doi.org/10.1523/JNEUROSCI.5429-03.2004>

- Ennion, S., Hagan, S., & Evans, R. (2000). The role of positively charged amino acids in ATP recognition by human P2X1 receptors. *The Journal of Biological Chemistry*, 275(45), 35656.
- European Medicines Agency. (2023). Lyfnua: Epar – medicine overview [Accessed: 2024-11-05]. <https://www.ema.europa.eu/en/medicines/human/EPAR/lyfnua>
- Fallah, G., Römer, T., Detro-Dassen, S., Braam, U., Markwardt, F., & Schmalzing, G. (2011). Tmem16a(a)/anoctamin-1 shares a homodimeric architecture with clc chloride channels. *Molecular & Cellular Proteomics*, 10(2), S1–S11. <https://doi.org/10.1074/mcp.M110.004697>
- Ford, A. P., Jarvis, M. F., Burgard, M. J., McGaraughty, M. S., Honore, M. J., Lynch, M., Brennan, M., Subieta, M., Biesen, M. V., Cartmell, M., Bianchi, M. B., Niforatos, M., Kage, M. K., Yu, M., Mikusa, M., Wismer, M. S., Zhu, M., Chu, M., Kohlhaas, M., ... Kowaluk, M. K. (2011). Af353, a novel, potent and orally bioavailable p2x3/p2x2/3 receptor antagonist, reduces chronic inflammatory and neuropathic pain in the rat. *British Journal of Pharmacology*, 160(6), 1387–1398. <https://doi.org/10.1111/j.1476-5381.2010.00796.x>
- Franke, H., Klimke, K., Brinckmann, U., Grosche, J., Francke, M., Sperlagh, B., Reichenbach, A., Liebert, U. G., & Illes, P. (2005). P2X(7) receptor-mRNA and -protein in the mouse retina; changes during retinal degeneration in BALBCrds mice. *Neurochemistry International*, 47(4), 235–242. <https://doi.org/10.1016/j.neuint.2005.04.022>
- Fryatt, A. G., Dayl, S., Cullis, P. M., Schmid, R., & Evans, R. J. (2016). Mechanistic insights from resolving ligand-dependent kinetics of conformational changes at ATP-gated P2X1R ion channels. *Scientific Reports*, 6, 32918. <https://doi.org/10.1038/srep32918>
- Gamper, N. (Ed.). (2013). *Ion channels* (Vol. 998). Humana Press. <https://doi.org/10.1007/978-1-62703-351-0>
- Gao, X., & Hwang, T.-C. (2016). Spatial positioning of CFTR's pore-lining residues affirms an asymmetrical contribution of transmembrane segments to the anion permeation pathway. *The Journal of General Physiology*, 147(5), 407–422. <https://doi.org/10.1085/jgp.201511557>
- GenomicsAgilent. (2007). Large insertions : Two simple steps using quikchange®ii site-directed mutagenesis kits.
- Giles, N. M., Watts, A. B., Giles, G. I., Fry, F. H., Littlechild, J. A., & Jacob, C. (2003). Metal and redox modulation of cysteine protein function. *Chem-*

- istry & Biology*, 10(8), 677–693. [https://doi.org/10.1016/s1074-5521\(03\)00174-1](https://doi.org/10.1016/s1074-5521(03)00174-1)
- Giniatullin, R., & Nistri, A. (2013). Desensitization properties of P2X3 receptors shaping pain signaling. *Frontiers in Cellular Neuroscience*, 7, 245. <https://doi.org/10.3389/fncel.2013.00245>
- Gloor, S., Pongs, O., & Schmalzing, G. (1995). A vector for the synthesis of cRNAs encoding myc epitope-tagged proteins in xenopus laevis oocytes. *Gene*, 160(2), 213–217. [https://doi.org/10.1016/0378-1119\(95\)00226-v](https://doi.org/10.1016/0378-1119(95)00226-v)
- Gray, V. E., Hause, R. J., & Fowler, D. M. (2017). Analysis of large-scale mutagenesis data to assess the impact of single amino acid substitutions. *Genetics*, 207(1), 53–61. <https://doi.org/10.1534/genetics.117.300064>
- Greger, I. H., Khatri, L., Kong, X., & Ziff, E. B. (2003). Ampa receptor tetramerization is mediated by q/r editing. *Neuron*, 40(4), 763–774. [https://doi.org/10.1016/S0896-6273\(03\)00668-8](https://doi.org/10.1016/S0896-6273(03)00668-8)
- Habermacher, C., Dunning, K., Chataigneau, T., & Grutter, T. (2016). Molecular structure and function of P2X receptors. *Neuropharmacology*, 104, 18–30. <https://doi.org/10.1016/j.neuropharm.2015.07.032>
- Hattori, M., & Gouaux, E. (2012). Molecular mechanism of ATP binding and ion channel activation in P2X receptors. *Nature*, 485(7397), 207–212. <https://doi.org/10.1038/nature11010>
- Hausmann, R., Bahrenberg, G., Kuhlmann, D., Schumacher, M., Braam, U., Bieler, D., Schlusche, I., & Schmalzing, G. (2014). A hydrophobic residue in position 15 of the rP2X3 receptor slows desensitization and reveals properties beneficial for pharmacological analysis and high-throughput screening. *Neuropharmacology*, 79, 603–615. <https://doi.org/10.1016/j.neuropharm.2014.01.010>
- Hausmann, R., Bodnar, M., Woltersdorf, R., Wang, H., Fuchs, M., Messemer, N., Qin, Y., Günther, J., Riedel, T., Grohmann, M., Nieber, K., Schmalzing, G., Rubini, P., & Illes, P. (2012). ATP binding site mutagenesis reveals different subunit stoichiometry of functional P2X2/3 and P2X2/6 receptors. *The Journal of Biological Chemistry*, 287(17), 13930–13943. <https://doi.org/10.1074/jbc.M112.345207>
- Hausmann, R., Günther, J., Kless, A., Kuhlmann, D., Kassack, M. U., Bahrenberg, G., Markwardt, F., & Schmalzing, G. (2013). Salt bridge switching from arg290/glu167 to arg290/ATP promotes the closed-to-open transition of the P2X2 receptor. *Molecular Pharmacology*, 83(1), 73–84. <https://doi.org/10.1124/mol.112.081489>

- Heymann, G., Dai, J., Li, M., Silberberg, S. D., Zhou, H.-X., & Swartz, K. J. (2013). Inter- and intrasubunit interactions between transmembrane helices in the open state of P2X receptor channels. *Proceedings of the National Academy of Sciences of the United States of America*, 110(42), E4045–54. <https://doi.org/10.1073/pnas.1311071110>
- Hille, B. (2001). *Ion channels of excitable membranes* (3rd ed.). Sinauer Associates is an imprint of Oxford University Press.
- Horovitz, A. (1996). Double-mutant cycles: A powerful tool for analyzing protein structure and function. *Folding & design*, 1(6), R121–6. [https://doi.org/10.1016/S1359-0278\(96\)00056-9](https://doi.org/10.1016/S1359-0278(96)00056-9)
- Illes, P., Müller, C. E., Jacobson, K. A., Grutter, T., Nicke, A., Fountain, S. J., Kennedy, C., Schmalzing, G., Jarvis, M. F., Stojilkovic, S. S., King, B. F., & Di Virgilio, F. (2021). Update of P2X receptor properties and their pharmacology: IUPHAR review 30. *British Journal of Pharmacology*, 178(3), 489–514. <https://doi.org/10.1111/bph.15299>
- invitrogen. (2012, September). pcDNA™5/FRT vector USER GUIDE.
- Jarvis, M. F., Burgard, M. J., McGaraughty, M. S., Honore, M. J., Lynch, M., Brennan, M., Subieta, M., Biesen, M. V., Cartmell, M., Bianchi, M. B., Niforatos, M., Kage, M. K., Yu, M., Mikusa, M., Wismer, M. S., Zhu, M., Chu, M., Kohlhaas, M., Alexander, M. K., ... Kowaluk, M. K. (2002). A-317491, a novel potent and selective non-nucleotide antagonist of p2x3 and p2x2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat. *Proceedings of the National Academy of Sciences*, 99(26), 17179–17184. <https://doi.org/10.1073/pnas.252537299>
- Jiang, L. H., Rassendren, F., Surprenant, A., & North, R. A. (2000). Identification of amino acid residues contributing to the ATP-binding site of a purinergic P2X receptor. *The Journal of Biological Chemistry*, 275(44), 34190–34196. <https://doi.org/10.1074/jbc.M005481200>
- Jiang, R., Taly, A., Lemoine, D., Martz, A., Cunrath, O., & Grutter, T. (2012). Tightening of the atp-binding sites induces the opening of p2x receptor channels. *The EMBO Journal*, 31(9), 2134–2143. <https://doi.org/10.1038/emboj.2012.75>
- Jiang, R., Taly, A., Lemoine, D., Martz, A., Specht, A., & Grutter, T. (2012). Intermediate closed channel state(s) precede(s) activation in the atp-gated p2x2 receptor. *Channels*, 6(5), 398–402. <https://doi.org/10.4161/chan.21520>
- Karasawa, A., & Kawate, T. (2016). Structural basis for subtype-specific inhibition of the P2X7 receptor. *eLife*, 5. <https://doi.org/10.7554/eLife.22153>

- Kasuya, G., Yamaura, T., Ma, X.-B., Nakamura, R., Takemoto, M., Nagumo, H., Tanaka, E., Dohmae, N., Nakane, T., Yu, Y., Ishitani, R., Matsuzaki, O., Hattori, M., & Nureki, O. (2017). Structural insights into the competitive inhibition of the ATP-gated P2X receptor channel. *Nature Communications*, 8(1), 876. <https://doi.org/10.1038/s41467-017-00887-9>
- Kawate, T. (2017). P2X receptor activation. *Advances in Experimental Medicine and Biology*, 1051, 55–69. https://doi.org/10.1007/5584_2017_55
- Kawate, T., Michel, J. C., Birdsong, W. T., & Gouaux, E. (2009). Crystal structure of the ATP-gated P2X(4) ion channel in the closed state. *Nature*, 460(7255), 592–598. <https://doi.org/10.1038/nature08198>
- Kawate, T., Robertson, J. L., Li, M., Silberberg, S. D., & Swartz, K. J. (2011a). Ion access pathway to the transmembrane pore in p2x receptor channels. *The Journal of General Physiology*, 137(6), 579–590. <https://doi.org/10.1085/jgp.201010593>
- Kawate, T., Robertson, J. L., Li, M., Silberberg, S. D., & Swartz, K. J. (2011b). Ion access pathway to the transmembrane pore in P2X receptor channels. *The Journal of General Physiology*, 137(6), 579–590. <https://doi.org/10.1085/jgp.201010593>
- Khakh, B. S., & North, R. A. (2012). Neuromodulation by extracellular ATP and P2X receptors in the CNS. *Neuron*, 76(1), 51–69. <https://doi.org/10.1016/j.neuron.2012.09.024>
- Li, M., Chang, T.-H., Silberberg, S. D., & Swartz, K. J. (2008). Gating the pore of P2X receptor channels. *Nature Neuroscience*, 11(8), 883–887. <https://doi.org/10.1038/nn.2151>
- Li, M., Kawate, T., Silberberg, S. D., & Swartz, K. J. (2010). Pore-opening mechanism in trimeric P2X receptor channels. *Nature Communications*, 1(4), 44. <https://doi.org/10.1038/ncomms1048>
- Li, M., Silberberg, S. D., & Swartz, K. J. (2013). Subtype-specific control of P2X receptor channel signaling by ATP and mg^{2+} . *Proceedings of the National Academy of Sciences of the United States of America*, 110(36), E3455–63. <https://doi.org/10.1073/pnas.1308088110>
- Li, M., Toombes, G. E. S., Silberberg, S. D., & Swartz, K. J. (2015). Physical basis of apparent pore-dilation of atp-activated p2x receptor channels. *Nature Neuroscience*, 18(11), 1577–1583. <https://doi.org/10.1038/nn.4120>
- Li, M., Wang, Y., Banerjee, R., Marinelli, F., Silberberg, S., Faraldo-Gómez, J. D., Hattori, M., & Swartz, K. J. (2019). Molecular mechanisms of human P2X3 receptor channel activation and modulation by divalent cation bound ATP. *eLife*, 8. <https://doi.org/10.7554/eLife.47060>

- Lin, Y. Y., Lu, Y., Li, C. Y., Ma, X. F., Shao, M. Q., Gao, Y. H., Zhang, Y. Q., Jiang, H. N., Liu, Y., Yang, Y., Huang, L. D., Cao, P., Wang, H. S., Wang, J., & Yu, Y. (2024). Finely ordered intracellular domain harbors an allosteric site to modulate physiopathological function of P2X3 receptors. *Nature Communications*, 15(1), 7652. <https://doi.org/10.1038/s41467-024-51815-7>
- Linsdell, P. (2015a). Metal bridges to probe membrane ion channel structure and function. *Biomolecular Concepts*, 6(3), 191–203. <https://doi.org/doi:10.1515/bmc-2015-0013>
- Linsdell, P. (2015b). Metal bridges to probe membrane ion channel structure and function. *Biomolecular concepts*, 6(3), 191–203. <https://doi.org/10.1515/bmc-2015-0013>
- Luckey, M. (2008). *Membrane structural biology: With biochemical and biophysical foundations*. Cambridge University Press. <https://doi.org/10.1017/{CBO9780511811098}>
- Mahler, J., & Persson, I. (2012). A study of the hydration of the alkali metal ions in aqueous solution. *Inorganic Chemistry*, 51(1), 425–438. <https://doi.org/10.1021/ic2018693>
- Mansoor, S. E., Lü, W., Oosterheert, W., Shekhar, M., Tajkhorshid, E., & Gouaux, E. (2016). X-ray structures define human P2X(3) receptor gating cycle and antagonist action. *Nature*, 538(7623), 66–71. <https://doi.org/10.1038/nature19367>
- Marquez-Klaka, B., Rettinger, J., Bhargava, Y., Eisele, T., & Nicke, A. (2007). Identification of an intersubunit cross-link between substituted cysteine residues located in the putative atp binding site of the p2x $\square$ receptor. *The Journal of Neuroscience*, 27(6), 1456–1466. <https://doi.org/10.1523/JNEUROSCI.3105-06.2007>
- McCarthy, A. E., Yoshioka, C., & Mansoor, S. E. (2019). Full-length P2X7 structures reveal how palmitoylation prevents channel desensitization. *Cell*, 179(3), 659–670.e13. <https://doi.org/10.1016/j.cell.2019.09.017>
- Merkley, E. D., Rysavy, S., Kahraman, A., Hafen, R. P., Daggett, V., & Adkins, J. N. (2014). Distance restraints from crosslinking mass spectrometry: Mining a molecular dynamics simulation database to evaluate lysine-lysine distances. *Protein Science*, 23(6), 747–759. <https://doi.org/10.1002/pro.2458>
- Migita, K., Haines, W. R., Voigt, M. M., & Egan, T. M. (2001). Polar residues of the second transmembrane domain influence cation permeability of the

- ATP-gated P2X(2) receptor. *The Journal of Biological Chemistry*, 276(33), 30934–30941. <https://doi.org/10.1074/jbc.M103366200>
- Mirus Bio. (2023). Antibiotic kill curve [Accessed August 1, 2023].
- Moffatt, L., & Hume, R. I. (2007). Responses of rat P2X2 receptors to ultrashort pulses of ATP provide insights into ATP binding and channel gating. *The Journal of General Physiology*, 130(2), 183–201. <https://doi.org/10.1085/jgp.200709779>
- Murrell-Lagnado, R. D., & Qureshi, O. S. (2008). Assembly and trafficking of P2X purinergic receptors (review). *Molecular membrane biology*, 25(4), 321–331. <https://doi.org/10.1080/09687680802050385>
- Nathan, D. (1987). The use of xenopus oocytes for the study of ion channel. *Critical Reviews in Biochemistry and Molecular Biology*.
- New England Biolabs. (2012). Protocol: Phusion high-fidelity pcr master mix with hf buffer (m0531) [Accessed: October 21, 2024].
- Nicke, A., Bäumer, H. G., Rettinger, J., Eichele, A., Lambrecht, G., Mutschler, E., & Schmalzing, G. (1998a). P2X1 and P2X3 receptors form stable trimers: A novel structural motif of ligand-gated ion channels. *The EMBO Journal*, 17(11), 3016–3028. <https://doi.org/10.1093/emboj/17.11.3016>
- Nicke, A., Bäumer, H. G., Rettinger, J., Eichele, A., Lambrecht, G., Mutschler, E., & Schmalzing, G. (1998b). P2x₁ and p2x₃ receptors form stable trimers: A novel structural motif of ligand-gated ion channels. *The EMBO Journal*, 17(11), 3016–3028. <https://doi.org/10.1093/emboj/17.11.3016>
- North, R. A. (2002). Molecular physiology of P2X receptors. *Physiological Reviews*, 82(4), 1013–1067. <https://doi.org/10.1152/physrev.00015.2002>
- North, R. A. (2016). P2X receptors. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 371(1700). <https://doi.org/10.1098/rstb.2015.0427>
- North, R. A., & Jarvis, M. F. (2013). P2X receptors as drug targets. *Molecular Pharmacology*, 83(4), 759–769. <https://doi.org/10.1124/mol.112.083758>
- Öjemalm, K., Watson, H. R., Roboti, P., Cross, B. C. S., Warwicker, J., von Heijne, G., & High, S. (2013). Positional editing of transmembrane domains during ion channel assembly. *Journal of Cell Science*, 126(Pt 2), 464–472. <https://doi.org/10.1242/jcs.111773>
- Oken, A. C., Krishnamurthy, I., Savage, J. C., Lisi, N. E., Godsey, M. H., & Mansoor, S. E. (2022). Molecular pharmacology of P2X receptors: Exploring druggable domains revealed by structural biology. *Frontiers in pharmacology*, 13, 925880. <https://doi.org/10.3389/fphar.2022.925880>

- Ormond, S. J., Barrera, N. P., Qureshi, O. S., Henderson, R. M., Edwardson, J. M., & Murrell-Lagnado, R. D. (2006). An uncharged region within the n terminus of the P2X6 receptor inhibits its assembly and exit from the endoplasmic reticulum. *Molecular Pharmacology*, 69(5), 1692–1700. <https://doi.org/10.1124/mol.105.020404>
- Paukert, M., Osteroth, R., Geisler, H. S., Brandle, U., Glowatzki, E., Ruppersberg, J. P., & Gründer, S. (2001). Inflammatory mediators potentiate ATP-gated channels through the P2X(3) subunit. *The Journal of Biological Chemistry*, 276(24), 21077–21082. <https://doi.org/10.1074/jbc.M101465200>
- Peris-Díaz, M. D., Guran, R., Zitka, O., Adam, V., & Krężel, A. (2020). Metal- and affinity-specific dual labeling of cysteine-rich proteins for identification of metal-binding sites. *Analytical Chemistry*, 92(19), 12950–12958. <https://doi.org/10.1021/acs.analchem.0c01604>
- Pratt, E. B., Brink, T. S., Bergson, P., Voigt, M. M., & Cook, S. P. (2005). Use-dependent inhibition of P2X3 receptors by nanomolar agonist. *The Journal of Neuroscience*, 25(32), 7359–7365. <https://doi.org/10.1523/JNEUROSCI.5189-04.2005>
- Pu, M., Xu, Z., Peng, Y., Hou, Y., Liu, D., Wang, Y., Liu, H., Song, G., & Liu, Z.-J. (2018). Protein crystal quality oriented disulfide bond engineering. *Protein & cell*, 9(7), 659–663. <https://doi.org/10.1007/s13238-017-0482-7>
- Rassendren, F., Buell, G., Newbolt, A., North, R. A., & Surprenant, A. (1997). Identification of amino acid residues contributing to the pore of a P2X receptor. *The EMBO Journal*, 16(12), 3446–3454. <https://doi.org/10.1093/emboj/16.12.3446>
- Rettinger, J., & Schmalzing, G. (2003). Activation and desensitization of the recombinant p2x1 receptor at nanomolar atp concentrations. *Journal of General Physiology*, 121(5), 451–461. <https://doi.org/10.1085/jgp.200208730>
- Rettinger, J., & Schmalzing, G. (2004). Desensitization masks nanomolar potency of ATP for the P2X1 receptor. *The Journal of Biological Chemistry*, 279(8), 6426–6433. <https://doi.org/10.1074/jbc.M306987200>
- Riedel, T., Schmalzing, G., & Markwardt, F. (2007). Influence of extracellular monovalent cations on pore and gating properties of p2x₁ receptor-operated single-channel currents. *Biophysical Journal*, 93(3), 846–858. <https://doi.org/10.1529/biophysj.106.103614>
- Ritter, J. M., Flower, R. J., Henderson, G., Loke, Y. K., MacEwan, D., & Rang, H. P. (2019). *Rang & dale's pharmacology* (9th ed.). Elsevier.
- Roberts, J. A., Vial, C., Digby, H. R., Agboh, K. C., Wen, H., Atterbury-Thomas, A., & Evans, R. J. (2006). Molecular properties of P2X receptors. *Pflugers*

- Archiv: *European Journal of Physiology*, 452(5), 486–500. <https://doi.org/10.1007/s00424-006-0073-6>
- Rokic, M. B., & Stojilkovic, S. S. (2013). Two open states of P2X receptor channels. *Frontiers in Cellular Neuroscience*, 7, 215. <https://doi.org/10.3389/fncel.2013.00215>
- Rokic, M. B., Stojilkovic, S. S., & Zemkova, H. (2014). Structural and functional properties of the rat P2X4 purinoreceptor extracellular vestibule during gating. *Frontiers in Cellular Neuroscience*, 8, 3. <https://doi.org/10.3389/fncel.2014.00003>
- Saier Lab. (2023). *TCDB: Transporter classification database* [Accessed July 2, 2023]. <http://www.tcdb.org>
- Samways, D. S. K., & Egan, T. M. (2007). Acidic amino acids impart enhanced Ca^{2+} permeability and flux in two members of the ATP-gated P2X receptor family. *The Journal of General Physiology*, 129(3), 245–256. <https://doi.org/10.1085/jgp.200609677>
- Samways, D. S. K., Khakh, B. S., Dutertre, S., & Egan, T. M. (2011). Preferential use of unobstructed lateral portals as the access route to the pore of human ATP-gated ion channels (P2X receptors). *Proceedings of the National Academy of Sciences of the United States of America*, 108(33), 13800–13805. <https://doi.org/10.1073/pnas.1017550108>
- Samways, D. S. K., Li, Z., & Egan, T. M. (2014). Principles and properties of ion flow in P2X receptors. *Frontiers in Cellular Neuroscience*, 8, 6. <https://doi.org/10.3389/fncel.2014.00006>
- Samways, D. S., & Egan, T. M. (2007). Acidic amino acids impart enhanced Ca^{2+} permeability and flux in two members of the atp-gated p2x receptor family. *The Journal of General Physiology*, 129(3), 245–256. <https://doi.org/10.1085/jgp.200609677>
- Saul, A., Hausmann, R., Kless, A., & Nicke, A. (2013). Heteromeric assembly of P2X subunits. *Frontiers in Cellular Neuroscience*, 7, 250. <https://doi.org/10.3389/fncel.2013.00250>
- Schmalzing, G., Gloor, S., Omay, H., Kröner, S., Appelhans, H., & Schwarz, W. (1991). Up-regulation of sodium pump activity in xenopus laevis oocytes by expression of heterologous beta 1 subunits of the sodium pump. *The Biochemical Journal*, 279 (Pt 2)(Pt 2), 329–336. <https://doi.org/10.1042/bj2790329>
- Schwehm, J. M., Fitch, C. A., Dang, B. N., E., B. G.-M., & Stites, W. E. (2003). Changes in stability upon charge reversal and neutralization substitution in

- staphylococcal nuclease are dominated by favorable electrostatic effects. *Biochemistry*, 42(4), 1118–1128. <https://doi.org/10.1021/bi0266434>
- Smith, F. M., Humphrey, P. P., & Murrell-Lagnado, R. D. (1999). Identification of amino acids within the P2X2 receptor c-terminus that regulate desensitization. *The Journal of Physiology*, 520 Pt 1(Pt 1), 91–99. <https://doi.org/10.1111/j.1469-7793.1999.00091.x>
- Sokolova, E., Skorinkin, A., Moiseev, I., Agrachev, A., Nistri, A., & Giniatullin, R. (2006). Experimental and modeling studies of desensitization of p2x3 receptors. *Molecular Pharmacology*, 70(1), 373–382. <https://doi.org/10.1124/mol.106.023564>
- Sokolova, E., Skorinkin, A., Fabbretti, E., Masten, L., Nistri, A., & Giniatullin, R. (2004). Agonist-dependence of recovery from desensitization of P2X(3) receptors provides a novel and sensitive approach for their rapid up or downregulation. *British Journal of Pharmacology*, 141(6), 1048–1058. <https://doi.org/10.1038/sj.bjp.0705701>
- Stelmashenko, O., Lalo, U., Yang, Y., Bragg, L., North, R. A., & Compan, V. (2012). Activation of trimeric P2X2 receptors by fewer than three ATP molecules. *Molecular Pharmacology*, 82(4), 760–766. <https://doi.org/10.1124/mol.112.080903>
- Stephan, G., Kowalski-Jahn, M., Zens, C., Schmalzing, G., Illes, P., & Hausmann, R. (2016). Inter-subunit disulfide locking of the human p2x3 receptor elucidates ectodomain movements associated with channel gating. *Purinergic Signal*, 12(2), 221–233. <https://doi.org/10.1007/s11302-016-9496-5>
- Stokes, L., Bidula, S., Bibič, L., & Allum, E. (2020). To inhibit or enhance? is there a benefit to positive allosteric modulation of P2X receptors? *Frontiers in pharmacology*, 11, 627. <https://doi.org/10.3389/fphar.2020.00627>
- Syed, N.-i.-H., & Kennedy, C. (2012). Pharmacology of P2X receptors. *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling*, 1(1), 16–30. <https://doi.org/10.1002/wmts.1>
- Tam, S. W., Huffer, K., Li, M., & Swartz, K. J. (2023). Ion permeation pathway within the internal pore of P2X receptor channels. *eLife*, 12. <https://doi.org/10.7554/eLife.84796>
- Thermo Fisher Scientific. (2019). Freezing cells.
- Torres, G. E., Egan, T. M., & Voigt, M. M. (1999). Hetero-oligomeric assembly of P2X receptor subunits. specificities exist with regard to possible partners. *The Journal of Biological Chemistry*, 274(10), 6653–6659. <https://doi.org/10.1074/jbc.274.10.6653>

- Tsushima, R. G., Li, R. A., & Backx, P. H. (1997). P-loop flexibility in na⁺ channel pores revealed by single- and double-cysteine replacements. *The Journal of General Physiology*, 110(1), 59–72. <https://doi.org/10.1085/jgp.110.1.59>
- Vial, C., & Evans, R. J. (2000). P2X receptor expression in mouse urinary bladder and the requirement of P2X(1) receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle. *British Journal of Pharmacology*, 131(7), 1489–1495. <https://doi.org/10.1038/sj.bjp.0703720>
- Vieira-Pires, R. S., & Morais-Cabral, J. H. (2010). 310 helices in channels and other membrane proteins. *Journal of General Physiology*, 136(6), 585–592. <https://doi.org/10.1085/jgp.201010508>
- Wang, J., Wang, Y., Cui, W.-W., Huang, Y., Yang, Y., Liu, Y., Zhao, W.-S., Cheng, X.-Y., Sun, W.-S., Cao, P., Zhu, M. X., Wang, R., Hattori, M., & Yu, Y. (2018). Druggable negative allosteric site of P2X3 receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 115(19), 4939–4944. <https://doi.org/10.1073/pnas.1800907115>
- Wang, J., & Yu, Y. (2016). Insights into the channel gating of P2X receptors from structures, dynamics and small molecules. *Acta Pharmacologica Sinica*, 37(1), 44–55. <https://doi.org/10.1038/aps.2015.127>
- Werner, P., Seward, E. P., Buell, G. N., & North, R. A. (1996). Domains of P2X receptors involved in desensitization. *Proceedings of the National Academy of Sciences of the United States of America*, 93(26), 15485–15490. <https://doi.org/10.1073/pnas.93.26.15485>
- Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T. L., Miller, E., Bache, S. M., Müller, K., Ooms, J., Robinson, D., Seidel, D. P., Spinu, V., ... Yutani, H. (2019). Welcome to the tidyverse. *Journal of Open Source Software*, 4(43), 1686. <https://doi.org/10.21105/joss.01686>
- Wickham, H., François, R., Henry, L., Müller, K., & Vaughan, D. (2023). *Dplyr: A grammar of data manipulation* [R package version 1.1.1].

A. I: Result Figures

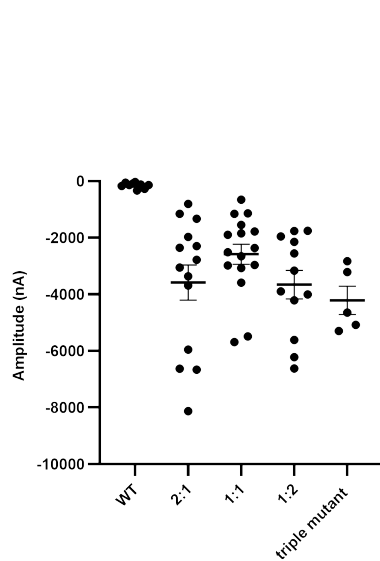


Figure A.1.1: The amplitudes from the first ATP application for each group, results are shown as mean $\pm$ SEM. The WT group has an overall amplitude of -147.4 ± 33 nA, the 2:1 group response is with -3583 ± 623 nA, the 1:1 group is at -2582 ± 355 nA, the 1:2 group yields -3657 ± 04 nA, and the triple mutant has an amplitude of -4213 ± 501 nA.

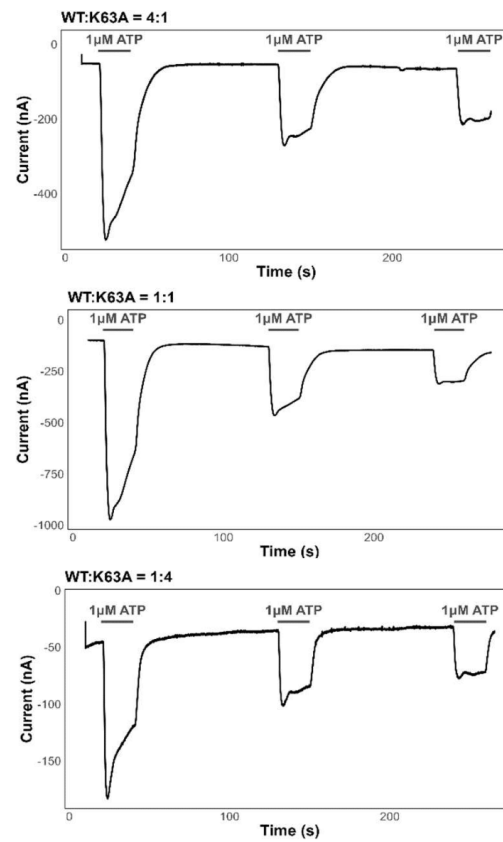


Figure A.1.2: Representative current traces of WT + K63A- triple mutant elicited by 1 μ M ATP. cRNAs encoding for the indicated constructs (WT with N^{WT} ; K63A with N^M) were mixed in ratios as indicated within the titles.

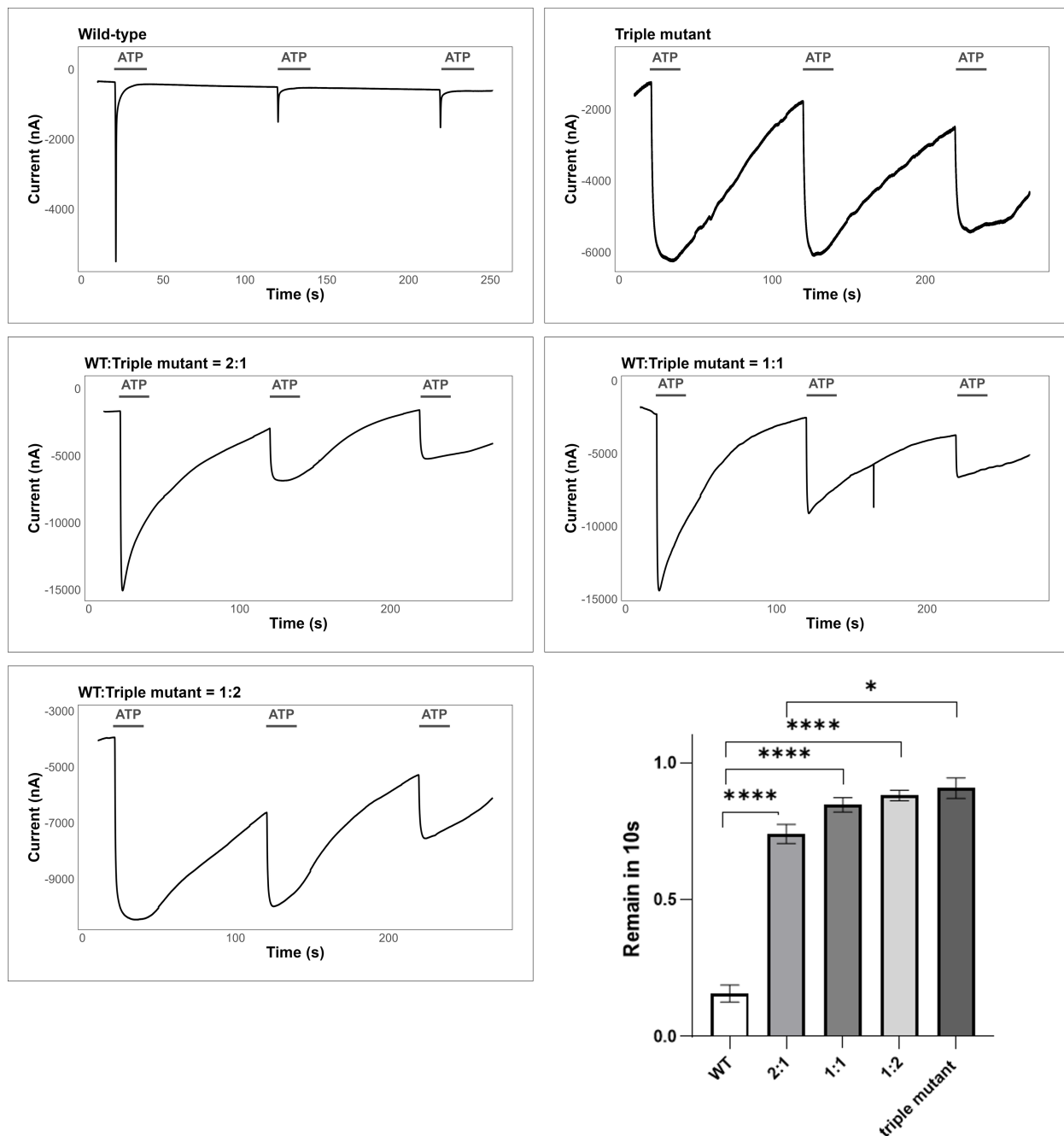


Figure A.1.3: The left panels, as well as the upper and middle right panels, give examples of traces for each group from TEVC. 10 μ M ATP application is noted. Constructs are specified in the titles. The right lower panel shows the comparison of the first response's remaining current in 10 seconds from wild-type, different ratios of RNA-mixed groups, and the triple mutant. The groups are as noted. From left to right, the ratios all refer to the RNA volume ratio of WT: triple mutant. Error bars mark the SEM. $n \geq 5$. The ratios of remain after 10 seconds of ATP activation are as follows: WT 0.16 $\pm$ 0.03; 2:1 groups 0.74 $\pm$ 0.03, 1:1 groups 0.85 $\pm$ 0.03; 1:2 groups 0.88 $\pm$ 0.02; triple mutant = 0.91 $\pm$ 0.04. Multi-comparisons were performed between every two groups; significant differences exist between: WT vs. 1:1 group (WT: triple mutant, $p = 0.0297$), WT vs. ratio = 1:2 group ($p = 0.024$), and WT vs. pure triple mutant group ($p = 0.0131$).

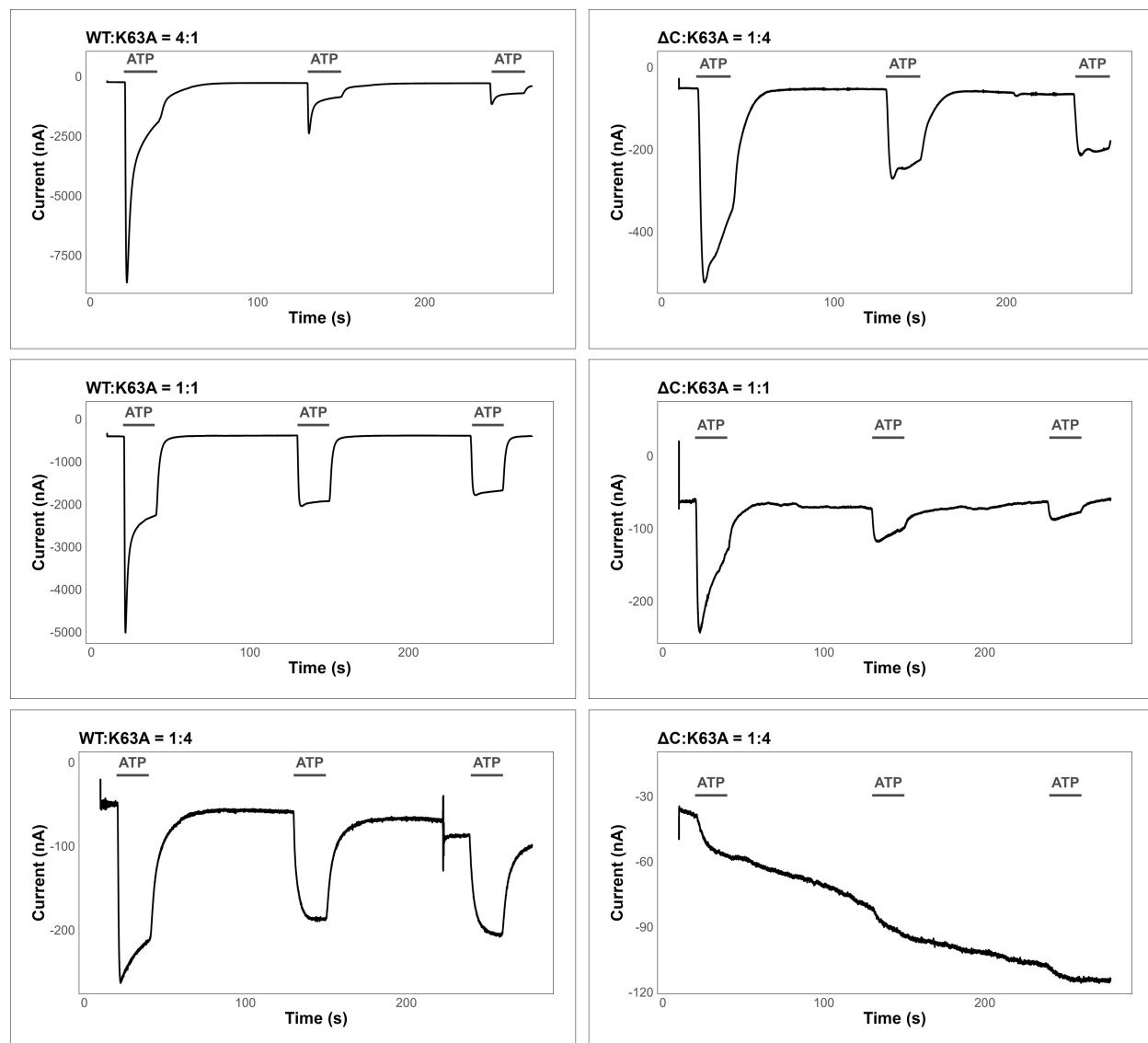


Figure A.1.4: Representative current traces for each group of mixed cRNAs encoding for the indicated constructs. The application of 10 μ M ATP is indicated by the horizontal bar.

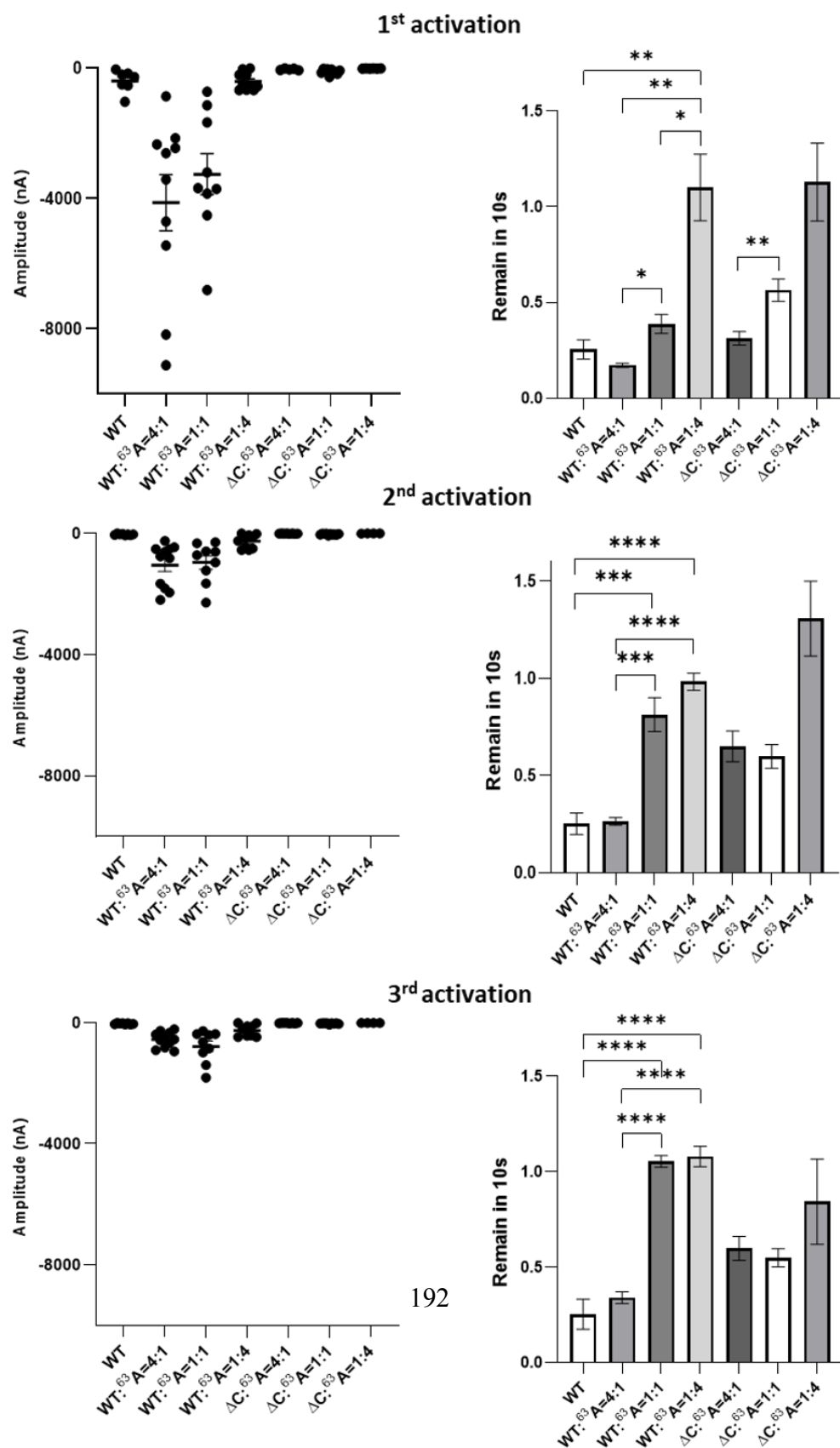


Figure A.1.5: Group comparison of amplitudes and ratios of remain from three subsequent ATP applications (1st application: upper panels; 2nd application: middle panels; 3rd application: lower panels). The y-axis is kept similar to highlight changes in absolute values across activations. Error bars represent SEM. Significant differences from the multiple comparisons are labeled. $n \geq 5$, except for the $\Delta C:K63A = 1:4$ group ($n = 4$) due to poor function. During the three activations, the WT group shows amplitudes of -396.6 ± 125.9 , -42.6 ± 7.5 , -30.6 ± 7.0 nA, with a remain of 0.25 ± 0.05 , 0.25 ± 0.06 , 0.26 ± 0.08 . The WT:K63A = 4:1 group has amplitudes of -4132 ± 860.8 , -1054.0 ± 212.2 , -552.8 ± 77.2 nA, with a remain of 0.17 ± 0.01 , 0.26 ± 0.02 , and 0.34 ± 0.03 . The WT:K63A = 1:1 group has amplitudes of -3259.0 ± 627.8 , -964.0 ± 219.5 , -785.0 ± 176.9 nA, with a remain of 0.39 ± 0.05 , 0.81 ± 0.09 , and 1.05 ± 0.03 . The WT:K63A = 1:4 group responds with -411.7 ± 84.1 , -265.1 ± 72.2 , -250.4 ± 65.4 nA, with a remain of 1.10 ± 0.17 , 0.98 ± 0.04 , and 1.08 ± 0.05 . The $\Delta C:K63A = 4:1$ group has amplitudes of -43.7 ± 12.1 , -12.6 ± 2.0 , -10.0 ± 2.7 nA, with a remain of 0.31 ± 0.04 , 0.65 ± 0.08 , and 0.60 ± 0.06 . The $\Delta C:K63A = 1:1$ group shows amplitudes of -109.9 ± 25.2 , -34.5 ± 6.8 , -23.8 ± 6.0 nA, with a remain of 0.56 ± 0.06 , 0.60 ± 0.06 , and 0.55 ± 0.05 . The $\Delta C:K63A = 1:4$ group responds with -16.1 ± 1.5 , -8.0 ± 1.6 , -4.4 ± 1.3 nA, with a remain of 1.13 ± 0.20 , 1.30 ± 0.19 , and 0.84 ± 0.22 . Multi comparisons in ANOVA tests were performed between every two groups within the WT/K63A groups and the ΔC /K63A groups.

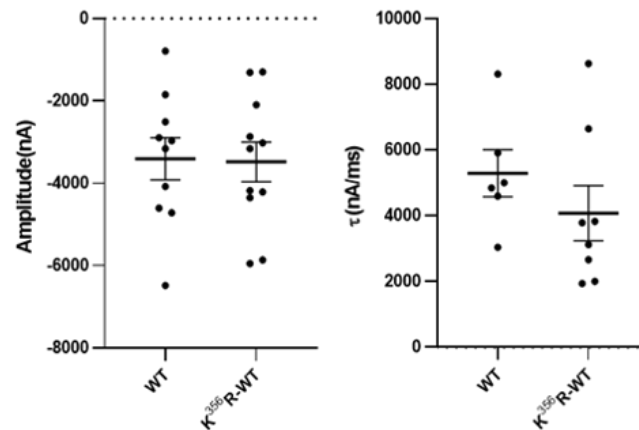


Figure A.1.6: Comparison of current amplitudes and desensitization time constants in scattered points between the WT and the K356R-WT. $n > 5$. The left panel describes the amplitudes from both groups. The WT yields an average response of -3405 ± 512 nA, and the K356R mutant has an amplitude of -3483 ± 483 nA. The right panel lays out the τ values from the exponential decay fitting of the desensitization. The τ of the WT is 5286 ± 715 nA/ms, while the K356R mutation brings a result of 4079 ± 837 nA/ms. No significant differences were detected.

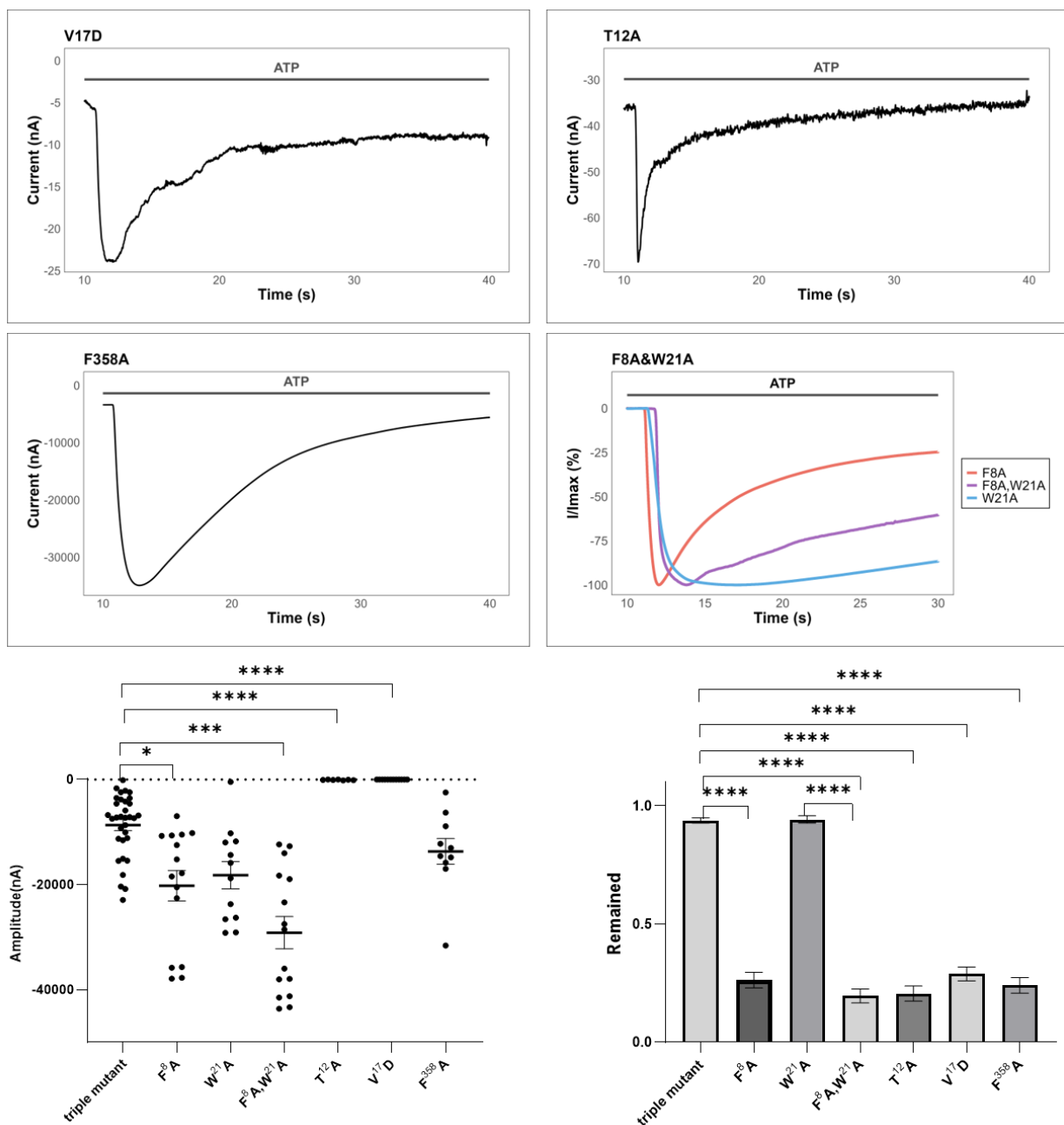


Figure A.1.7: Representative traces are shown in the upper panel, with mutants indicated in the titles. ATP applications are as noted. The paired mutant group F8-W21 is overlapped, and amplitudes are normalized to the maximal responses. The lower panels display the statistics of amplitudes and ratios of remain in comparison to the triple mutant, with mean $\pm$ SEM noted. Significant differences from multiple comparisons are labeled. $n \geq 5$. The amplitudes and ratios of remain for each group are as follows: triple mutant = -8683 ± 1037 nA, remain = 0.93 ± 0.01 ; F8A = -20215 ± 2890 nA, remain = 0.26 ± 0.03 ; W21A = -18201 ± 2589 nA, remain = 0.92 ± 0.02 ; F8A,W21A = -29167 ± 3049 nA, remain = 0.19 ± 0.03 ; T12A = -72.4 ± 20.1 nA, remain = 0.21 ± 0.03 ; V17D = -26.2 ± 4.8 nA, remain = 0.29 ± 0.03 ; F358A = -13681 ± 2457 nA, remain = 0.24 ± 0.03 . Multiple comparisons were conducted (1) between the triple mutant and each group, and (2) within mutants targeting the same residue. Significant differences are labeled.

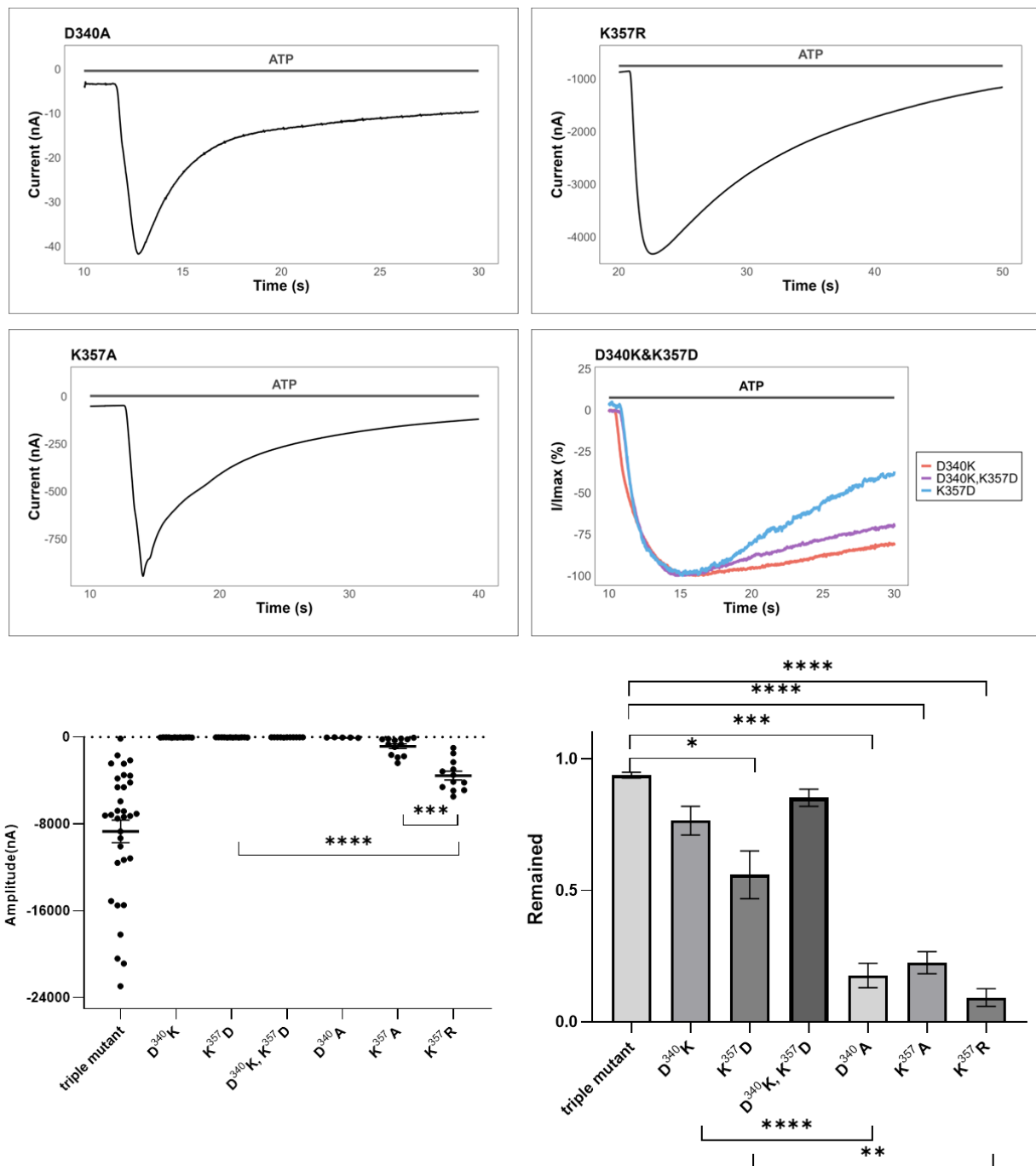


Figure A.1.8: Representative traces are shown in the upper panel. ATP applications are as noted. Charge-reversing and -swapping mutants are grouped and overlapped, with amplitudes normalized to the maximal responses. The bottom panels display the statistics of amplitudes and ratios of remain at 30 seconds in comparison to the triple mutant, with averages $\pm$ SEM marked. Significant differences from multiple comparisons are labeled. $n \geq 5$. The amplitudes for each group are: triple mutant = -8683 ± 1037 nA, D340A = -39 ± 12 nA, K357D = -22 ± 4 nA, double mutant D340K,K357D = -14 nA. Within mutants targeting the same residue, significant differences are noted. In the kinetics bar graph, multiple comparisons were applied: (1) between the triple mutant and all other mutants, (2) within all D340 mutants, and (3) within all K357 mutants. In the amplitude scatter plot, multiple comparisons were applied within D340 mutants and within K357 mutants. Significant differences are noted.

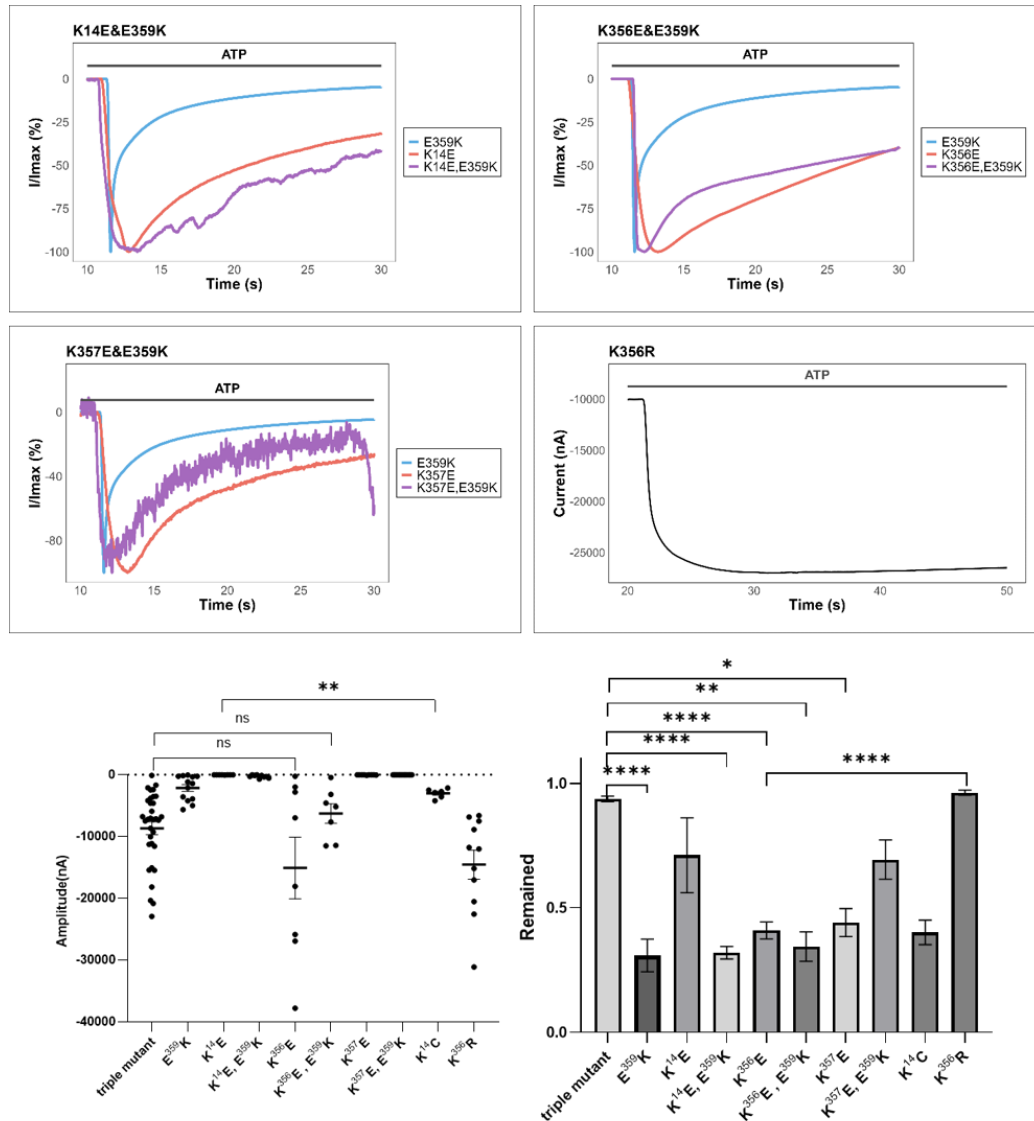


Figure A.1.9: Representative traces are shown in the upper panel. ATP applications are as noted. Charge-reversing and -swapping mutants are grouped and overlapped, with amplitudes normalized to the maximal responses. The lower panels display the statistics of amplitudes and ratios of remain at 30 seconds in comparison to the triple mutant, with averages $\pm$ SEM marked. Significant differences from multiple comparisons are labeled. $n \geq 5$. The amplitudes and ratios of remain for each group are as follows: triple mutant = -15093 ± 4984 nA, remain = 0.93 ± 0.01 ; E359K = -2158 ± 566.9 nA, remain = 0.31 ± 0.07 ; K14E = -23 ± 9.4 nA, remain = 0.72 ± 0.15 ; K14E, E359K = -278.8 ± 79.7 nA, remain = 0.32 ± 0.02 ; K356E = -15903 ± 4984 nA, remain = 0.41 ± 0.03 ; K356E, E359K = -6284 ± 1570 nA, remain = 0.34 ± 0.06 ; K357E = -34.7 ± 11.7 nA, remain = 0.44 ± 0.06 ; K357E, E359K = -18.9 ± 4.08 nA, remain = 0.38 ± 0.08 ; K14C = -4028 ± 1058 nA, remain = 0.40 ± 0.05 ; K356R = -14563 ± 2337 nA, remain = 0.96 ± 0.01 . Multiple comparisons were conducted: (1) between the triple mutant and each group (significance not noted), (2) within mutants targeting the same residue, where significant differences are labeled. In the kinetics bar graph, multiple comparisons were applied: (1) between the triple mutant and all mutants, (2) within all D340 mutants, and (3) within all K357 mutants. In the amplitude scatter plot, multiple comparisons were applied within D340 mutants and within K357 mutants. Significant differences are noted.

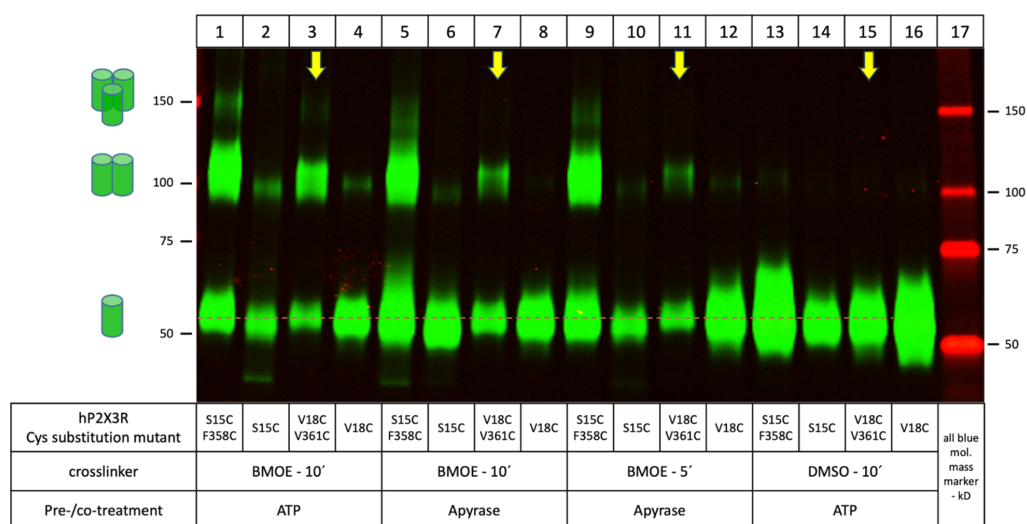


Figure A.1.10: Crosslinking of N- and C-terminal residues of the V15C-F358C and V16C-V361C groups. SDS-PAGE analysis of the samples was performed in parallel with a molecular mass marker (MM). Yellow asterisks (**) highlight the dimeric bands of the 18C/361C-hP2X3R, which could be cross-linked to a greater extent in the presence of 300 μ M ATP as compared to apyrase (compare lane 3 versus lanes 7 and 11). Without BMOE, no crosslinking was observed (DMSO control, lanes 13-16).

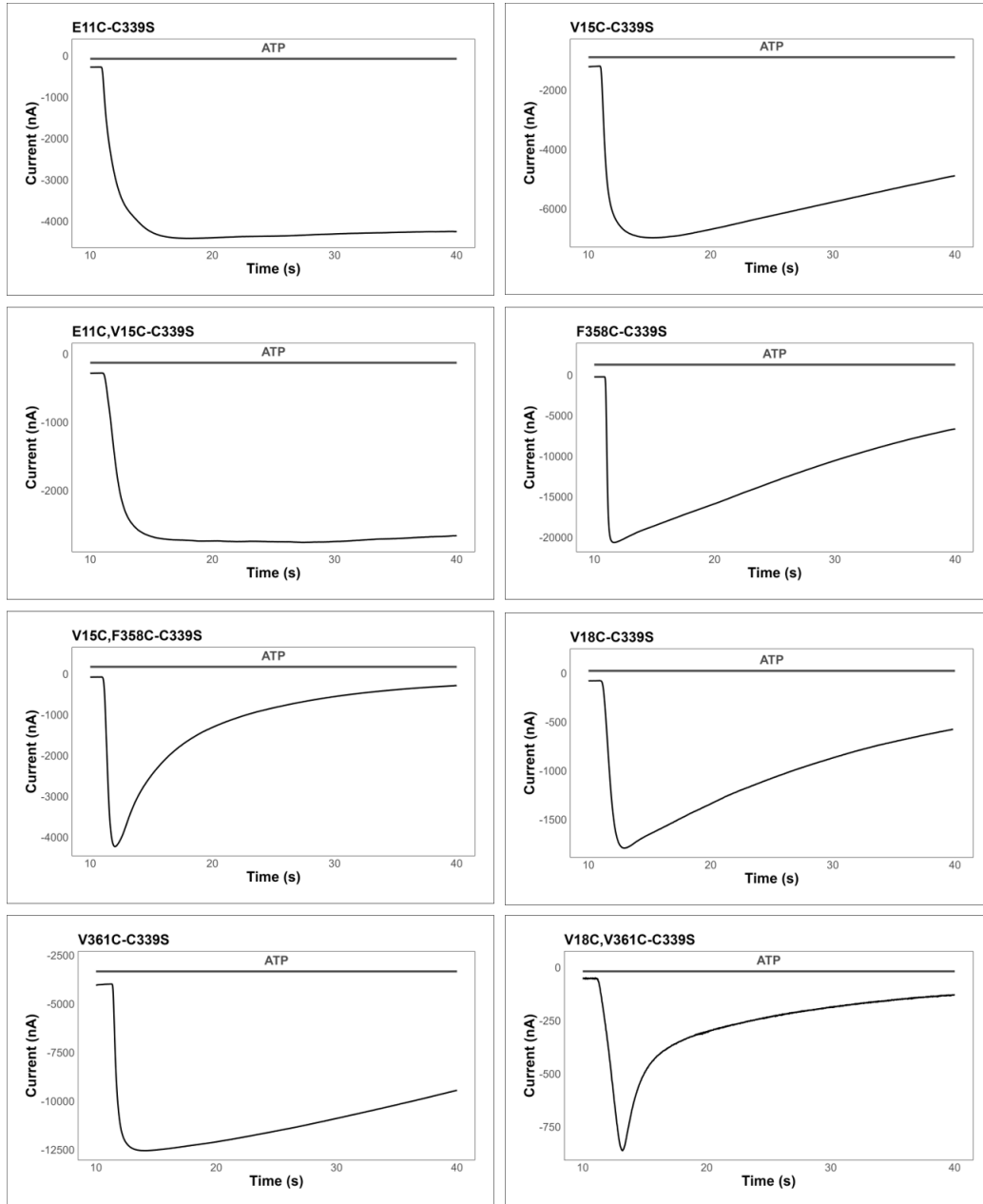


Figure A.1.11: Representative traces from the mutants. Each mutant is specified in the title, and ATP applications are noted.

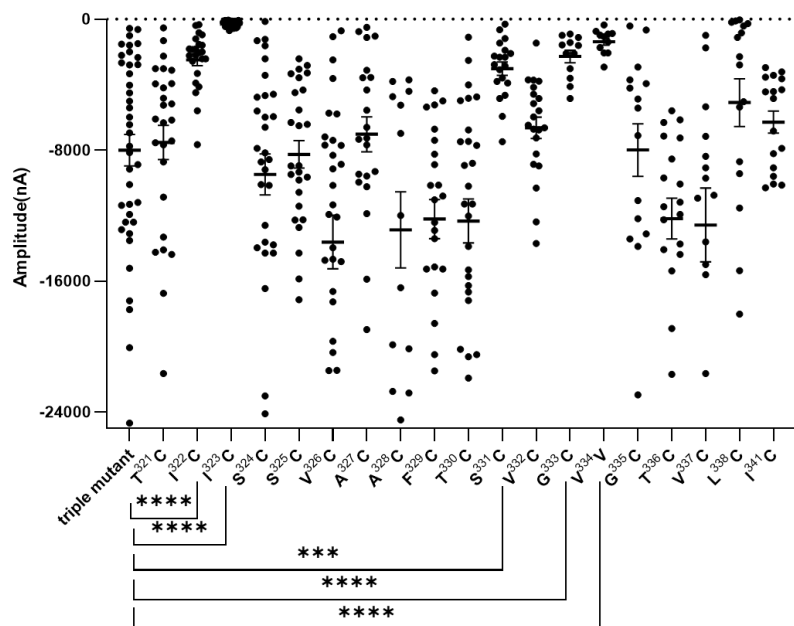


Figure A.2.1: The TEVC results of current amplitude elicited by 10 μ M ATP from the cysteine mutations and the control group, $n > 10$. All constructs are as indicated. The mean $\pm$ SEM is specified for each group. 10 μ M ATP yielded the following amplitudes (nA): -8009 $\pm$ 961.7 from the triple mutant; -7523 $\pm$ 1038 from 321C; -2488 $\pm$ 359.3 from 322C; -297.4 $\pm$ 29.6 from 323C; -9488 $\pm$ 1256 from 324C; -8265 $\pm$ 839.1 from 325C; -13618 $\pm$ 1633 from 326C; -7034 $\pm$ 1072 from 327C; -12875 $\pm$ 2326 from 328C; -12215 $\pm$ 1203 from 329C; -12331 $\pm$ 1304 from 330C; -3022 $\pm$ 414.5 from 331C; -6632 $\pm$ 653.6 from 332C; -2280 $\pm$ 378.4 from 333C; 1369 $\pm$ 226.7 from 334C; -7996 $\pm$ 1611 from 335C; -12188 $\pm$ 1247 from 336C; -12574 $\pm$ 2253 from 337C; -5096 $\pm$ 1462 from 338C; -6288 $\pm$ 682.2 from 341C. All constructions were compared to the triple mutant, significant differences were indicated. 322C, 323C; 331C, 333C, and 334C were significantly different from the control, with P values of <0.0001, <0.0001, 0.0003, <0.0001, and <0.0001, respectively.

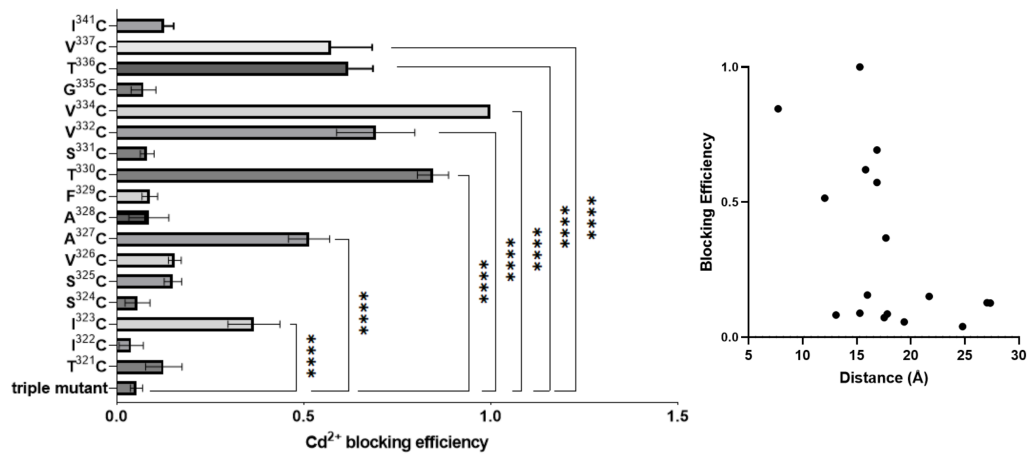


Figure A.2.2: The left panel shows the Cd^{2+} blocking efficiency of each mutant in a column chart. The mean $\pm$ SEM are indicated ($n > 3$). The Cd^{2+} -induced blocking efficiency values are as follows: 0.05 ± 0.02 for the triple mutant; 0.13 ± 0.05 for 321C; 0.04 ± 0.03 for 322C; 0.37 ± 0.07 for 323C; 0.06 ± 0.03 for 324C; 0.15 ± 0.02 for 325C; 0.16 ± 0.02 for 326C; 0.51 ± 0.06 for 327C; 0.09 ± 0.05 for 328C; 0.09 ± 0.02 for 329C; 0.85 ± 0.04 for 330C; 0.08 ± 0.02 for 331C; 0.70 ± 0.10 for 332C; 1.00 ± 0 for 334C; 0.07 ± 0.03 for 335C; 0.62 ± 0.07 for 336C; 0.57 ± 0.11 for 337C; and 0.13 ± 0.03 for 341C. Each group was compared to the control using multiple comparisons. Significant differences were observed for 323C, 327C, 330C, 332C, 334C, 336C, and 337C, all with a P value < 0.0001 . All significant differences are noted. The right panel shows the plot of blocking efficiency against the simulated Ca-Ca distance. A non-parametric Spearman correlation was calculated, revealing a correlation between the efficiency and the distance with a P value of 0.0252 (correlation $r^2 = -0.51$)

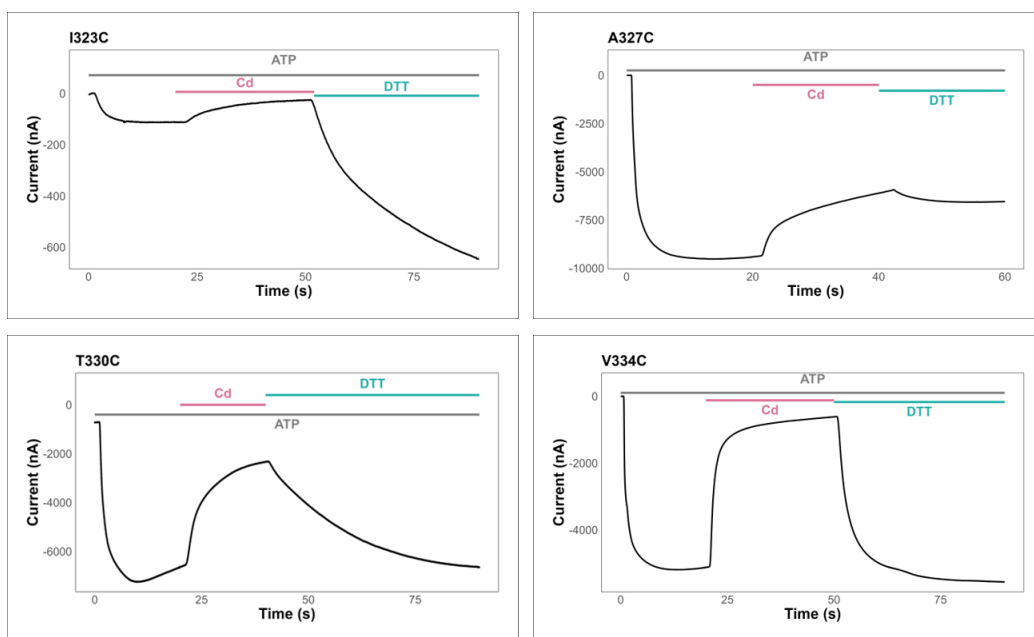


Figure A.2.3: Traces of example showing different DTT-induced reaction after Cd²⁺ blocking. Mutants are as indicated in the titles. ATP, Cd²⁺, and DTT applications are marked and color-coded.

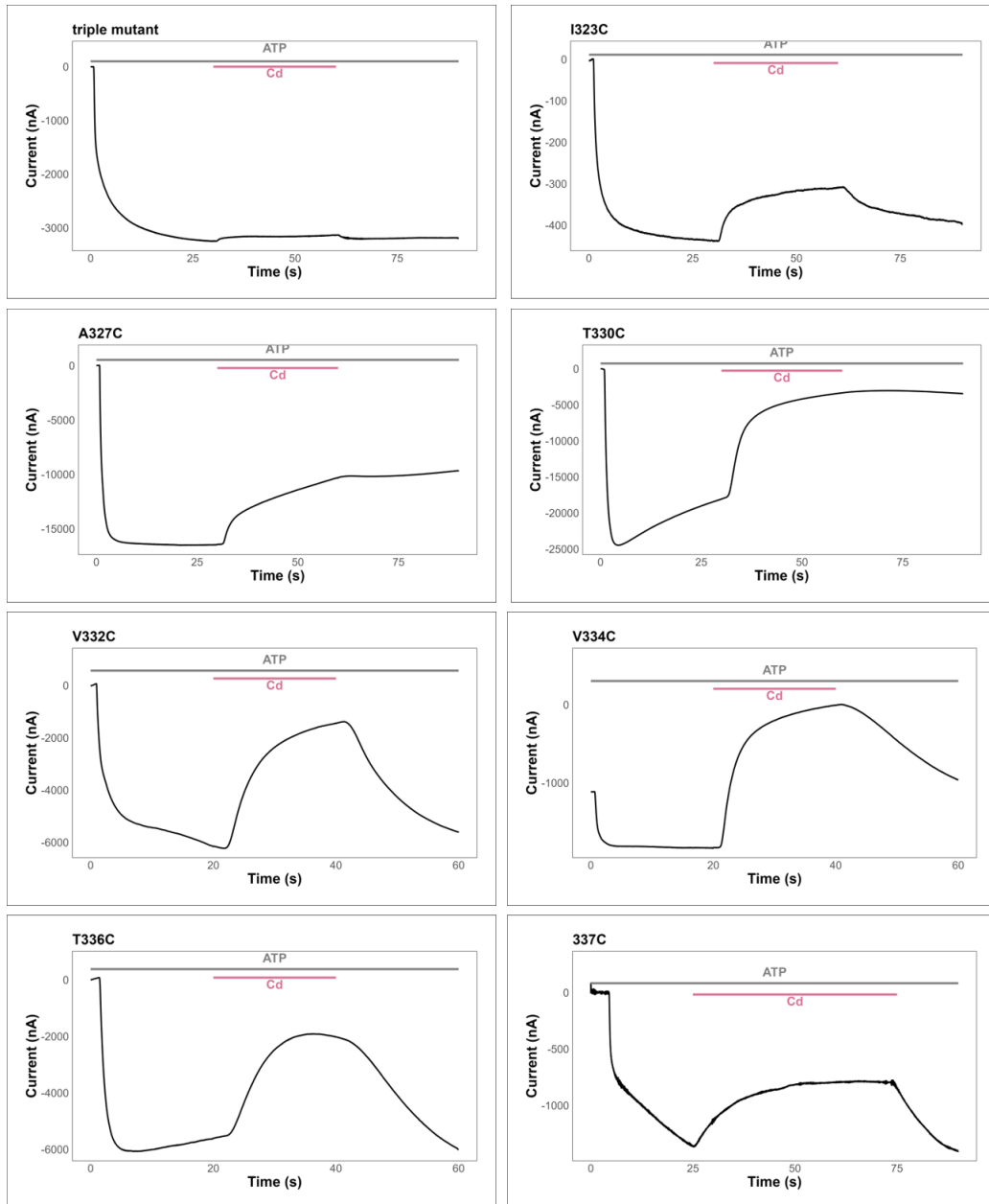


Figure A.2.4: Recording traces from mutants exhibit a significant blocking effect in response to Cd²⁺. The constructions are as described in the titles. The ATP and Cd²⁺ applications are indicated and color-coded.

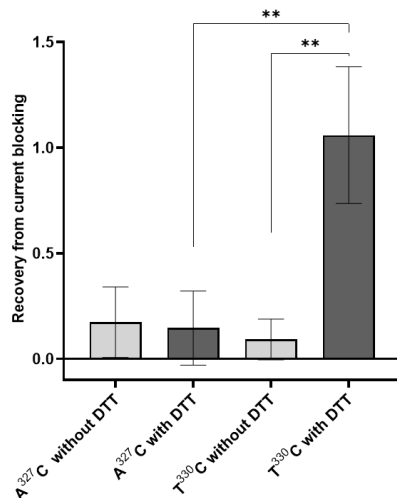


Figure A.2.5: The comparisons between recover with or without DTT for A327C and T330C. The condition and constructs are indicated. Results are shown in columns with mean±SEM. After Cd²⁺ induced inhibitory, 327C with pure ATP washing recovered 0.17 ±0.07, with DTT recovered 0.15±0.07. 330C recovered without 0.09±0.02, with DTT recovered with 1.06±0.13. Multiple comparisons were performed within the same constructs and the same conditions. Significant differences were indicated.

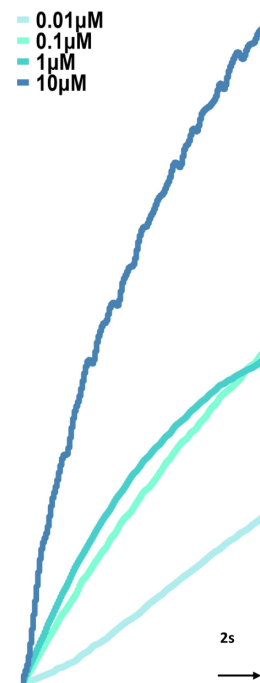


Figure A.2.6: Representative traces of Cd²⁺ blocking effect onset on 327C. Traces are normalized to the maximal amplitude. Concentrations of Cd²⁺ are color-coded and indicated. The time scale is noted.

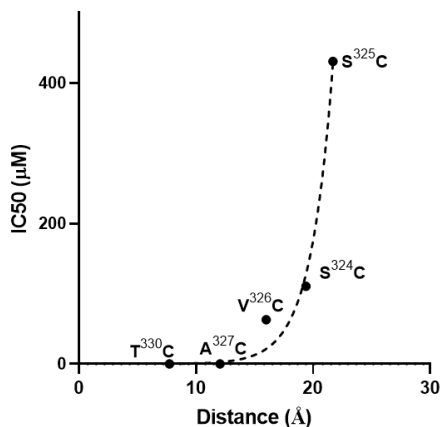


Figure A.2.7: Plotting of α-C distance against IC₅₀ values from the tested mutants. The details about the axis indication and each data point's origin are noted. A correlation of exponential growth is indicated as a fitted curve.

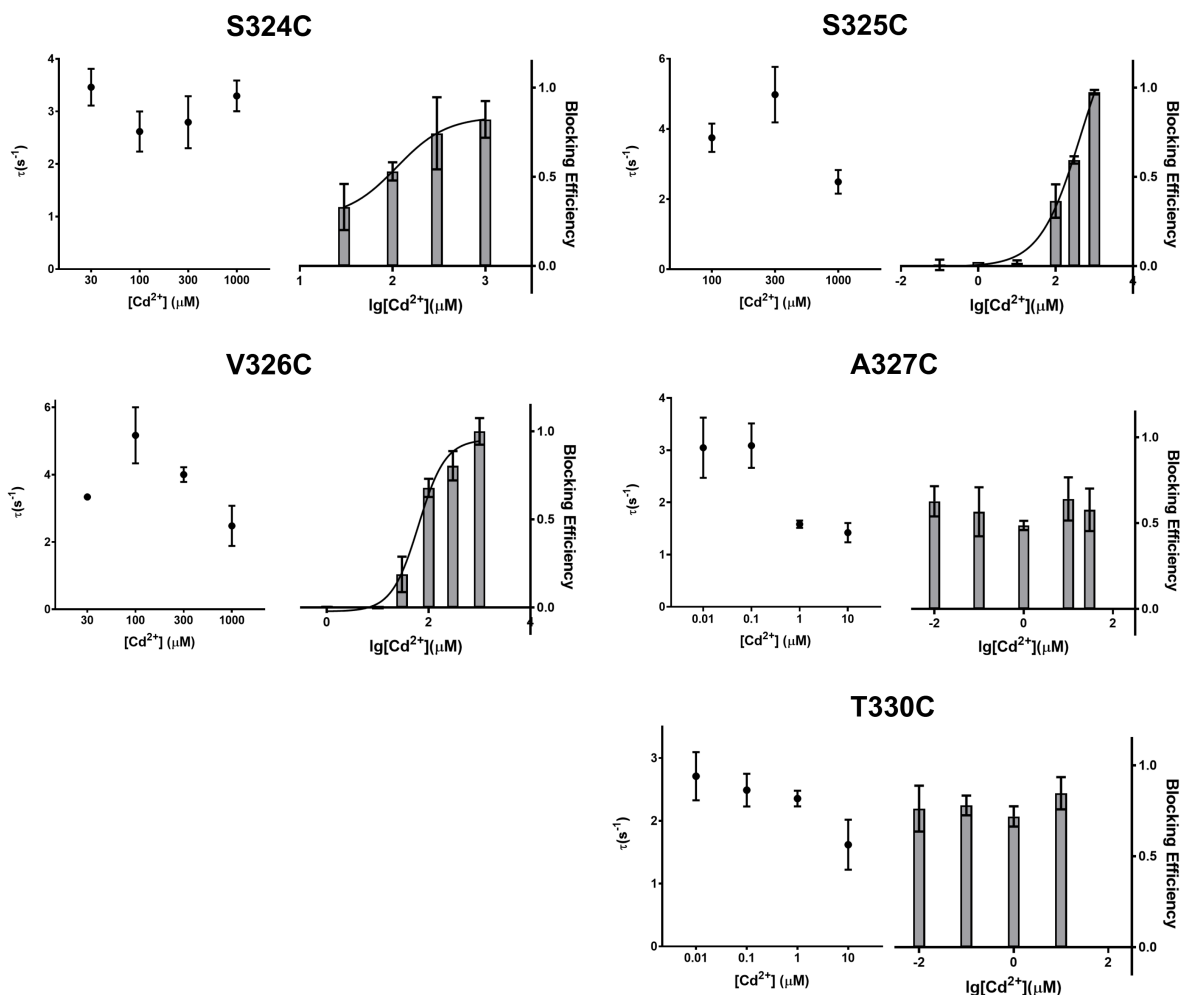


Figure A.2.8: The onset kinetics and dose-related blocking efficiency for the mutants. The left panel depicts the change in τ by different concentrations; the concentration of Cd^{2+} is specified on the x-axis. Results are shown in mean $\pm$ SEM. The right panel shows different blocking efficiencies varied by Cd^{2+} concentration. Cd^{2+} concentrations are logarithms of concentration in μM . Results are shown in the column with mean $\pm$ SEM specified. The constructions are noted. $n \geq 3$. Results of 324C were obtained under $[Cd^{2+}] = 30/100/300/1000 \mu M$, with corresponding $\tau = 3.46 \pm 0.35$, 2.62 ± 0.39 , 2.80 ± 0.50 , 3.30 ± 0.29 nA/s, and blocking efficiency of 0.33 ± 0.13 , 0.53 ± 0.05 , 0.74 ± 0.20 and 0.82 ± 0.10 ; Results of 325C were obtained under $[Cd^{2+}] = 0.1/1/10/100/300/1000 \mu M$. τ were collected from 100/300/1000 μM , with a value of 3.74 ± 0.40 , 4.98 ± 0.79 and 2.49 ± 0.34 nA/s. Corresponding blocking efficiencies were 0.01 ± 0.03 , 0.02 ± 0.00 , 0.01 ± 0.01 , 0.36 ± 0.09 , 0.60 ± 0.02 and 0.98 ± 0.01 ; V326C results were from 1/10/30/100/300/1000 μM . τ of 1 and 10 μM Cd^{2+} could not be measured, the rest resulted in 3.34 , 5.17 ± 0.83 , 4.00 ± 0.22 and 2.48 ± 0.60 nA/s. The blocking efficiencies were 0.00 , 0.00 , 0.19 ± 0.01 , 0.68 ± 0.05 , 0.80 ± 0.08 and 1 ± 0.08 ; A327C was tested for 0.01/0.1/1/10 μM , with τ values of 3.05 ± 0.58 , 3.09 ± 0.43 , 1.58 ± 0.07 and 1.42 ± 0.18 nA/s, and blocking efficiencies of 0.63 ± 0.09 , 0.57 ± 0.14 , 0.49 ± 0.03 and 0.64 ± 0.13 . T330C was tested under the same conditions, with τ values of 2.71 ± 0.38 , 2.49 ± 0.23 , 2.36 ± 0.13 and 1.62 ± 0.40 nA/s, and blocking efficiencies of 0.76 ± 0.13 , 0.78 ± 0.05 , 0.72 ± 0.06 and 0.85 ± 0.09 . Multiple comparisons were performed 1) for the onset kinetics, every value with the adjacent one; 2) for blocking efficiency, all values within A327C and T330C. The significant difference was as indicated.

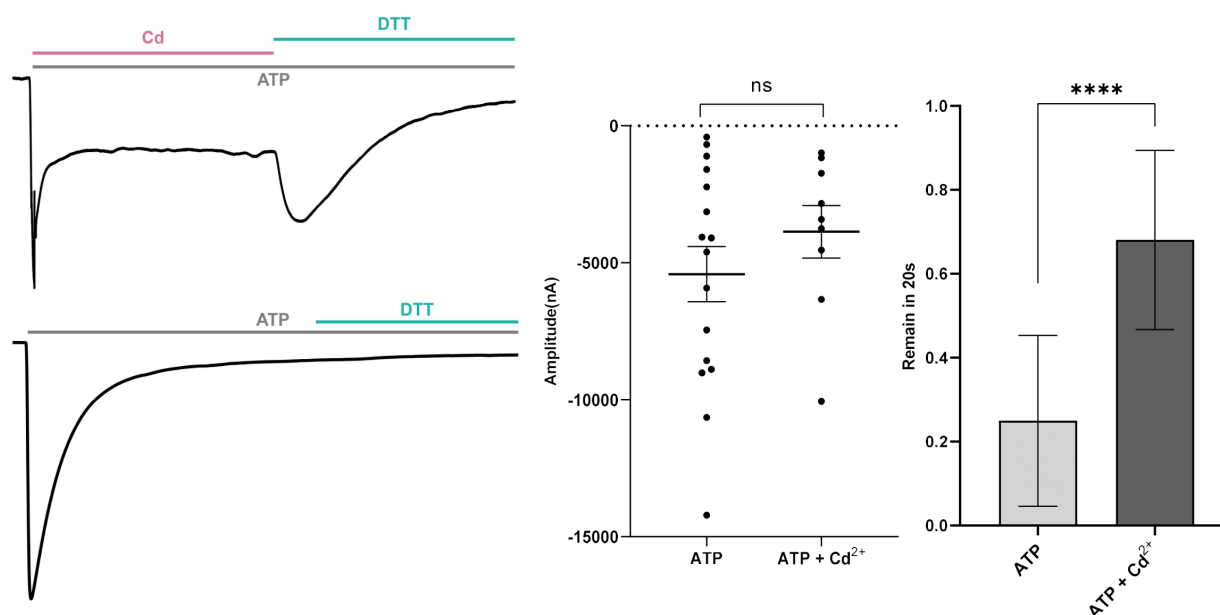


Figure A.2.9: The left panel shows representative traces of G333C, being directly activated by ATP in Cd²⁺ and subsequently DTT solution, or solely ATP and subsequently ATP+DTT as indicated. The middle panel shows amplitudes of 333C in different conditions (indicated)in scattered points, mean±SEM are marked. 333C activated by ATP yielded overall -5414±1009nA, and -3868±959.4nA. The unpaired t-test detected no differences between them. The right panel exhibits the remaining currents after 20 seconds of constant activation by ATP. Different conditions are noted. Results are shown in columns with mean±SEM marked. ATP group remained 0.25±0.05 in the 20s, while ATP+Cd²⁺ group remained 0.68±0.08. Unpaired t-tests reveal significant differences between the two groups with a P value < 0.0001. All statistical results are noted.

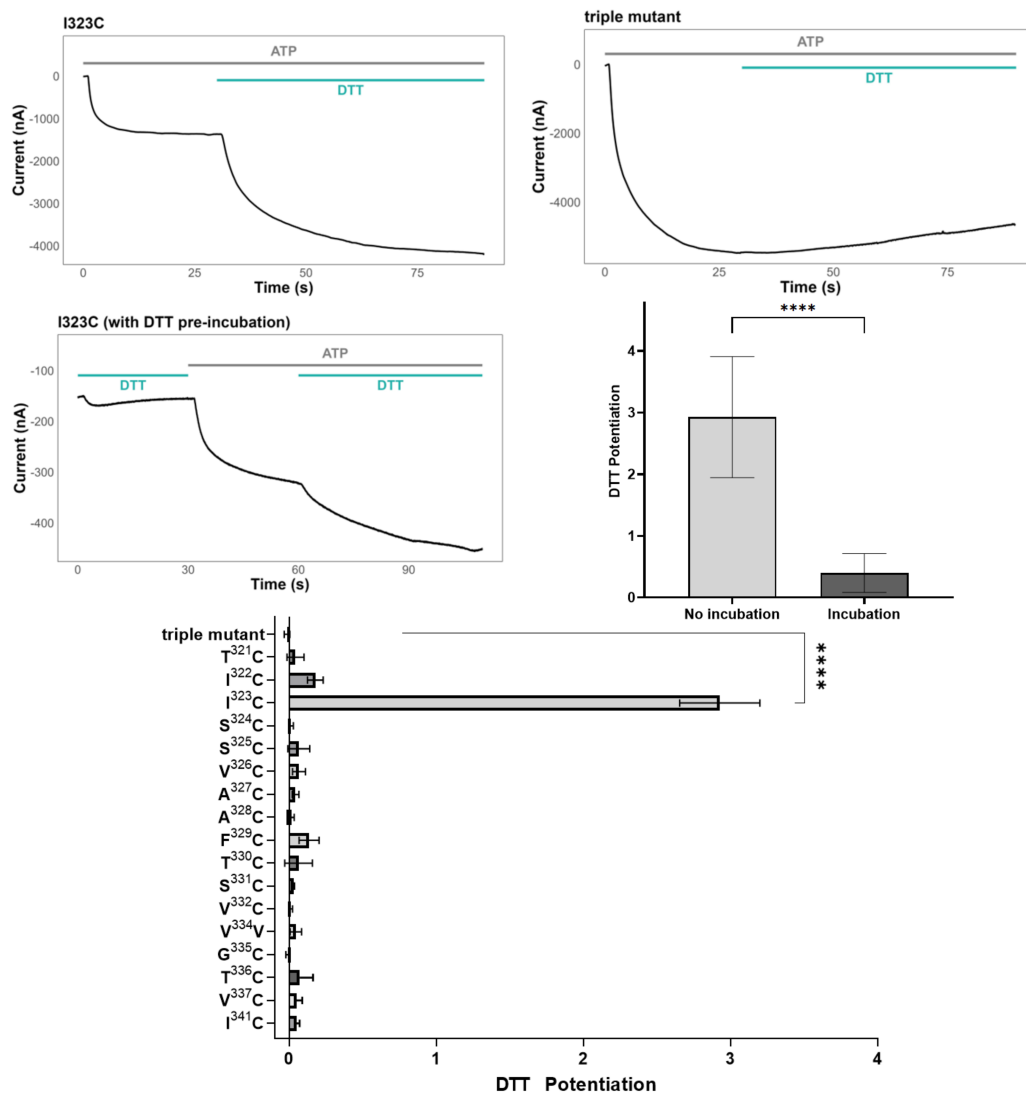


Figure A.2.10: 14 The upper and the left panels in the middle are to show representative traces of I323C and the control from TEVC. The constructions are noted in the title, chemical applications are color-coded and marked. The right panel in the middle demonstrates the effect of DTT preincubation on the overall potentiation. The conditions are described. The results are shown in columns with mean±SEM (n>7). The amplitudes from the groups that underwent preincubation recorded a DTT potentiation by an additional current increase of 2.93 ± 0.27 , while the non-incubated group was potentiated by 0.40 ± 0.11 . T-test detected a significant difference with p-value < 0.0001 as marked. The bottom panel depicts the overall results of the DTT potentiation experiments. The results are shown in columns with mean±SEM (n>3). Results are presented in additional current growth: triple mutant = -0.01 ± 0.02 , $321C = 0.04 \pm 0.06$, $322C = 0.18 \pm 0.05$, $323C = 2.93 \pm 0.27$, $324C = 0.01 \pm 0.02$, $325C = 0.07 \pm 0.07$, $326C = 0.06 \pm 0.04$, $327C = 0.04 \pm 0.02$, $328C = 0.01 \pm 0.02$, $329C = 0.14 \pm 0.07$, $330C = 0.06 \pm 0.09$, $331C = 0.03 \pm 0.01$, $332C = 0.01 \pm 0.01$, $334C = 0.05 \pm 0.04$, $335C = -0.01 \pm 0.01$, $336C = 0.07 \pm 0.09$; $337C = 0.05 \pm 0.04$, $341C = 0.05 \pm 0.02$. Multi-comparisons were performed between the triple mutant and every other mutant and detected only a significant difference between I323C and the control with a P value < 0.0001. The test result is marked.

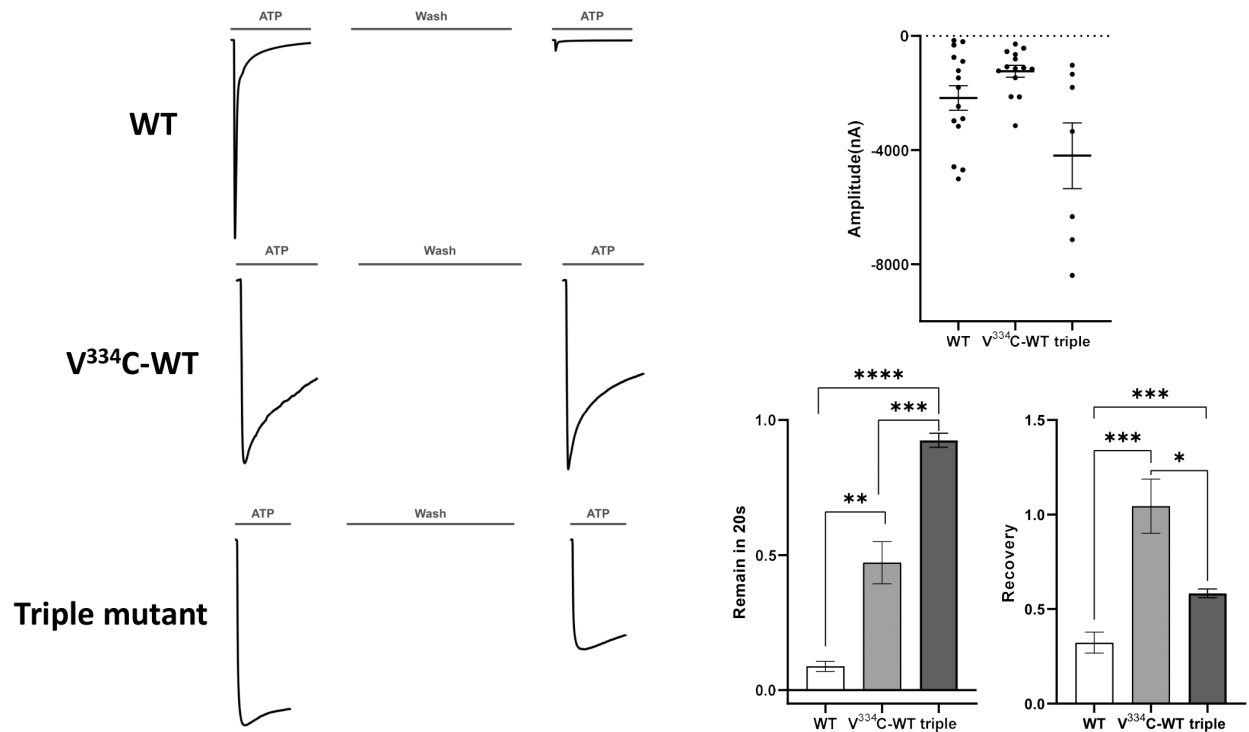


Figure A.2.11: Panel A shows representative traces. The solutions are indicated in the trace above. The constructions are as noted on the left. Panel B shows the statistical results, $n > 6$. The upper panel describes the amplitudes from the first activations of the constructions. The results are shown in scattered points with mean $\pm$ SEM marked. The WT yielded -2173 ± 431.8 nA, while the V334C-WT yielded -1236 ± 208.6 nA, and the triple mutant yielded -4197 ± 1150 nA. Multi-comparisons found a significant difference with P-value = 0.0463, with no differences found between every two constructs during multiple comparisons. The left bottom panel describes the remaining currents in 20 seconds in columns with mean $\pm$ SEM. The WT remained 0.09 ± 0.02 , the V334C-WT remained 0.47 ± 0.09 , and the triple mutant remained 0.93 ± 0.03 in 20 seconds. Multi-comparisons found a significant difference with $P < 0.0001$, multi-comparisons revealed differences between every 2 groups, and results are noted. The right bottom panel showed recovery between two activations, results represented in columns with mean $\pm$ SEM. The WT recorded 0.32 ± 0.06 during repetitive activations, V334-WT recovered 1.04 ± 0.14 , and the triple mutant recovered 0.54 ± 0.06 . The one-way ANOVA test found a significant difference with $P < 0.0001$, multiple comparisons revealed differences between every 2 groups, and results are noted.

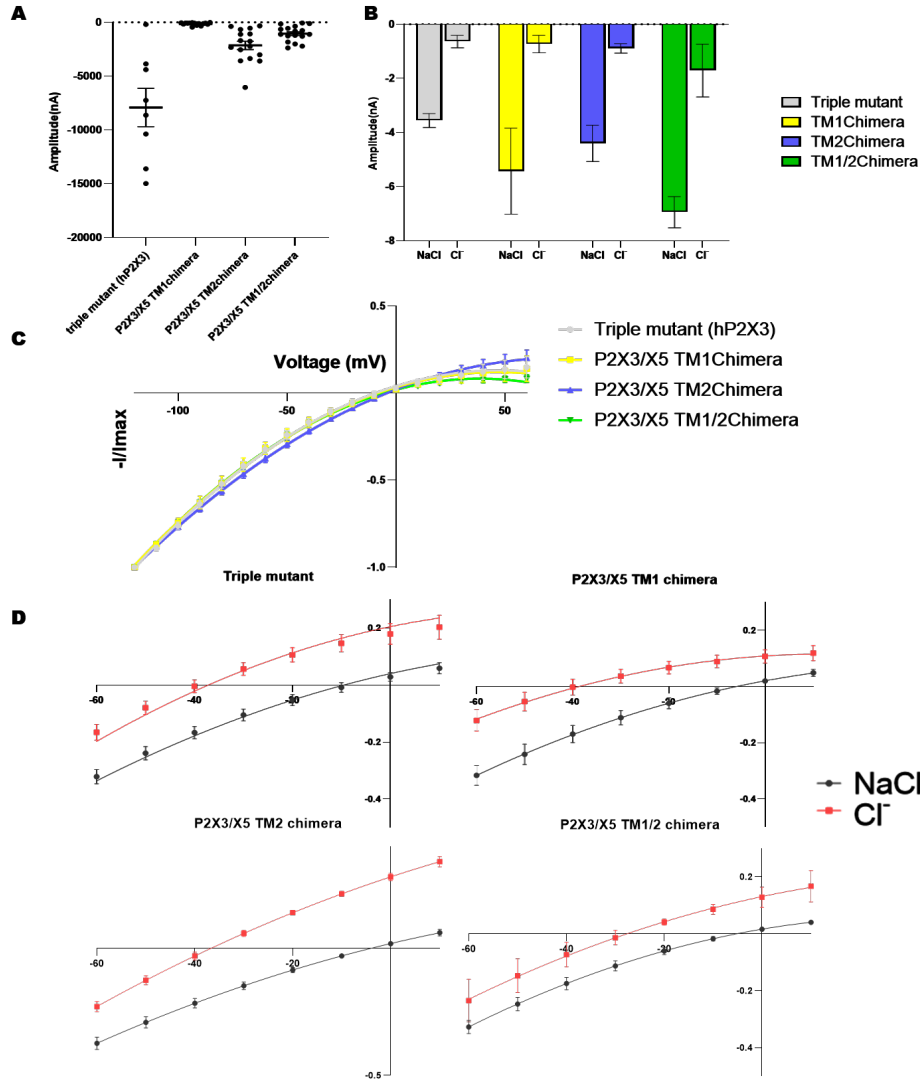


Figure A.3.1: shows the current amplitude on TEVC (oocytes) under 10 μ M ATP; results are scatter points with mean $\pm$ SEM ($n > 8$). Triple mutant: -7903 ± 1781 nA; TM1 chimera: -96.37 ± 20.0 nA; TM2 chimera: -2139 ± 384.8 nA; TM1/2 chimera: -1047 ± 176.6 nA. Panel B addresses the current amplitudes on the patch clamp. Results are grouped by constructs (color-coded as indicated by legends) with different solutions indicated on the axis. Results are shown in columns with specified SEM. The triple mutant yields $-3.56 \times 10^{-9} \pm 2.59 \times 10^{-10}$ A in NaCl and $-6.35 \times 10^{-10} \pm 2.3 \times 10^{-10}$ A in Choline-Cl (Cl^-); the TM1 chimera yields $-5.43 \times 10^{-9} \pm 1.59 \times 10^{-9}$ A in NaCl and $-7.31 \times 10^{-10} \pm 3.2 \times 10^{-10}$ A in Cl^- ; the TM2 chimera yields $-4.40 \times 10^{-9} \pm 6.73 \times 10^{-10}$ A in NaCl and $-8.90 \times 10^{-10} \pm 1.78 \times 10^{-10}$ A in Cl^- ; and the TM1/2 chimera yields $-6.95 \times 10^{-9} \pm 5.79 \times 10^{-10}$ A in NaCl and $-1.71 \times 10^{-10} \pm 9.75 \times 10^{-10}$ A in Cl^- . Panel C shows the I/V curve obtained from the patch clamp of the four constructs, with results normalized to the maximal current. Panel D shows normalized I/V curves (-60 mV to $+10$ mV) under NaCl and Cl^- conditions (color-coded).

Figure A.3.2

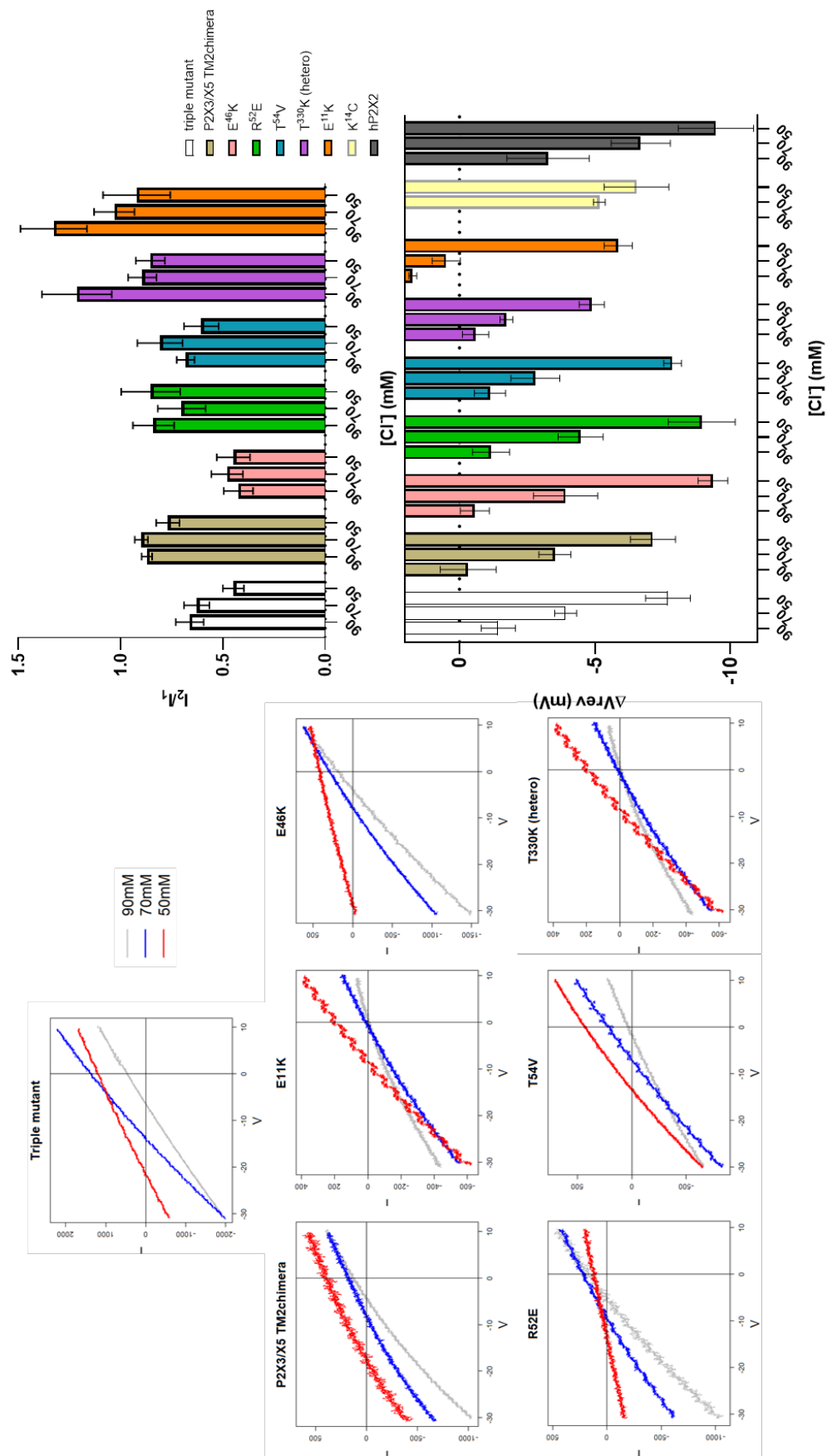


Figure A.3.2: The left panel from the observing orientation describes the representative I/V curves showing shifting of the V_{rev} during the decrease of $[Cl^-]$. The different concentrations are color-coded and noted. Details of the constructs are indicated in the titles. The curves are zoomed in at -30 to +10 mV intervals for a clear view. The upper right panel shows grouped I_2/I_1 values of each construct under different conditions, all constructs are color-coded and indicated in the legend, and the conditions are specified on the axis. The triple mutant defined the baseline of current recovery, apart from 2 minutes of rinse, as the I_2/I_1 of the group 90mM/90mM NaCl with a ratio of 0.66 ± 0.07 . During the decrease of Cl^- to 70mM and 50mM, the ratio reduced to 0.63 ± 0.06 and 0.45 ± 0.05 , respectively. The TM2 chimera yielded an I_2/I_1 of 0.87 ± 0.03 in 90/90 solution, 0.90 ± 0.03 in 90/70 Cl^- and 0.77 ± 0.06 in 90/50 Cl^- . The E46K mutation resulted in ratios of 0.43 ± 0.07 , 0.48 ± 0.08 , and 0.45 ± 0.08 ; while the R52E variant showed 0.84 ± 0.10 , 0.70 ± 0.12 , and 0.85 ± 0.14 . The T54V mutant ratios were 0.68 ± 0.04 , 0.81 ± 0.11 , and 0.61 ± 0.08 . The T330K mutant demonstrated a higher I_2/I_1 ratio in general, with 1.22 ± 0.17 , 0.89 ± 0.07 , and 0.86 ± 0.07 . E11K had ratios of 1.33 ± 0.49 , 1.03 ± 0.31 , and 0.92 ± 0.55 . The lower right panel shows grouped ΔV_{rev} values of each construct under different conditions, all constructs are color-coded and indicated in the legend, and the conditions are specified on the axis. The triple mutant establishes the baseline of ΔV_{rev} after a 2-minute rinse, with a value of 0.66 ± 0.07 in the group exposed to 90mM/90mM NaCl. When the Cl^- concentration is decreased to 70mM and then to 50mM, the ΔV_{rev} diminishes correspondingly to 0.63 ± 0.06 and 0.45 ± 0.05 . The TM2 chimera shows ΔV_{rev} values of 0.87 ± 0.03 for 90/90 NaCl, 0.90 ± 0.03 for 90/70 Cl^- , and 0.77 ± 0.06 for 90/50 Cl^- . For the E46K mutation, the ΔV_{rev} values are 0.43 ± 0.07 , 0.48 ± 0.08 , and 0.45 ± 0.08 across the decreasing Cl^- concentrations. The R52E variant yields ΔV_{rev} values of 0.84 ± 0.10 , 0.70 ± 0.12 , and 0.85 ± 0.14 . The ΔV_{rev} values for the T54V mutant are 0.68 ± 0.04 , 0.81 ± 0.11 , and 0.61 ± 0.08 , while the T330K mutant demonstrates higher overall ΔV_{rev} values of 1.22 ± 0.17 , 0.89 ± 0.07 , and 0.86 ± 0.07 . Lastly, the E11K mutation presents ΔV_{rev} values of 1.33 ± 0.49 , 1.03 ± 0.31 , and 0.92 ± 0.55 for the respective Cl^- concentrations.

Figure A.3.3

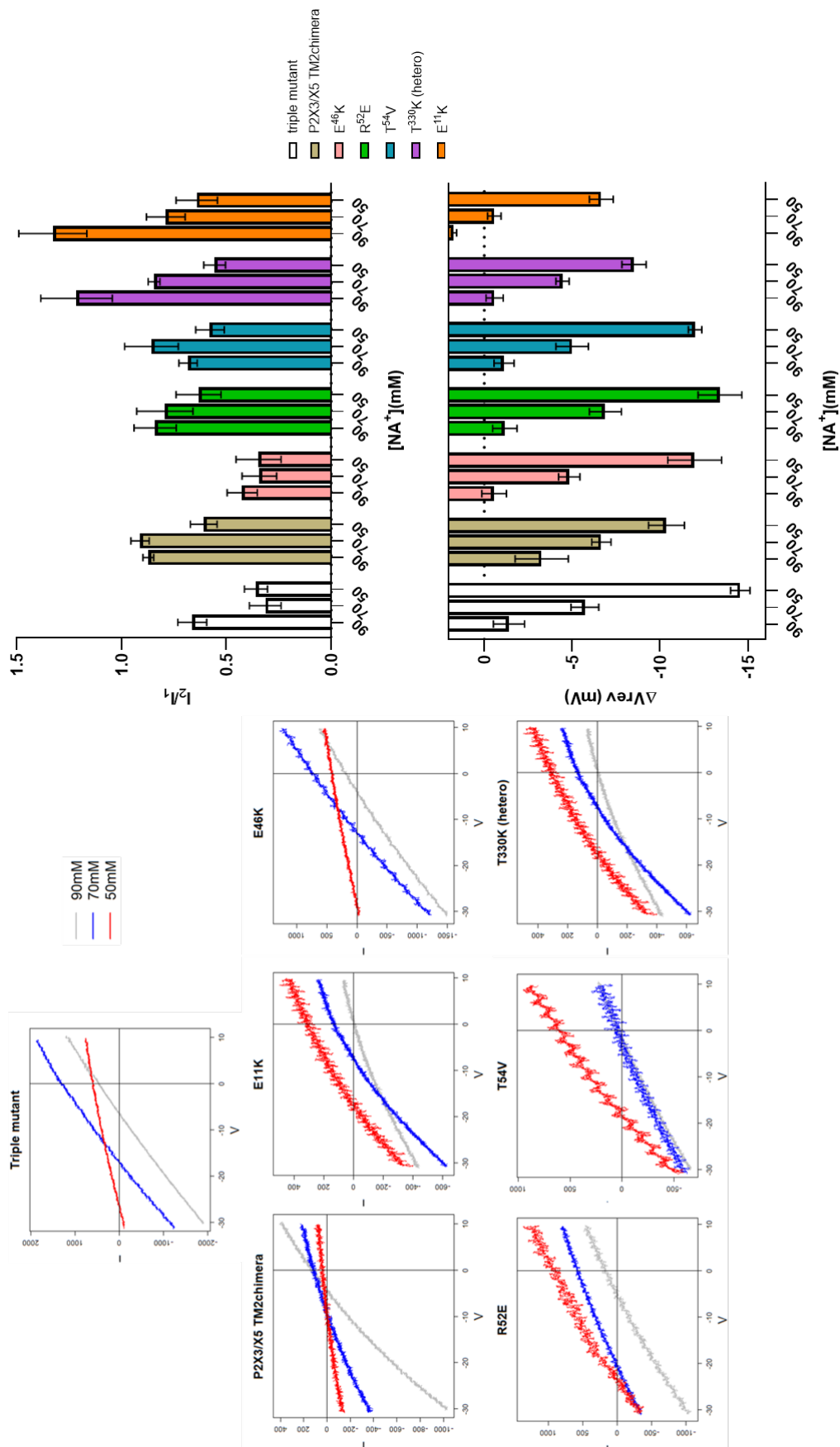


Figure A.3.3: The left panel from the observing orientation describes the representative I/V curves showing shifting of the V_{rev} during the decrease of $[Na^+]$. The different concentrations are color-coded and noted. Details of the constructs are indicated in the titles. The curves are zoomed in at -30 to +10 mV intervals for a clear view. The upper right panel shows grouped I₂/I₁ values of each construct under different conditions, all constructs are color-coded and indicated in the legend, and the conditions are specified on the axis. The triple mutant defined the baseline of current recovery, apart from 2 minutes of rinse, as the I₂/I₁ of the group 90mM/90mM NaCl with a ratio of 0.66 ± 0.07 . During the decrease of Cl^- to 70mM and 50mM, the I₂/I₁ ratio reduced to 0.63 ± 0.06 and 0.45 ± 0.05 respectively. The TM2 chimera yielded an I₂/I₁ of 0.87 ± 0.03 in 90/90 solution, 0.90 ± 0.03 in 90/70 Cl^- and 0.77 ± 0.06 in 90/50 Cl^- . The E46K mutation resulted in I₂/I₁ ratios of 0.43 ± 0.07 , 0.48 ± 0.08 , and 0.45 ± 0.08 ; while the R52E variant showed 0.84 ± 0.10 , 0.70 ± 0.12 , and 0.85 ± 0.14 . The T54V mutant I₂/I₁ ratios were 0.68 ± 0.04 , 0.81 ± 0.11 , and 0.61 ± 0.08 . The T330K mutant demonstrated a higher I₂/I₁ ratio in general, with 1.22 ± 0.17 , 0.89 ± 0.07 , and 0.86 ± 0.07 . E11K had I₂/I₁ ratios of 1.33 ± 0.49 , 1.03 ± 0.31 , and 0.92 ± 0.55 . The lower right panel shows grouped ΔV_{rev} values of each construct under different conditions, all constructs are color-coded and indicated in the legend, and the conditions are specified on the axis. The triple mutant showed a mean ΔV_{rev} of -1.414 ± 0.8863 at 90mM Na^+ , which progressively decreased to -14.57 ± 0.5519 at 50mM Na^+ . E46K exhibited a less negative ΔV_{rev} at higher Na^+ levels (-0.5611 ± 0.6992 at 90mM Na^+) but became more negative as Na^+ decreased (-11.97 ± 1.526 at 50mM Na^+). The R52E mutation followed a similar trend with a ΔV_{rev} of -1.17 ± 0.6856 at 90mM Na^+ and -13.41 ± 1.245 at 50mM Na^+ . T54V had a ΔV_{rev} of -1.128 ± 0.575 at 90mM Na^+ and -12 ± 0.3809 at 50mM Na^+ . The P2X3/X5 TM2 chimera's ΔV_{rev} decreased from -3.276 ± 1.519 at 90mM Na^+ to -10.37 ± 1.02 at 50mM Na^+ . The T330K (hetero) construct showed an increase in the negative ΔV_{rev} with a decrease in Na^+ concentration, from -0.5927 ± 0.4846 at 90mM Na^+ to -8.521 ± 0.6891 at 50mM Na^+ . Lastly, E11K had a ΔV_{rev} of 1.722 ± 0.1464 at 90mM Na^+ , turning more negative with decreasing Na^+ to -6.665 ± 0.676 at 50mM Na^+ .

A. II: Result Tables

Pairing residues	340 - 357	14 - 359	356 - 359	357 - 359
K0	0.943±0.0113			
K(X)	0.765±0.0542	0.711±0.151	0.409±0.0343	0.441±0.0561
K(Y)	0.559±0.0908	0.441±0.0561		
K(XY)	0.852±0.0326	0.320±0.0249	0.345±0.0594	0.694±0.0793
P values	<0.0001	0.0760	0.0030	0.0009
Summary	****	ns	*	***

Table A.1.1: Double mutant cycle analysis of pairing residues potentially forms salt bridges. K0 refers to the triple mutant, K(X), K(Y), and K(XY) represent single and double mutant constructs, respectively. Calculated values correspond to the coupling energies of indicated residue pairs, with the summary column denoting statistical significance of differences.

Pairing residues	8 – 21
K0	0.943±0.0113
K(X)	0.289±0.0407
K(Y)	0.967±0.0077
K(XY)	0.227±0.0343
P values	0.0250
Summary	*

Table A.1.2: Double mutant cycle analysis of pairing residues with potential hydrophobic interactions. K0 refers to the triple mutant, K(X), K(Y), and K(XY) represent single and double mutant constructs, respectively. Calculated values correspond to the coupling energies of indicated residue pairs, with the summary column denoting statistical significance of differences.

	Triple mutant			P2X3/X5 TM2chimera			E11K			11K-X3/X5 TM2chimera		
	mean	SEM	n	mean	SEM	n	mean	SEM	n	mean	SEM	n
Na ⁺	0.07904	0.01208	8	0.2468	0.05432	9	0.2173	0.06422	10	0.1559	0.02945	10
Cl ⁻	0.03492	0.006078	9	0.1542	0.02196	9	0.1943	0.04485	10	0.1889	0.05039	9

Table A.3.1: I2/I1 ratio of each construct in either Na⁺ or Cl⁻ Ori⁻.

	Triple mutant			P2X3/X5 TM2chimera			E11K			11K-X3/X5 TM2chimera		
	mean	SEM	n	mean	SEM	n	mean	SEM	n	mean	SEM	n
Na ⁺	-26.49	1.617	11	-32.67	3.56	11	-8.981	2.9	9	-21.69	3.834	7
Cl ⁻	-44.95	1.493	11	-45.36	1.265	11	-9.115	5.153	11	-33.48	1.917	8

Table A.3.2: ΔV_{rev} value of each construct in either Na⁺ or Cl⁻ Ori⁻.

	I2/I1		ΔV_{rev}	
	<i>P</i> value	summary	<i>P</i> value	summary
Component	0.3033	ns	<0.0001	****
Construct	0.0033	**	<0.0001	****
Component x Construct	0.5144	ns	0.0286	*

Table A.3.3: Summary of 2-way ANOVA results where all constructs are analyzed collectively. I2/I1 and ΔV_{rev} values of all constructs were compared, respectively, as indicated in the headings.

	Triple mutant		P2X3/X5 TM2chimera		E11K	
	Summary	<i>P</i> Value	Summary	<i>P</i> Value	Summary	<i>P</i> Value
P2X3/X5 TM2chimera	**	0.0097	ns	>0.9999	ns	0.96
E11K	**	0.0053				
11K-X3/X5 TM2chimera	ns	0.0538	ns	0.9846	ns	0.96

Table A.3.4: Summary of Sidak's multiple comparisons between I2/I1 values of every two constructs. Comparison results are shown in the summary and *P* values.

	Triple mutant		P2X3/X5 TM2chimera		E11K	
	Summary	P Value	Summary	P Value	Summary	P Value
P2X3/X5 TM2chimera	ns	0.853				
E11K	****	<0.0001	****	<0.0001		
11K-X3/X5 TM2chimera	ns	0.0809	**	0.0041	****	<0.0001

Table A.3.5: Summary of Sidak's multiple comparisons between ΔV_{rev} values of every two constructs. Comparison results are shown in the summary and P values.

Triple mutant									
	mean	SEM	n						
90mM Cl ⁻	0.663	0.0689	16						
70mM Cl ⁻	0.6288	0.0616	13						
50mM Cl ⁻	0.4498	0.0514	13						
E46K				R52E			T54V		
	mean	SEM	n	mean	SEM	n	mean	SEM	n
90mM Cl ⁻	0.4251	0.0723	14	0.8405	0.1007	14	0.6835	0.0439	11
70mM Cl ⁻	0.4799	0.0768	20	0.7027	0.1165	16	0.8086	0.111	11
50mM Cl ⁻	0.4495	0.0816	20	0.8536	0.1441	17	0.6066	0.0842	9
P2X3/X5 TM2chimera				T330K (hetero)			E11K		
	mean	SEM	n	mean	SEM	n	mean	SEM	n
90mM Cl ⁻	0.8727	0.0253	8	1.215	0.1707	11	1.328	0.1627	9
70mM Cl ⁻	0.8993	0.0324	17	0.8946	0.0692	16	1.032	0.099	10
50mM Cl ⁻	0.7704	0.0571	17	0.8556	0.0712	15	0.9222	0.1646	11

Table A.3.6: I2/I1 values from each construct under 90/70/50mM [Cl⁻], results presented in mean, SEM, and n (number of measurements).

	vs. E46K		vs. R52E		vs. T54V	
	P value	Summary	P value	Summary	P value	Summary
[Cl ⁻]	0.3062	ns	0.4119	ns	0.0317	*
Construct	0.0383	*	0.0259	*	0.0757	ns
[Cl ⁻] x Construct	0.2855	ns	0.2367	ns	0.4734	ns
	vs. P2X3/X5 TM2 chimera		vs. T330K (hetero)		vs. E11K	
	P value	Summary	P value	Summary	P value	Summary
[Cl ⁻]	0.0087	**	0.0107	*	0.0151	*
Construct	<0.0001	****	<0.0001	****	<0.0001	****
[Cl ⁻] x Construct	0.6686	ns	0.2391	ns	0.4245	ns

Table A.3.7: 2-way ANOVA results of comparing I2/I1 values between triple mutant and mutants, indicated as (triple mutant) vs. constructs as indicated. Each comparison is composed of P values and summaries regarding different effects as indicated.

	Triple mutant			hP2X2			K14C		
	mean	SEM	n	mean	SEM	n	mean	SEM	n
90mM Cl ⁻	-1.44	0.63	7	-3.28	1.52	6			
70mM Cl ⁻	-3.92	0.40	13	-6.70	1.09	7	-5.17	0.21	3
50mM Cl ⁻	-7.70	0.83	14	-9.47	1.39	7	-6.54	1.19	3
	E46K			R52E			T54V		
	mean	SEM	n	mean	SEM	n	mean	SEM	n
90mM Cl ⁻	-0.57	0.54	7	-1.17	0.69	9	-1.13	0.58	10
70mM Cl ⁻	-3.92	1.18	13	-4.47	0.83	13	-2.80	0.91	9
50mM Cl ⁻	-9.36	0.55	14	-8.95	1.24	8	-7.87	0.33	9
	E11K			T330K (hetero)			P2X3/X5 TM2 chimera		
	mean	SEM	n	mean	SEM	n	mean	SEM	n
90mM Cl ⁻	1.72	0.15	10	-0.59	0.48	10	-0.33	1.04	6
70mM Cl ⁻	0.49	0.52	10	-1.74	0.24	10	-3.53	0.59	13
50mM Cl ⁻	-5.87	0.52	9	-4.89	0.47	13	-7.15	0.83	15

Table A.3.8: ΔV_{rev} values from each construct under 90/70/50mM [Cl⁻], results presented in mean, SEM, and n (number of measurements).

	vs. hP2X2		vs. T54V	
	<i>P value</i>	<i>Summary</i>	<i>P value</i>	<i>Summary</i>
[Cl ⁻]	<0.0001	****	<0.0001	****
Construct	0.0111	*	0.4685	ns
[Cl ⁻] x Construct	0.8079	ns	0.6197	ns
	vs. E46K		vs. R52E	
	<i>P value</i>	<i>Summary</i>	<i>P value</i>	<i>Summary</i>
[Cl ⁻]	<0.0001	****	<0.0001	****
Construct	0.7019	ns	0.4527	ns
[Cl ⁻] x Construct	0.3075	ns	0.6896	ns
	vs. P2X3/X5 TM2 chimera		vs. T330K (hetero)	
	<i>P value</i>	<i>Summary</i>	<i>P value</i>	<i>Summary</i>
[Cl ⁻]	<0.0001	****	<0.0001	****
Construct	0.442	ns	0.0002	***
[Cl ⁻] x Construct	0.843	ns	0.2696	ns
	vs. E11K		vs. K14C	
	<i>P value</i>	<i>Summary</i>	<i>P value</i>	<i>Summary</i>
[Cl ⁻]	<0.0001	****	0.0193	*
Construct	<0.0001	****	0.0737	ns
[Cl ⁻] x Construct	0.0973	ns	0.083	ns

Table A.3.9: Δ2-way ANOVA results of comparing ΔV_{rev} values between triple mutant and mutants, indicated as (triple mutant) vs. constructs as indicated. Each comparison is composed of P values and summaries regarding different effects as indicated.

Triple mutant									
	mean	SEM	n						
90mM Na ⁺	0.663	0.0689	16						
70mM Na ⁺	0.3147	0.0758	6						
50mM Na ⁺	0.3593	0.055	10						
E46K			R52E			T54V			
	mean	SEM	n	mean	SEM	n	mean	SEM	n
90mM Na ⁺	0.4251	0.0723	14	0.8405	0.1007	14	0.6835	0.0439	11
70mM Na ⁺	0.3437	0.0825	9	0.7941	0.1344	12	0.8573	0.1278	9
50mM Na ⁺	0.3468	0.1073	10	0.6334	0.1076	10	0.5791	0.0682	8
P2X3/X5 TM2chimera			T330K (hetero)			E11K			
	mean	SEM	n	mean	SEM	n	mean	SEM	n
90mM Na ⁺	0.8727	0.0253	8	1.215	0.1707	11	1.328	0.1627	9
70mM Na ⁺	0.9116	0.0434	9	0.8455	0.028	6	0.7894	0.0925	11
50mM Na ⁺	0.6084	0.0635	11	0.5561	0.0518	10	0.6414	0.0987	10

Table A.3.10: I2/I1 values from each construct under 90/70/50mM [Na⁺], results presented in mean, SEM, and n (number of measurements).

	vs. E46K		vs. R52E		vs. T54V	
	P value	Summary	P value	Summary	P value	Summary
[Na ⁺]	0.0309	*	0.0354	*	0.0431	*
Construct	0.3535	ns	0.0005	***	0.0002	***
Na ⁺] x Construc	0.1536	ns	0.3479	ns	0.008	**
	vs. P2X3/X5 TM2 chimera		vs. T330K (hetero)		vs. E11K	
	P value	Summary	P value	Summary	P value	Summary
[Na ⁺]	0.0003	***	<0.0001	****	<0.0001	****
Construct	<0.0001	****	<0.0001	****	<0.0001	****
Na ⁺] x Construc	0.0259	*	0.1339	ns	0.1351	ns

Table A.3.11: 2-way ANOVA results of comparing I2/I1 values between triple mutant and mutants, indicated as (triple mutant) vs. constructs as indicated. Each comparison is composed of P values and summaries regarding different effects as indicated.

Triple mutant												
	mean	SEM	n									
90mM Na ⁺	-1.414	0.8863	8									
70mM Na ⁺	-5.727	0.789	9									
50mM Na ⁺	-14.57	0.5519	11									
	E46K			R52E			T54V					
	mean	SEM	n	mean	SEM	n	mean	SEM	n			
90mM Na ⁺	-0.561	0.6992	12	-1.17	0.6856	9	-1.128	0.575	10			
70mM Na ⁺	-4.842	0.6024	10	-6.891	0.9077	6	-5.01	0.9214	8			
50mM Na ⁺	-11.97	1.526	3	-13.41	1.245	3	-12	0.3809	8			
	P2X3/X5 TM2chimera			T330K (hetero)			E11K					
	mean	SEM	n	mean	SEM	n	mean	SEM	n			
90mM Na ⁺	-3.276	1.519	6	-0.593	0.4846	10	1.722	0.1464	10			
70mM Na ⁺	-6.67	0.5472	3	-4.457	0.3688	7	-0.585	0.3837	6			
50mM Na ⁺	-10.37	1.02	7	-8.521	0.6891	9	-6.665	0.676	9			

Table A.3.12: ΔVrev values from each construct under 90/70/50mM [Na⁺], results presented in mean, SEM, and n (number of measurements).

	vs. E46K		vs. R52E		vs. T54V	
	P value	Summary	P value	Summary	P value	Summary
[Na ⁺]	<0.0001	****	<0.0001	****	<0.0001	****
Construct	0.0269	*	0.9092	ns	0.0423	*
Na ⁺] x Construc	0.4851	ns	0.426	ns	0.2236	ns
	vs. P2X3/X5 TM2 chimera		vs. T330K (hetero)		vs. E11K	
	P value	Summary	P value	Summary	P value	Summary
[Na ⁺]	<0.0001	****	<0.0001	****	<0.0001	****
Construct	0.5735	ns	<0.0001	****	<0.0001	****
Na ⁺] x Construc	0.0039	**	0.0002	***	0.0011	**

Table A.3.13: 2-way ANOVA results of comparing ΔVrev values between triple mutant and mutants, indicated as (triple mutant) vs. constructs as indicated. Each comparison is composed of P values and summaries regarding different effects as indicated.

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First and for the most, I would like to express my sincere gratitude to Prof. Dr. med. Ralf Hausmann for offering me the opportunity to work on this project. Throughout the study, his consistent support, insightful scientific guidance, and open, constructive discussions have been invaluable. I am especially grateful for his patience and his genuine willingness to help. While always generous in providing guidance and support, he also ensured that I always had the freedom to explore independently. I truly appreciate his guidance throughout this journey.

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Eidesstattliche Erklärung

erkläre hiermit, dass diese Dissertation und die darin dargelegten Inhalte die eigenen sind und selbstständig, als Ergebnis der eigenen originären Forschung, generiert wurden.

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3. Wenn immer andere eigene- oder Veröffentlichungen Dritter herangezogen wurden, wurden diese klar benannt;
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