

Modulatory Influence of PCOL on ASIC1a: Electrophysiological Investigation of Key
Functional Domains

Von der Medizinischen Fakultät
der Rheinisch-Westfälischen Technischen Hochschule Aachen
zur Erlangung des akademischen Grades eines Doktors der Medizin
genehmigte Dissertation

vorgelegt von

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Tag der mündlichen Prüfung: 04.03.2026

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Abbreviations

AA	-	Amino acid
ASIC	-	Acid-sensing ion channel
BASIC	-	Bile acid-sensitive ion channel
bp	-	Base pair
cASIC	-	ASIC from <i>Gallus gallus</i> (chicken)
C-base	-	Cytosin
CHO	-	Chinese hamster ovary (cell line)
CNS	-	Central nervous system
C-terminus	-	Carboxy-terminus (COOH); end of a protein
DEG	-	Degenerin (ion-channels)
DMEM	-	Dulbecco's modified eagle medium
DNA	-	Desoxyribonucleic acid
dNTPs	-	desoxyribose nucleoside tri-phosphates
EC ₅₀	-	Half-maximal effective concentration
E. coli	-	Escherichia coli
EDTA	-	Ethylenediaminetetraacetic acid
EGTA	-	Ethylene glycol tetraacetic acid
ENaC	-	Epithelial sodium channel
FAD	-	Flavin adenine dinucleotide
FBS	-	Fetal bovine serum
GFP	-	Green fluorescent protein
H ₂ O or aqua	-	Water
hASIC	-	ASIC from <i>Homo sapiens sapiens</i>

HEK293	-	Human embryonic kidney 293 (cell line)
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC50	-	Half-maximal inhibitory concentration
KCl	-	Potassium chloride
KOH	-	Potassium hydroxide
LB-medium	-	Lysogeny broth (bacteria medium)
MES	-	2-(N-morpholino)ethane sulfonic acid
NAD	-	Nicotinamide adenine dinucleotide
N-terminus	-	amine-terminus (NH ₂); start of a protein
PCO	-	Prenylcysteine oxidase 1
PCOL	-	Prenylcysteine oxidase 1-like
PCR	-	Polymerase chain reaction
PcTx1	-	Psalmotoxin
PCYOX1	-	Prenylcysteine oxidase 1
PCYOX1-L	-	Prenylcysteine oxidase 1-like
PDZ	-	PSD-95/Dlg/ZO-1 (protein-interaction domain)
PICK1	-	Protein interacting with C kinase 1
PNS	-	Peripheral nervous system
PSD95	-	Postsynaptic density protein 95
rASIC	-	ASIC of <i>Rattus norvegicus</i>
RFP	-	Red fluorescent protein
RNA	-	Ribonucleic acid
SB-solution	-	Standard bath solution for electrophysiological measurements
SD	-	Standard deviation
SEM	-	Standard error of the mean

SSD	-	Steady-state desensitization
TEA	-	Tetraethylammonium
WT <i>or</i> wt	-	Wildtype

1 Introduction

1.1 Acid-sensing Ion Channels

Low pH can cause pain (Reeh & Steen, 1996; Steen et al., 1995; Steen et al., 1996). Thus the question arises how acid can activate depolarizing currents in peripheral sensory neurons, leading to the hypothesis of a receptor for protons (Bevan & Yeats, 1991; Krishtal & Pidoplichko, 1980, 1981). In 1997 the “Acid-sensing Ion Channel” alpha and beta (1a and 1b) were among the first channels of this group to be identified in a rat brain (Waldmann et al., 1997).

Acid-sensing Ion Channels (ASICs) are proton gated Na⁺-channels that belong to the voltage-insensitive Degenerin/Epithelial Na⁺-Channel (DEG/ENaC) gene superfamily (Chalfie & Wolinsky, 1990; Kellenberger & Schild, 2002). ASICs bind protons with high affinity, leading to a rapid opening of the channel (Babini et al., 2002; Sutherland et al., 2001). ASICs and DEG/ENaC channels share a homology of 15-20% (**Figure 1**) and all members of the ASIC subfamily share an amino acid sequence that is at least 45% identical (Saugstad et al., 2004).

All channels within the DEG/ENaC gene superfamily share a common membrane topology with each subunit consisting of two hydrophobic transmembrane domains, short intracellular N and C termini (around 35-90 aa), and a large extracellular region (around 370 aa) (Jasti et al., 2007; Saugstad et al., 2004).

In mammals, the ASIC gene family is composed of five genes (*Accn1-Accn5*), two of which (*Accn1* and *Accn2*) can be subject to alternative splicing, resulting in a difference of the large first exon (Bässler et al., 2001). Other reported splice variants are not functional or of unknown significance (Delaunay et al., 2012; Hoagland et al., 2010; Ugawa et al., 2001).

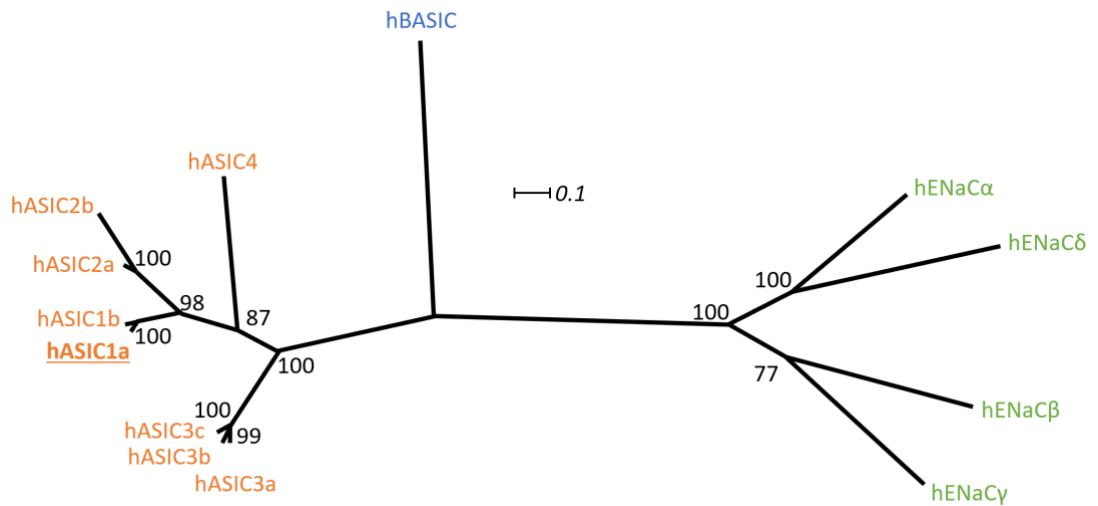


Figure 1: Phylogenetic tree of the degenerin (DEG)/human epithelial sodium channel (ENaC) family. The organization into subfamilies and the evolutionary distance between members of the Deg/ENaC gene family that are expressed in humans is displayed. The channels are divided into three groups: α -, β -, γ - and δ -epithelial sodium channels (ENaC) on the right, ASIC1a,-1b,-2a,-2b,-3a,-3b,-3c and 4 on the left and BASIC in the middle. Digits indicate the number of substitutions in the gene.

ASIC1a subunits can assemble as homo- or heterotrimers with a flexible stoichiometry to form ASIC1a, ASIC1a/ASIC2a (Baron et al., 2002), ASIC1a/ASIC2b, and ASIC1a/ASIC2a/ASIC3 (Bartoi et al., 2014; Hesselager et al., 2004). ASIC1a is expressed in the CNS and PNS (Waldmann et al., 1997; Wemmie et al., 2002). In the CNS ASIC1a is primarily localized in brain regions with high synaptic density, such as the amygdala (Wemmie et al., 2003) While ASIC1a channels have been shown to be predominantly localized in the endoplasmic reticulum (Chai et al., 2010), they can be trafficked to the plasma membrane of the soma and cellular processes (Alvarez de la Rosa et al., 2002) and to the postsynaptic membrane (Kreple et al., 2014).

Since the resolution of the crystal structure of cASIC1a (ASIC1a from *Gallus gallus*) in 2007 (**Figure 2 A**) it is known that functional ASICs are composed of three subunits (Jasti et al., 2007). The extracellular loop is often compared to a clenched fist, comprising domains such as the thumb, finger, knuckle, palm, and β -ball (Jasti et al., 2007) (**Figure 2 B**). Formed by the finger, thumb and β -ball domain, the acidic pocket with its proton binding residues such as D238, D346, and D350 is thought to be the key region for proton binding (Jasti et al., 2007) (**Figure 2 C**). Additionally, residues L415 and N416, commonly referred to as the

β 11-12 linker, play a pivotal role in the desensitization mechanism of the channel (Rook et al., 2020) (**Figure 2 B**). Another important structural motif is the GAS-belt, characterized by the presence of glycine (G), alanine (A), and serine (S) residues, typically located at the bend of the second transmembrane helix. The GAS-belt is presumed to contribute to ion selectivity, gating and protein stability (Baconguis et al., 2014).

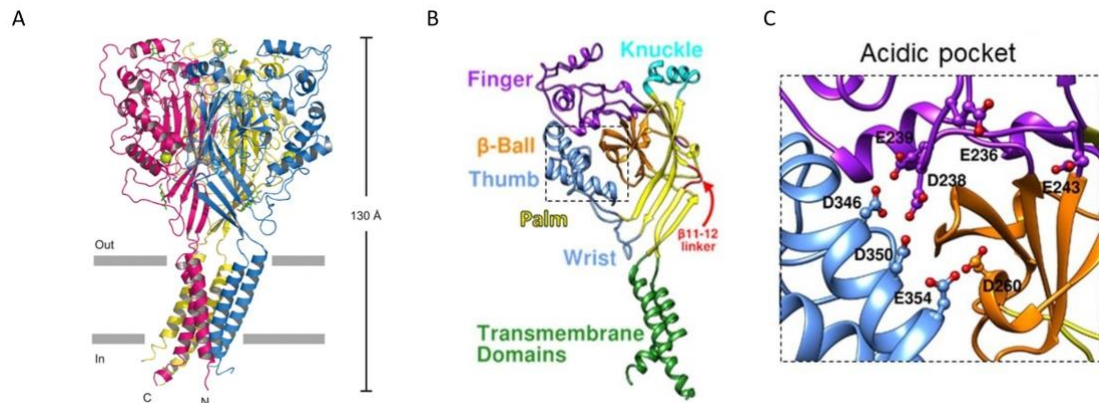


Figure 2: Chrystal Structure of cASIC1a. **A:** ASIC1a is a trimeric channel (different colours for individual subunits). Each subunit consists of two transmembrane domains, one large extracellular loop and short intracellular N- and C-termini (Jasti et al., 2007). **B:** Clenched fist model of ASIC1a with marked β 11-12 linker. **C:** The acidic pocket of ASIC1a. Proton binding residues are highlighted.

1.2 Functional and Biophysical Properties of ASIC1a

Although chemically diverse, acids share the common property of releasing hydrogen ions (protons, H^+). Depending on the environment of the protein and on the presence of water, protons play a significant role in protein structure and function (Schönichen et al., 2013) and can be bound to amino acids such as Glu, Asp, Arg, Lys and His. However, their size and omnipresence make protons a unique class of ligands that require highly cooperative gating mechanisms allowing for a dose-dependent response. Under physiological conditions, the pH is buffered to approximately 7.4 with a relatively low concentration of protons and low probability of ASIC activation.

ASIC1a is activated by mild acidification to pH ~6.9 – equivalent to a threefold increase of protons from 40 nM (pH 7.4) to 125 nM (pH 6.9) – with a full saturation of response at pH ~6.0 (Babini et al., 2002; Sutherland et al., 2001). Variations in the activation pH values across ASIC subtypes and trimers, enable the detection of a wide range of extracellular pH concentrations. Upon rapid extracellular acidification and consecutive proton binding to the extracellular domain, ASIC1a is activated and a fast rising ($T_{\text{activation}}$ 6-13 ms at pH 6.0) (Hesselager et al., 2004) and quickly desensitized ($T_{\text{desensitization}}$ 1.1-3.5 ms) (Babini et al., 2002; Bässler et al., 2001; Sutherland et al., 2001) transient current is elicited (Chen et al., 2006). Homomeric ASIC1a channels conduct a cation flux ($Na^+ > Ca^{2+} > K^+$) (Waldmann et al., 1997), and are the only Ca^{2+} permeable ASIC subunits ($P_{Na}/P_{Ca} \sim 18.5$) (Bässler et al., 2001; Chen et al., 1998). All ASICs are more permeable for Na^+ than for K^+ ($P_{Na^+} / P_{K^+} \sim 7.8$ to 15) with ASIC1a discriminating less between Na^+ and K^+ than other DEG/ENaC channels ($P_{Na}/P_{K} \sim 7.8$) (Bässler et al., 2001). After stimulation and upon restoration of pH to physiological levels, ASIC1a undergoes a conformational transition from the desensitized state back to the closed state. The recovery time constant T_{recovery} for ASIC1a homotrimers ranges between 5 to 13 seconds (Askwith et al., 2004; Babini et al., 2002; Sutherland et al., 2001). Successive acidic stimulations of ASIC1a result in diminished responses, a phenomenon known as tachyphylaxis, which may be mediated by H^+ and Ca^{2+} permeation through ASIC1a (Chen & Grunder, 2007). An overview of the biophysical properties of ASIC1a is presented in **Table 1**.

Extracellular divalent cations (Ca^{2+} , Mg^{2+}) as well as the polyvalent cation spermine shift steady-state desensitization of ASIC1a to more acidic values leading to an extended dynamic range due to a stabilized resting state (Babini et al., 2002; Paukert et al., 2004) and an open channel block (IC_{50} approximately 4 mM at pH 5.5) (Babini et al., 2002; Immke & McCleskey, 2003).

Table 1: Biophysical properties of ASIC1a. Overview of the pH-dependent activation profile (pH_{50} activ.), activation kinetics (characterized as time constant $T_{\text{act.}}$), steady-state desensitization parameters (pH_{50} desens.), desensitization kinetics (characterized as time constant $T_{\text{desens.}}$), time constant for recovery from steady-state desensitization (T_{recovery}), and single channel current amplitude for homomeric ASIC1a channels. Properties can vary between species and pH_{50} values are modified by extracellular Ca^{2+} . Adapted from (Grunder & Pusch, 2015). References: 1(Sherwood et al., 2011); 2(Babini et al., 2002); 3(Sutherland et al., 2001); 4(Bässler et al., 2001); 5(Li et al., 2010); 6(Bässler et al., 2001); 7(Askwith et al., 2004).

	ASIC1a
pH_{50} activ.	6.4 - 6.5 ^{1,2,3}
$T_{\text{act.}}$ [sec] at pH 6.0	6 - 13 ^{4,3,5}
pH_{50} desens.	7.2 - 7.3 ^{1,2}
T_{desens} [sec]	1.1 - 3.5 ^{1,3,6}
T_{recovery} [sec] at pH 7.4	5 - 13 ^{2,3,7}
Single channel amplitude [pA]	1.3

1.3 Physiological and Pathophysiological Role of ASIC1a

Together with heteromeric ASIC1a/ASIC2a channels, homomeric ASIC1a channels are the main ASICs in the CNS (Askwith et al., 2004; Baron et al., 2002; Grunder & Pusch, 2015). ASIC1a mRNA is expressed in most parts of the brain, particularly highly in cortex, cerebellum, hippocampus, amygdala, and olfactory bulb (Grunder & Pusch, 2015; Lingueglia et al., 1997; Waldmann et al., 1997; Wemmie et al., 2002).

While any change in proton concentration will affect ASIC1a activity, activation requires a rapid pH drop. In the case of ASIC1a channels on the post-synaptic membrane this means that a rapid pH drop in the synaptic cleft is necessary to elicit an excitatory postsynaptic potential. Such a rapid pH drop can result from the release of acidic secretory vesicles (Grunder & Pusch, 2015) (**Figure 3**). Non-peptide neurotransmitters, such as acetylcholine, γ -aminobutyric acid, and monoamines, are stored in secretory vesicles via secondary active transporters. The electrochemical gradient for this transport is generated by hydrolysis through vesicular ATPases that actively pump protons into the vesicle, effectively lowering the intravesicular pH to about 5.5 (Gasnier, 2000; Miesenböck et al., 1998). As shown in models of the synaptic cleft, the pH in the synaptic cleft rapidly drops to pH values as low as 6.65 upon vesicle release, low enough to activate ASIC1a channels (Dietrich & Morad, 2010). This process has led to the recognition of protons as neurotransmitters or co-agonists (Bi & Schuldiner, 1987; Du et al., 2014). It is involved in physiological processes such as synaptic plasticity, long term potentiation, learning and memory, and fear conditioning, all of which have been shown to be reduced when ASIC1a is blocked or knocked-out or to be increased when ASIC1a is overexpressed in mice (Chesler, 2003; Kreple et al., 2014; Mogil et al., 2005; Ugawa et al., 2001; Vralsted et al., 2011; Wemmie et al., 2003; Wemmie et al., 2002). In accordance with these findings, ASICs have been linked to psychiatric disorders that involve pathological synaptic plasticity, such as fear, anxiety, depression, and addiction (Kreple et al., 2014; Sherwood et al., 2012), as well as chronic pain syndromes (Chen & Wong, 2013). Moreover, some ASIC1a variants have been implicated in the pathogenesis of epilepsy by enhancing neuronal excitability (Cheng et al., 2021; Lv et al., 2011)

Some ASIC1a/2a heteromers have been shown to desensitize slowly and deactivate unusually fast, enabling them to sustain postsynaptic responses to high-frequency bursts when other receptors (e.g., glutamate receptors) may desensitize (MacLean & Jayaraman, 2016).

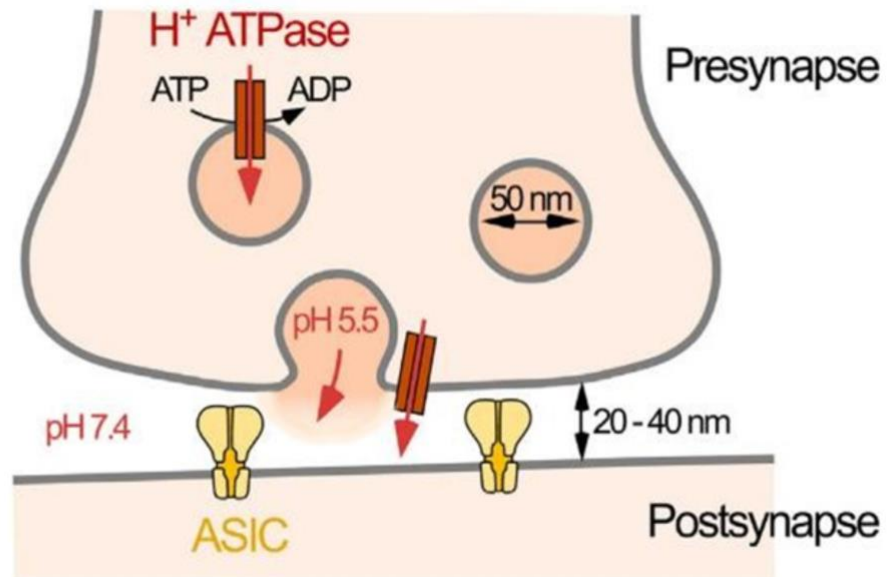


Figure 3: Presynaptic release of neurotransmitters can lead to acidification of the synaptic cleft and activation of ASICs. Illustration of presynapse with vesicles and proton pump (red), synaptic cleft, and postsynapse with membraneous ASICs (yellow). Through the activation of ASICs protons contribute to synaptic transmission. Adapted from (Grunder & Pusch, 2015).

While rapid acidosis activates ASIC1a, slow acidosis has been discussed to either lead to an increased likelihood of the channel to transition into the desensitized state and thereby reduce ASIC1a ion conductance to a negligible level, or to be activated more frequently through the higher proton concentration (Lipton & Rosenberg, 1994; Vats et al., 2018; Yermolaieva et al., 2004). The latter may result in increased intracellular calcium concentrations and the activation of the inflammasome, a process known as excitotoxicity, that causes a unique form of cell death, which is independent of caspase pathways (Lipton & Rosenberg, 1994; Vats et al., 2018; Yermolaieva et al., 2004). It remains unclear which of these mechanisms is dominant or more relevant for the observed neurotoxic effects of ASIC1a in acidosis.

Slow changes in pH can have several contributors, including glycolysis in neurons, lactate and CO₂ production in glial cells and neurons, and changed transporter activities in both neurons and glia (Chesler, 1990; Deitmer, 2002; Du et al., 2014; Grunder & Pusch, 2015; Krishtal et al., 1987; Miesenböck et al., 1998; Obara et al., 2008; Xiong et al., 2008). Among other scenarios, these changes occur under pathological conditions, such as tissue inflammation, ischemic stroke, neurotrauma, and epileptic seizures and can lead to a reduction of tissue pH to below 6.0 (Chesler, 1990, 2003; Rehnrcrona, 1985). A low pH can cause severe and irreversible neuronal damage by disrupting the synthesis and degradation of cellular components, impairing mitochondrial function, altering cell volume, reducing blood flow, and promoting the formation of harmful free radicals (Chesler & Kaila, 1992; Rehnrcrona, 1985). Additionally, low pH affects ASIC1a channel activity, as demonstrated by reduced infarction volumes in mice undergoing middle cerebral artery occlusion when ASIC1a was blocked pharmacologically or knocked out, or when putative positive allosteric modulators such as glutamate were blocked (Lai et al., 2024; Xiong et al., 2004; Yermolaieva et al., 2004). Conversely, increased ASIC1a activity has been linked to larger infarction volumes after middle cerebral artery occlusion (Yermolaieva et al., 2004).

In neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease the effects of ASIC1a on both synaptic plasticity and cell damage through acidosis in neuroinflammation come into play and the ASIC blocker amiloride has been shown to potentially exert neuroprotective effects in these conditions (Frieze et al., 2007; Mango & Nisticò, 2021; Mango & Nisticò, 2022).

Moreover, in slow acidosis, ASIC1a may activate a current-independent, RIPK1 dependent pathway which leads to necroptosis (Clusmann et al., 2022).

The involvement of ASICs in a broad range of neurological diseases and disorders makes them a clinically relevant potential pharmacological target (Vats et al., 2018; Xiong et al., 2008).

1.4 Pharmacology of ASIC1a

Several pharmacological and endogenous substances are known to interact with ASIC channels. A selection of these agents is introduced below.

Amiloride is a K⁺-retaining and natriuretic diuretic that unselectively blocks channels of the DEG/ENaC family (Sun & Sever, 2020). In the clinical setting it is mostly used as an adjunct to thiazide or loop diuretics in oedematous states (Sun & Sever, 2020). Amiloride is known to reversibly inhibit ASIC currents (IC₅₀ = 10 μM) by blocking the pore of the channel (Chen et al., 1998; Waldmann et al., 1997; Xiong et al., 2008).

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) like diclofenac, and ibuprofen are a class of medications that block the inflammatory cascade, primarily by inhibiting the enzyme cyclooxygenase (Bacchi et al., 2012; Moses & Bertone, 2002). Clinically, they are widely used for their anti-inflammatory, analgesic, and antipyretic properties (Bacchi et al., 2012). NSAIDs have been reported to inhibit the inflammation-induced increase in ASIC expression in sensory neurons, and to inhibit ASIC currents at therapeutic doses (Voilley et al., 2001). Interestingly, this interaction is independent of cyclooxygenase and suggests that NSAIDs may have direct analgesic effects through the modulation of ASIC activity (Dorofeeva et al., 2008; Voilley, 2004).

The tarantula (*Psalmopoeus cambridgei*) polypeptide toxin Psalmotoxin1 (PcTx1) specifically and reversibly inhibits ASIC1a and 1b with an IC(50) of 0.9 nM (Escoubas et al., 2003; Escoubas et al., 2000). This inhibition is based on an increase in apparent H⁺ affinity, leading to a shift to the desensitized state at resting pH (pH=7.4) (Chen et al., 2005).

MitTx, a dimeric protein derived from the venom of the Texas coral snake (*Micrurus tener tener*) binds to ASIC1a and activates the channel, through stabilizing the channel in the open state and thereby potentiating ASIC currents and shifting pH-dependent activation to less acidic values (Baconguis et al., 2014; Bohlen et al., 2011; Bohlen & Julius, 2012; Verkest et al., 2022). In vivo MitTx can contribute to pain (Bohlen & Julius, 2012).

1.5 Endogenous Interaction Partners of ASIC1a

ASIC1a interacts with several proteins that regulate its function via modulation of the trafficking of the channel, internalization, surface expression, or scaffolding. These proteins typically engage with many other proteins, contributing to a dynamic regulatory network. Unlike many other channels, stable complexes involving ASIC1a and its regulatory partners are not well studied, underscoring the transient nature of these interactions and highlighting gaps in our understanding of the ASIC1a interactome.

The Protein Interacting with C Kinase 1 (PICK1) has been shown to directly interact with ASIC1a via its C-terminal PDZ-domain (Duggan et al., 2002; Hruska-Hageman et al., 2002). It acts as a scaffolding protein that facilitates phosphorylation of ASIC1a through protein kinase A and protein kinase C at the KRXS-sequence, a key determinant of the function and localization of ASICs (Duggan et al., 2002; Hruska-Hageman et al., 2002; Hu et al., 2010; Leonard et al., 2003; Schwartz et al., 2015). ASIC1a-mediated acidotoxicity has been shown to be magnified in the presence of PICK1, highlighting the significance of this interaction in the modulation of ASIC1a (Jin et al., 2010).

Recent studies have also highlighted the significance of dynorphin and FMRFamide-related peptides in the modulation of ASIC1a. Dynorphin, a member of the endogenous opioid peptide family, has been found to decrease the apparent proton affinity of ASIC1a by occluding its acidic pocket, primarily through electrostatic forces (Borg et al., 2019; Leisle et al., 2021). This interaction stabilizes a resting conformation of ASIC and shifts pH sensitivity so that a steady-state desensitization becomes less likely, thereby leading to enhanced activity during prolonged acidosis and enhanced neuronal damage following ischemia (Leisle et al., 2021; Sherwood & Askwith, 2009).

In addition, ASIC1a activity and expression are modulated by Annexin II light chain/p11 in conjunction with Annexin II, which increases the surface expression and current density of the channel (Donier et al., 2005). Further important interactions partners of ASICs include regulatory chaperones like Hsp90, Grp4, and Calnexin (Vila-Carriles et al., 2007). Moreover, α -actinin, interacting with the C-terminal LDDVK motif of ASIC1a, alters its biophysical properties by shifting

the pH-dependence of activation to higher proton concentrations, increasing current density, and accelerating desensitization kinetics (Schnizler et al., 2009).

ASIC1a, when activated by mild acidosis, is involved in the recruitment and activation of RIPK1, a kinase important in the necroptosis signalling pathway (Clusmann et al., 2022). A specific region of the ASIC1a C-terminus is implicated in this interaction, indicating a direct link between ASIC1a activation and necroptosis induction (Clusmann et al., 2022). It has been demonstrated that the activation of ASIC1a by mild acidosis significantly reduces tumorsphere formation in glioblastoma stem cells through a necroptosis pathway that is sensitive to Nec-1 (a necroptosis inhibitor) and to PcTx1 (an ASIC1a antagonist) (Clusmann et al., 2022). The ASIC1a-RIPK1 interaction indicates ASIC1a to act as a metabotropic death-receptor on glioblastoma stem cells that triggers cell death in addition to its properties as an ion channel (Clusmann et al., 2022).

These interactions underline the complexity of the regulatory network surrounding ASICs. A better knowledge of the interactome is crucial for understanding the role of ASIC1a in physiological neuronal activity and its potential as a target in the treatment of neurological disorders. The emerging paradigm of pharmacologically targeting protein complexes further illuminates the promise held by a deeper insight into the interactome of ASIC1a. Such advancements could pave the way for the discovery of innovative drugs that are not only more selective but also bear the potential for reduced side effects (Rosenbaum et al., 2020).

1.6 Prenylcysteine Oxidase 1-like (PCOL)

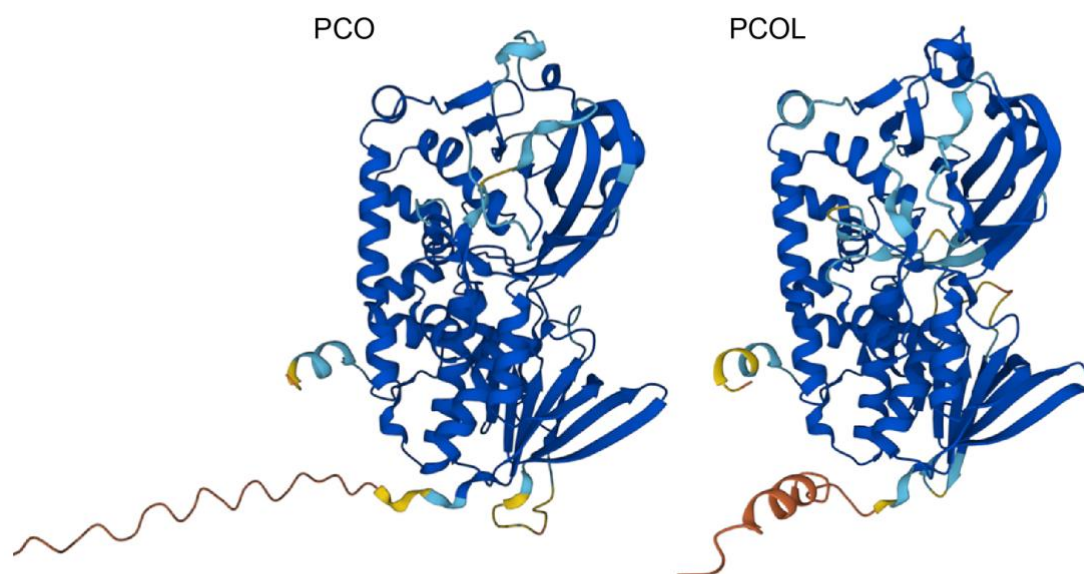
A recent immune-purification screen to identify the proteome of ASIC1a, performed by Logopharm, identified prenylcystein oxidase 1-like (PCOL), encoded by the PCYOX1L gene, as a potential interaction partner of ASIC1a. For the screen, wild-type and ASIC1a knock-out mice brains were homogenized and immunoprecipitated with a set of antibodies and further controlled with an anti-IgG antibody (Kuspiel et al., 2025).

The PCYOX1 and PCYOX1L genes are 40% identical at the amino acid level (UniProt alignment) and have retained largely overlapping enzymatic and structural characteristics (Barone et al., 2024) (**Figure 4A**).

PCOL has a total length of 497 amino acids corresponding to approximately 56 kDa (rPCOL from *rattus norvegicus*). Based on sequence homology analysis (HomoloGene) and recent findings by Barone et al., PCOL may feature a N-terminal signal peptide of 20 amino acids, followed by a confirmed NAD/FAD binding domain of 50 amino acids and a confirmed lyase domain of about 300 amino acids (Barone et al., 2024). The predicted domains of PCOL resemble the observed domains of PCO (**Figure 4B**).

Based on features of the secondary and tertiary structure, PCOL has previously been subdivided into four regions (Kuspiel et al., 2025), the “whirled β -sheet domain”, the “antiparallel β -sheet domain”, the “aligned α -helix domain”, and the “ α -helix domain” (**Figure 5**).

A



B

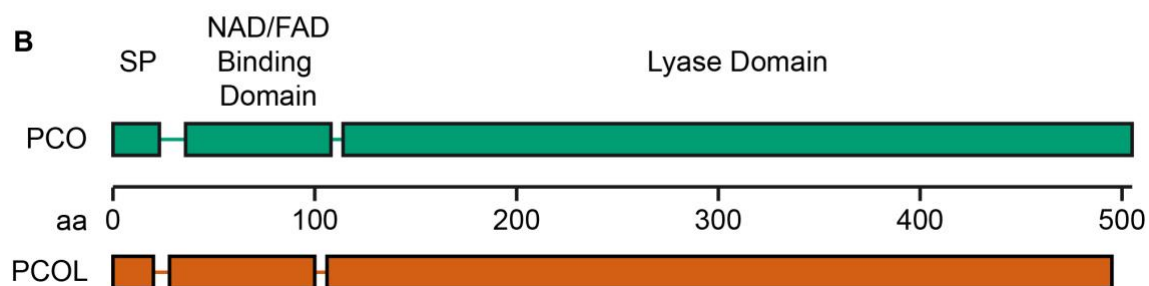


Figure 4: PCOL and PCO show a high level of structural homology. A: Predicted structures of rPCOL (right) and rPCO (left), generated by AlphaFold. Colours indicate model confidence, with navy indicating a per-residue model confidence score >90 and orange indicating a score below 50. **B:** Primary structure of PCO (green) and PCOL (red). Based on sequence homology screening (HomoloGene), both proteins encompass a signal peptide, a NAD/FAD binding domain, and a lyase domain. *Abbreviations: aa: amino acids; SP: signal peptide.*

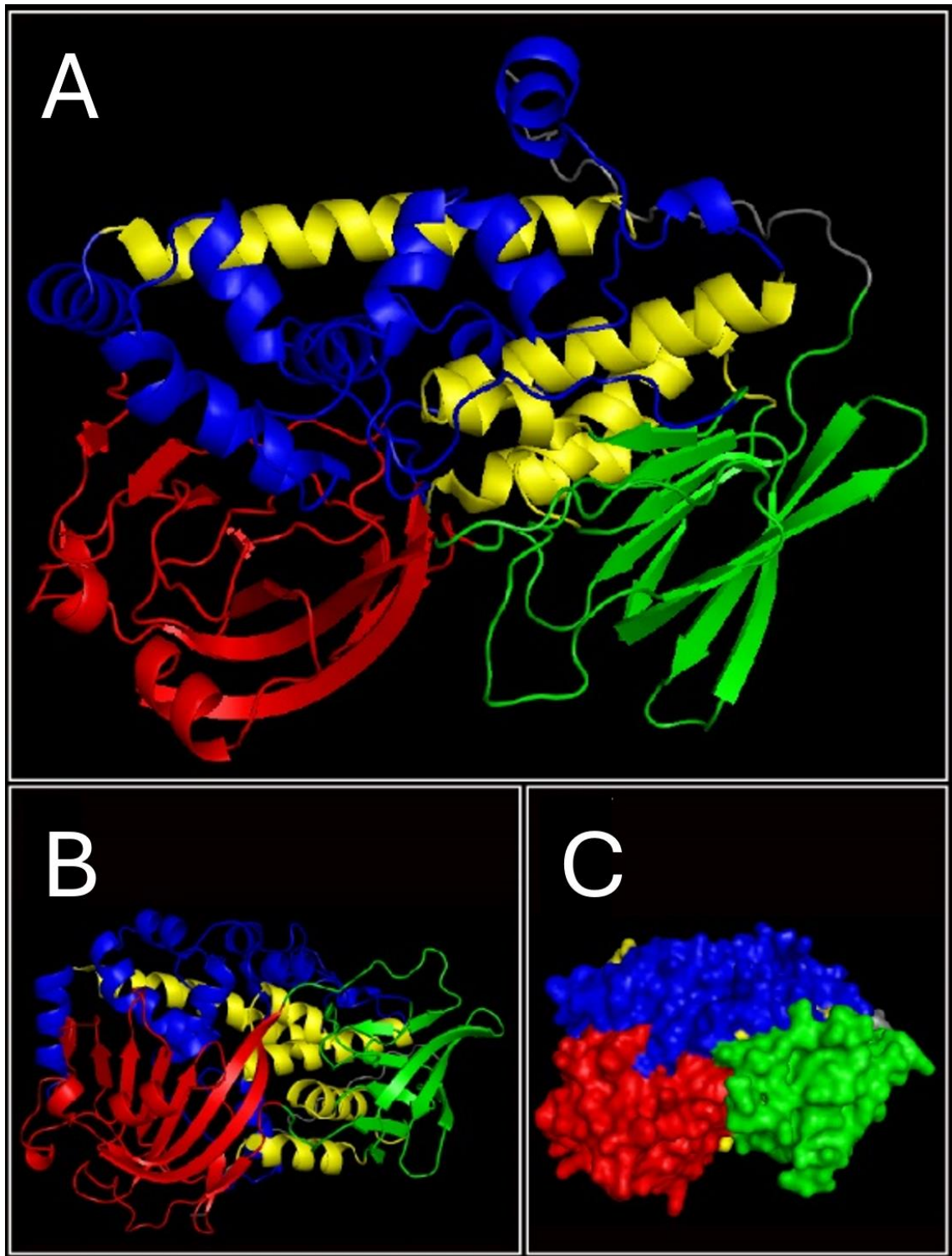


Figure 5: Structural domains of PCOL. PyMol graphics of structural domains of PCOL as proposed by Sven Kuspiel (unpublished, personal communication with Sven Kuspiel) **A:** Line structure of PCOL as predicted by AlphaFold with color-coded domains. “Whirled β -sheet domain” marked in green, “antiparallel β -sheet domain” marked in red, “aligned α -helix domain” marked in yellow, and “ α -helix domain” marked in blue. **B:** Same as A, turned for better view on “antiparallel β -sheet domain”. **C:** Same as A but with surface labelling.

Previous assumptions about the oxidoreductase function of PCOL (**Figure 6**) were based mainly on its homology to PCO. New findings solidify that PCOL is indeed an active oxidase that. Both PCO and PCOL oxidize long-chain prenyl-cysteines such as farnesyl-cysteine by cleaving the thioether bond in a FAD- and oxygen-dependent reaction (Barone et al., 2024). This metabolic activity has been shown to extend to farnesyl-containing drugs such as salirasib (Barone et al., 2024).

Moreover, the putative presence of a signal peptide hints towards a secretion of the protein. In addition, the presence of five confirmed N-glycosylation sites indicates a possible glycosylation of the protein (unpublished, personal communication with Felicitas Weiß).

The PCOL proteins in mice and humans share ~94% homology, suggesting a highly conserved role (Petenkova et al., 2023). Absence of PCOL impairs the mevalonate pathway, autophagy, and neutrophil bactericidal capacity (Petenkova et al., 2023). In the context of Alzheimer's disease, PCOL has recently been suggested as a diagnostic biomarker and may be involved in the pathogenesis of this disease (Lian et al., 2024). The PCYOX1L gene was identified as one of the feature genes in a necroptosis-related molecular classification of Alzheimer's disease. Moreover, He et al. have documented an upregulation of PCOL expression in cases of deep vein thrombosis through alterations in regulatory RNA mechanisms (He et al., 2023). Diminished PCOL expression correlated with a decrease in vascular inflammatory injury, consequently leading to reduced thrombus formation in mouse models (He et al., 2023). The presented strategy of manipulating long non-coding RNA and microRNA interactions presents a potential therapeutic strategy for modulating PCOL expression (He et al., 2023).

Preliminary electrophysiological work indicates that PCOL enhances rASIC1a maximal current amplitude by a factor of 3 to 5, while biophysical properties, such as pH dependence of activation, steady-state desensitization, or ion conductance, remain unchanged (Kuspiel et al., 2025). This effect seems to involve increased surface expression of ASIC1a and is specific to certain ASIC subunits (notably ASIC1a and partially ASIC1b) while it does not affect ASIC2a, ASIC3, or BASIC (Kuspiel et al., 2025).

When expressed in HEK293 cells, PCOL alone does not elicit an electrophysiological response to protons (Kuspiel et al., 2025). The potentiation of current density was observed with endogenous hASIC1a and rASIC1a channels in HEK cells, as well as in rASIC1a channels in CHO cells.

1.7 Prenylcysteine Oxidase 1 (PCO)

PCO is an enzyme involved in the degradation of prenylated proteins (Barone et al., 2024; Tschantz et al., 1999). It catalyses the cleaving of the thioether bond of prenyl-L-cysteines, such as farnesyl-cysteine and geranylgeranyl-cysteine, releasing hydrogen peroxide, cysteine, and isoprenoid aldehyde, as displayed in **Figure 6** (Digits et al., 2002; Herrera-Marcos et al., 2018). In humans, PCO RNA expression was observed to be most pronounced in fatty tissue (60.6 ± 6.7 reads per kilobase of transcript per million mapped reads (RPKM)), and in kidney tissue (49.5 ± 11.1 RPKM) (Fagerberg et al., 2014; Herrera-Marcos et al., 2018). Additionally, RNA expression was detected in brain tissue, albeit at a lower level (15.9 ± 1.9 RPKM) (Fagerberg et al., 2014). PCO comprises three functional domains, a N-terminal signal peptide followed by a NAD/FAD binding domain and a lyase domain (**Figure 4B**). Recent crystallographic work on an ancestral form of PCO confirmed that the substrate-binding region of the enzyme is characterized by a wide, tunnel-like pocket that may accommodate bulky long-chain prenylcysteines for cleavage (Barone et al., 2024)

Studies both in vitro and in vivo have demonstrated delayed thrombus formation in PCYOX1^{-/-} mice, attributed to a hypo-reactivity of platelets (Banfi et al., 2022). Additionally, the identification of PCO within low-density lipoproteins and very-low-density lipoproteins has led to the hypothesis that PCO may further play a role in hydrogen peroxide driven atherogenesis. It has been shown that PCO deficiency in mice retards atheroprogession and decreases lesion vulnerability (Banfi et al., 2021; Banfi et al., 2009; Herrera-Marcos et al., 2018). These findings suggest that PCO may be a novel therapeutic target in the treatment of atherosclerosis and atherogenesis.

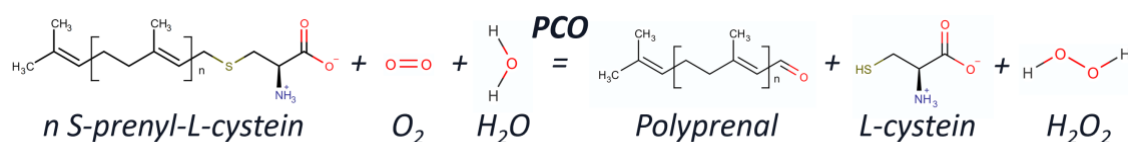


Figure 6: Catalytic activity of PCO. PCO hydrolytically cleaves thioether bonds of prenyl-L-cysteines. Adapted from Uniprot.

2 Aim of this Thesis

ASICs play an important role in many physiological and pathophysiological processes, such as synaptic plasticity and necroptosis. Despite the critical relevance of ASICs, their regulatory networks – particularly how they are modulated by interacting proteins – remain largely unexplored. This gap hinders our ability to fully exploit the therapeutic potential of ASICs.

Preliminary research conducted in the laboratory of Stefan Gründer identified PCOL as previously unrecognized potential interaction partner of ASIC1a. The presence of PCOL has been shown to potentiate ASIC1a current density without changing the biophysical properties of the channel, potentially by increasing surface expression. However, it remains unclear how ASIC1a and PCOL interact, and which structural elements are involved in this interaction. The present study is aimed at identifying functional structural elements of PCOL that may regulate ASIC1a current density.

To this end, ASIC1a ion conductance elicited by acidic stimulation was recorded as peak current density via whole-cell patch-clamp measurements in HEK293 ASIC^{-/-} cells that were co-transfected with rASIC1a and chimeric mutants of PCOL. This technique allows for the detection of subtle changes in ion channel.

The main goal of this research is to identify and characterize the key structural elements of PCOL that are essential for its ability to modulate ASIC1a activity. Ultimately, these findings might contribute to the development of new therapeutic strategies targeting ASIC functionality that may find an application in the treatment or prevention of conditions that involve ASIC1a, such as stroke and neurodegenerative diseases.

3 Materials and Methods

Based on information from protein structure repositories and prior insights into the functional domains of PCO (Herrera-Marcos et al., 2018), chimeras between PCOL and PCO were designed and synthesized. Subsequently, these chimeras were transfected into cultured immortalized human embryonic kidney (HEK293) cells, which were used as a heterologous expression system due to their robust and scalable nature. Whole-cell patch clamp recordings were acquired to assess electrophysiological properties of the cells. Peak current amplitudes were measured and adjusted to cell capacitance to obtain current density. The resulting data was tested for statistical significance and processed graphically to facilitate interpretation.

Recognizing the endogenous expression of hASIC1a in HEK293 cells (Gunthorpe et al., 2001), cells with targeted knockouts for this channel were employed (gift from Stefan Pless, University of Copenhagen, Center for Biopharmaceuticals, Department of Drug Design and Pharmacology), thus ensuring a controllable and isolated system for all experiments.

To maintain consistency and reproducibility across the present study, a uniform protocol for chimera construction, cell culture, transfection, electrophysiological measurements, and data processing was adhered to.

Given the working group's extensive experience in working with rASIC (ASIC from *rattus norvegicus*) and the near identical nature of rASIC1a to hASIC1a (ASIC from *human*) (98.1% homology per UniProt alignment), rASIC1a was used for the present study. Similarly, rat counterparts for PCO (rPCO) and PCOL (rPCOL), sharing 77.4% and 93.7% sequence identity with their human versions (as per UniProt alignments) were employed. This choice was based on the goal of maximizing the relevance and applicability of findings.

3.1 Computational Biology

Sophisticated algorithms were employed to predict, describe, and visualise PCOL and PCO in their primary, secondary, and tertiary conformations, facilitating a detailed structural analysis as well as the strategic design and evaluation of chimeric proteins.

A deep learning version of artificial intelligence (AI) the open-source software AlphaFold2 (Alphabet Inc.) was used. This software leverages an extensive neuronal network to predict the structure of proteins with a high level of accuracy, based on amino acid sequences (Jumper et al., 2021). The model focuses on pattern recognition and refines predictions, through the minimization of free energy gradients to elucidate the three-dimensional conformation of the protein (Callaway, 2020; Jumper et al., 2021; Service, 2020). AlphaFold2 was applied to ascertain the predicted structures of rPCO and rPCOL. Structures were subsequently rendered and adjusted for analytical clarity in the AlphaFold Web Application and the molecular visualization tool PyMOL (Schrödinger Inc.).

Further, Benchling (Benchling) was utilized as a digital laboratory notebook for the design of chimeric proteins and respective primers and for alignment and interpretation of sequencing results. Additionally, Benchling was used to perform amino acid alignment using the Clustal Omega algorithm (Sievers et al., 2011).

This approach allowed for the combination of AI-driven predictions and established techniques of molecular biology to provide a comprehensive understanding of protein structure.

3.2 Molecular Genetics

The cDNA sequences of rASIC1a, rPCOL and rPCO were all obtained from *rattus norvegicus* and cloned into a pBudCE4.1 vector, to facilitate expression of the genes. To discriminate between transfected and non-transfected cells, a co-transfected approach with Green Fluorescent Protein (GFP), cloned into a pcDNA 3.1 vector, was used.

3.2.2 Plasmid Vectors

Two different vectors were used simultaneously when transfecting HEK cells to enhance efficiency and reliability of gene expression. For the transfection of GFP a pcDNA 3.1 vector was used. Conventional methods using separate plasmids for the co-expression of rASIC1a and rPCOL, rPCO, or chimeric proteins resulted in strong overexpression artefacts. Thus, the pBudCE4.1 mammalian expression vector (gift from Eric Lingueia, Université Côte d'Azur, CNRS, Institut de Pharmacologie Moléculaire et Cellulaire, France) was used (**Figure S1**). The utility of this vector lies in the possibility of concurrently expressing two genes from a single plasmid in mammalian cells, thus assuring a stable ratio between the synthesized proteins. The rASIC1a gene was cloned into an expression cassette containing the CMV promoter, while the respective protein of interest was cloned into an expression cassette regulated by the EF-1 α -Promotor.

3.2.3 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used for the amplification of target DNA (Saiki et al., 1985).

In the present study, thermal cycling was performed with a thermocycler (T 3000, Biometra) and utilized the *KAPA HiFi HotStart DNA Polymerase* (Roche). The initial phase involved a high-temperature activation of the polymerase at 95°C, followed by the denaturation of template DNA at 98°C for 20 seconds to separate the double helix strands. Subsequently, annealing of primers occurred at a calculated temperature between 45-60°C for 20 seconds, contingent on the

melting temperature of the primer to be just below melting temperature T_m of the primer. Melting temperature was either calculated in Benchling or specified by Eurofins Genomics. The elongation phase at 72°C allowed for the synthesis of complementary DNA strands, starting at the primers. The duration of this step was set to 45 sec/kb. The cycle was repeated for 20 to 30 iterations. To allow short term storage and subsequent analysis of the PCR products, the reaction chamber was cooled down to 4°C in the final step. The reagents that constituted the PCR solution are listed in **Table 2**.

Table 2: Composition of the PCR solution.

Template DNA (cDNA, 100 to 200 µg)	1	µl
Forward primer	1.5	µl
Reverse primer	1.5	µl
KAPA-polymerase (1 U/µl)	1	µl
dNTPs (10 mM)	1	µl
KAPA buffer	10	µl
DEPC water	34	µl
Total volume	50	µl

Oligonucleotide primers were designed using the Benchling bioinformatics platform. Subsequently, primers were produced by Eurofins Genomics and diluted to a concentration of 10 picomoles per microliter (pmol/µl) in RNase-free water (Roth) according to the instructions of the provider, to prevent RNA degradation. A comprehensive list of these primer, including their sequences, is provided in **Table S1**.

3.2.4 Creation of Chimeric DNA by Recombinant PCR

The creation of chimeric mutants between PCO and PCOL required the construction of recombinant DNA molecules. This was achieved in a series of three PCRs with overlap extension. Initially, fragments intended for fusion were amplified via PCR with overlapping primers that included a complementary palindromic 5' overhang (**Figure 7 A**). The resulting expanded amplicons (**Figure**

7 B) were mixed and the overlapping complementary sequences hybridized – fusing the two molecules– and served as primers (**Figure 7 C**). Lastly, a PCR with only the primers for the far ends was performed to amplify the DNA that contained the desired chimeric border.

The resulting mutated fragment was then inserted into a vector, by treating both vector and PCR-product with the same restriction enzyme and subsequent ligation. The vector then contained the desired DNA and could be used for transfection and RNA synthesis. Pipetting scheme and thermocycler settings for recombinant PCR were the same as for non-recombinant PCR.

Chimeric borders were carefully designed to be located in putative loop regions, as identified by secondary structure analysis (AlphaFold2, Alphabet Inc.) to preserve structural features.

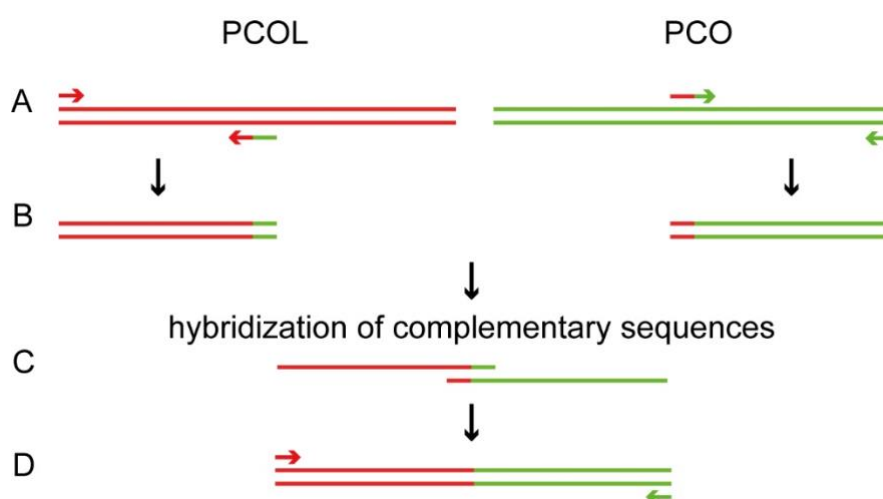


Figure 7: Construction of recombinant DNA fragments using recombinant PCR. Complementary recombinant primers containing sequence fragments of both genes that will constitute the chimera were used to determine the chimeric border. Steps **A to D** are detailed in section 3.2.5.

3.2.6 Agarose Gel Electrophoresis

Gel electrophoresis was used to identify, quantify, and purify fragments of nucleic acid fragments after PCR. For gel preparation, agarose was added to 1xTAE buffer in a concentration of either 1 or 1.5%, followed by heating to ensure complete solubilization. To this mixture, 0.005% of red safe nucleic acid staining

solution (HiSS Diagnostic) was added to allow visualization. The solution was cast into the gel chamber, utilizing a plastic comb to form wells, and put to solidify at room temperature for one hour.

The solidified gel was positioned in a horizontal electrophoresis chamber (BioRad) and covered with 1xTAE buffer. Loading buffer (Thermo Fischer) was added to the PCR products (10 µl of loading buffer for 50 µl of sample or 5 µl of loading buffer for 25 µl of sample) and the solution was loaded into the wells. During electrophoresis, the negatively charged DNA fragments migrated towards the anode within the electric field, generated by a power supply unit (Bio-Rad) set at 100 V. The duration of electrophoresis varied between 15 to 45 minutes, depending on the required resolution and the size of DNA fragments. To determine sizes of individual fragments a DNA ladder (Life Technologie) was concurrently run alongside the samples to provide a molecular size reference.

After agarose gel electrophoresis DNA was visualized using a UV-transilluminator (Vilber Lourmat). The resulting bands were captured using an imaging system and analyzed using the BioVision software (Vilber Lourmat).

3.2.7 Purification of DNA

DNA-fragments of interest were excised from the agarose gel and subsequently purified using a High Pure PCR Product Purification Kit (Roche) according to the protocol of the manufacturer. During the purification process, nucleic acids were selectively immobilized in the presence of a pH dependant chaotropic salt to separate DNA from contaminating substances. After two washing steps, the purified and concentrated DNA was eluted using elution buffer.

3.2.8 Ligation of PCR Fragments into Plasmid Vectors

The DNA Rapid Ligation Kit (TakaRa) was used according to the instructions of the manufacturer for the directional insertion of PCR-fragments into a plasmid vector. This process involved the utilization of overlap extension PCR to ensure that – once cleaved – the amplicon could integrate into the vector at a specific site. The restriction endonuclease NotI-HF (New England BioLabs) was used to cleave both vector and amplicon at a specific DNA sequence, yielding

complementary 'sticky' ends. These ends facilitated the precise alignment and subsequent fusion of amplicon and vector.

The formation of phosphodiester bonds between the 3'-hydroxyl group and the 5'-phosphate of the double stranded DNA-fragments was catalysed by the enzyme NotI and allowed for the creation of a continuous, stable DNA-molecule.

To optimize ligation efficiency and to ensure a stoichiometry favourable for the reaction, concentration and volume ratio between vector and insert were calculated with the "In-Fusion molar ratio calculator" (TaKaRa).

3.2.9 Transformation in TOP10 Heat-Competent *Escherichia coli*

Top10 Competent Cells derived from the *Escherichia coli* strain XL-1 Blue were employed for the transformation of purified, double-stranded, circular plasmid vectors. These cells took up plasmid DNA through the cell wall and subsequently amplified the vector concomitant to bacterial growth and division.

The infusion solution was composed of 2 µl of infusion reagent (Biolabs), 50 - 200 ng of the vector and 10 - 200 ng of the corresponding insert. Diethyl pyrocarbonate (DEPC)-treated water was added to the solution to a total volume of 10 µl. The solution was incubated at 50°C on a heating block (Biometra) for 15 minutes to facilitate annealing and ligation processes. Subsequently, the mixture was either stored at low temperature for future use or immediately used for transformation.

Prior to transformation, TOP10 cells were gradually thawed on ice from their storage conditions at -80°C. The prepared infusion solution was applied to these cells and incubated for 30 minutes on ice to allow DNA uptake. A subsequent heat shock at 42°C for 45 seconds (Eppendorf Thermomixer) was applied to further enhance the transformation efficiency. Immediately after the heat shock, 200 µl of pre-cooled Luria-Bertani (LB)-Medium was added to the cells to facilitate recovery.

Cells were incubated in a shaking incubator (Infors AG) for 1 hour at 37°C, allowing for the expression of antibiotic resistance markers. Subsequently, bacterial cells were plated out on agar plates containing either Zeocin for the selection of pBud vector transformants, or Ampicillin for the selection of pEGFP

C1 vector transformants. After 17 hours of incubation at 37°C (Memmert) colonies were harvested from the plates. Selected colonies were transferred to culture tubes containing 5 ml of LB-medium supplemented with the appropriate antibiotic to inhibit the growth of non-transformed cells or other contaminants. These culture tubes were left to incubate at 37°C over night in a shaking incubator to achieve plasmid amplification through bacterial growth.

The result of this process was an *E. coli* population containing the recombinant plasmid of interest.

3.2.10 Harvesting of Plasmid DNA with Miniprep

To isolate purified plasmid DNA from *E. coli* cultures a commercially available High Pure Plasmid Isolation Kit (Roche) was used according to the instructions of the manufacturer.

The bacterial cells underwent alkaline lysis to disrupt cellular integrity and denature chromosomal DNA and proteins, while preserving plasmid DNA. Intact plasmid DNA was bound onto a silica membrane. This was followed by two washing steps to remove residual cellular debris. Subsequently, the purified plasmid DNA was eluted in a buffer, desorbing it from the silica membrane.

3.2.11 DNA Sequencing

Post-construction validation of plasmids was performed by Sanger sequencing (Eurofins Genomics). For this, a 15 µl aliquot containing the amount of purified PCR product specified by Eurofins Genomics was sent in. The sequencing was performed using EF1 as forward and pCR3.1-BGHrev as reverse primer. Details on sequencing primers are presented in **Table S2**.

3.2.12 Storage and Dilution of DNA

DNA purity and concentration were assessed by evaluation of absorbance ratios at a wavelength of 260nm/280nm and 260nm/230nm. These measurements were conducted using a micro volume spectrophotometer (Thermo Fisher

Scientific) and the corresponding software (Thermo Fisher Scientific). The storage conditions of plasmids were adjusted to frequency of usage. Plasmids in regular use were kept at 4°C for short-term preservation, while plasmids that were used less frequently were kept at -20°C.

3.3 Cell Culture

Any manipulations that involved the opening of a cell container were performed within a biological safety cabinet (Thermo Fisher Scientific) to ensure aseptic conditions and prevent contamination. The cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with stable L-Alanyl-Glutamine (Gibco) and 10% fetal bovine serum (FBS; Capricorn Scientific). Prior to use, all solutions were warmed to 37°C in a water bath (GFL) for a minimum duration of 5 minutes.

Confluency levels were regularly assessed with a light microscope (Carl Zeiss). Cells were routinely passaged upon reaching 80-90% confluence. For passaging, any supernatant medium was aspirated with a vacuum aspirator (Integra) and the cellular layer was rinsed with 5ml of calcium free Dulbecco's Phosphate-Buffered Saline (DPBS) (PAN Biotech). Subsequently, 1ml of 0,05% Trypsin/EDTA solution (PAN Biotech) was applied to facilitate detachment of adherent cells. Following trypsinization, 5ml of calcium supplemented DMEM serum was added to inhibit the enzymatic activity of trypsin. The cell suspension was then transferred into a screw cap tube (Sarstedt AG) and centrifuged at 1200g for 90 seconds (Centrifuge 5702, Eppendorf AG). The supernatant was discarded, leaving behind a cell pellet, which was resuspended in 1ml of medium.

The resulting cell-suspension was seeded on tissue culture dishes with a regular surface (Sarstedt AG) containing 10ml of the medium. The seeding density was adjusted based on the desired growth period: 1:2 for 1 day, 1:5 for 2 days, 1:10 for 3 days, and 1:20 for 4 days to achieve 80-90% confluence. The culture dishes were incubated in a humidified incubator (Binder) at 37°C with a controlled atmosphere of 5% CO₂ and 95% air.

For cryopreservation, cells were suspended in a cryoprotective solution containing 5% dimethyl sulfoxide (SIGMA) and gradually cooled in an isopropanol container before storage in a liquid nitrogen vapor tank (AIR LIQUIDE Medical). Thawing was performed rapidly in a water bath at 37°C and cells were promptly transferred to pre-warmed medium, centrifuged, and resuspended in growth medium. Post-thaw, the cells were routinely tested for mycoplasma

contamination and cultured to a maximum of 25 passages to ensure genomic stability.

3.3.1 Transfection

To establish an isolated heterologous transient expression system for the examination of whole-cell currents via patch-clamp technique, HEK293 cells with a targeted knock-out for the hASIC1a gene were used. This genetic modification allowed for the establishment of a well-controlled system with elicited currents in an optimal range for recording and analysis (0.2-20nA).

Prior to cell seeding, 10-mm coverslips were distributed among six-well chambers (Falcon) and coated with Poly-D-Lysin (Sigma-Aldrich) to enhance cell adhesion. The cells were plated onto these coverslips at a dilution ratio of 1:20 (10 μ l/2ml) and incubated for 120 minutes before transfection.

For the transfection 1 μ g of the pcDNA 3.1 vector containing the GFP gene and 1 μ g of a pBudCE4.1 vector containing the DNA for rASIC1a and for the respective protein of interest or RFP (serving as a control to balance the amount of genetic material used for transfection) were used. These plasmids were combined with 100 μ l of 250mM calcium chloride, followed by the addition of 100 μ l of HEPES Buffer Solution (HEBS) (Gibco) and agitation to ensure mixing. The solution was left to rest for 30 minutes at room temperature to allow for the formation of calcium-phosphate-DNA co-precipitates, before being dispersed onto the cells. All reagents were brought to 37°C before their application to the cells.

Subsequent to the transfection, cells were incubated for 12 to 36 hours to allow for gene expression before conducting electrophysiological measurements.

3.4 Electrophysiological Measurements

The electrophysiological properties of transfected HEK293 ASIC1a^{-/-} cells were investigated utilizing continuous single-electrode voltage-clamp technique in a whole-cell configuration (Hamill et al., 1981; Jackson, 2001). All measurements were performed at 20-24°C. Once retrieved from the incubator, the prepared coverslips with adherent cells were used for measurements for a maximum of one hour.

3.4.1 Solutions used for Patch-Clamping

All chemicals were produced from Carl Roth GmbH, Sigma-Aldrich or VWR International. For each experiment the formulation of three distinct solutions, a bath solution, a stimulation solution, and a pipette solution, was needed. These solutions were based on deionized water (MilliporeSigma) as the solvent. The osmolality of each solution was determined using a quick-reading cryoscopic osmometer (Gonotec) and meticulously adjusted to the required concentration using glucose. The pH of each solution was titrated using a precision pH meter (Metrohm), with continuous agitation provided by a magnetic stirrer (Heidolph Instruments) to ensure homogeneity.

Pipette solution was composed to mimic physiological intracellular ionic composition, pH and osmolarity. It contained 10 mM NaCl, 10 mM HEPES, 121 mM KCl, 5 mM EGTA and 2 mM MgCl₂. The osmolality was standardized to 280 mOsmol/kg. To achieve a target pH of 7.25 either 10 M or 1 M KOH was utilized for incremental adjustments. This solution was distributed into 1.5 ml Eppendorf tubes, stored at -20°C, and thawed immediately before use to maintain integrity.

The standard bath solution was designed to replicate the ionic environment of culture mediums and consisted of 128 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES. The pH was adjusted to 7.4 using either 10 M or 1 M NaOH. For the acidic stimulation solution, HEPES was substituted with MES buffer, and the pH was adjusted to 6.0. The osmolality of bath solutions was consistently maintained at a minimum of 10 mOsmol/kg above that of the pipette

solution to ensure proper diffusion gradients. These solutions were stored at 5°C and used within two weeks post-preparation.

3.4.2 Preparation of Micropipettes

Micropipettes were fabricated from thin wall borosilicate glass capillaries embedded with a filament, possessing an outer diameter of 1.2 mm, an inner diameter of 0.94 mm and a length of 100 mm (Harvard Apparatus). Due to logistical constraints in supply chain, there was a temporary transition to standard wall capillaries with an outer diameter of 1.5 mm and an inner diameter of 0.8 mm (Harvard Apparatus). Pipettes were manufactured just before utilization in a biphasic pulling sequence, followed by fire polishing. This process was conducted using an automated dual-carriage horizontal pipette puller (Zeitz). The micropipette tip was relatively blunt with a diameter in the micrometre range, optimized to facilitate the formation of a patch configurations and to avoid the piercing of the cell.

3.4.3 Setup of the Measuring Station

The same setup was used for all patch clamp recordings.

An Amplifier (AXON CNS Molecular Devices) was used to measure magnitude and polarity of electrical currents or changes in membrane potential and to transmit them as analogue signals. To digitize these signals for computational analysis a digitizer (AXON CNS Molecular Devices) was employed, serving as an interface between the amplifier and the computer. A headstage (AXON CNS Molecular Devices) served as pipette holder with build-in circuitry to relay information to the amplifier. It offered precise mechanical control in all three dimensions, facilitated by the micromanipulator (Luigs & Neumann) which allowed for nanometer-level adjustments. This unit was combined with a pressure/suction system, essential for avoiding contamination of the micropipette tip, forming seals and executing patch rupturing. The use of an inverted Microscope (Carl Zeiss AG), featuring an integrated fluorescent filter and contrast enhancement, facilitated electrode access from above. Bath solutions were

gravity perfused and rapidly switched using an electronically valve-controlled pressure perfusion system (ALA Scientific Instruments).

A faraday cage was used to mitigate electromagnetic interference, such as radio waves. Additionally, to buffer against external vibrations that could disrupt recordings, an Air-Anti-Vibration Table was used to ensure stability and integrity of the experimental environment.

3.4.4 Whole-Cell Patch Clamping

Whole-cell patch clamp recordings were performed as previously described (Cole & Curtis, 1941; Hodgkin & Huxley, 1952; Neher & Sakmann, 1976).

For the construction of pipette and bath electrodes, silver wires were chlorinated through electrolysis in a 3 M KCL solution at a current of 2.50 mA. During this process the anionic chloride ions were drawn towards the silver anode and coated it, resulting in a chlorinated coating on the wire.

In preparation for electrophysiological recordings, coverslips with transfected cells were placed in the recording chamber (Warner Instruments), containing the standard bath solution. The chamber was mounted on the stage of the optical microscope. Irradiation of the cells with blue UV light was used to identify the transfected cells by the emission of green fluorescence.

The micropipette was filled air bubble free with pipette solution to establish contact to the pipette-electrode of the headstage. Once affixed in the holder, the tip of the micropipette was lowered into the bath solution, initiating an electrical circuit between the recording and reference electrodes. The command potential of 2 mV, applied at a frequency of 6.7 Hz, elicited a square waveform response, serving as an indicator to evaluate the resistance of the pipette. Any pipette resistances outside the range of 2 to 5 MΩ lead to the discarding of the respective pipette. The pipette was maneuvered towards the targeted cell. Prior to cellular contact, baseline normalization was conducted, and the operational mode was transitioned from bath to patch. In patch mode, 10 mV pulses at a frequency of 100 Hz were employed to both establish and assess the patch configuration. An increase in resistance, signifying pipette tip occlusion, indicated cellular contact. Giga-seal formation (resistance ≥ 1 GΩ) was achieved through gentle suction.

With a holding potential set at -70 mV, the cell configuration was activated and continuously monitored using 10 mV pulses at a frequency of 6.7 Hz to ascertain the integrity of the configuration. Upon successful establishment of the whole-cell configuration, a direct pathway between the pipette solution and the cytoplasm was formed, while preserving the integrity of the cellular membrane, separating the two electrodes. Capacitive transients of the pipette, whole-cell capacitance, and series resistance were nullified analogously via the amplifier. During the recording phase, the application of pulses was stopped to maintain the recording state.

During the recordings, cells were continuously superfused. After 10 seconds of initial superfusion with the standard bath solution at pH 7.4, the stimulation solution at pH 6.0 was applied for 10 seconds. Subsequently, cells were incubated for an additional 60 seconds in the pH 7.4 standard bath solution to restore baseline conditions and give sufficient recovery time before a second stimulation at pH 6.0 took place (**Figure S2**). To facilitate rapid pH changes, the application pipette was positioned in close proximity to the target cell. Current amplitude was measured at the peak of the cellular response elicited by the second pH 6.0 stimulation to ensure consistency considering tachyphylaxis and to thereby to obtain reliable and comparable data (Chen & Grunder, 2007). Another reason for measuring the second stimulation is that initial stimulations are more likely to elicit artefacts.

Recordings were captured using Clampex 10.1, operating at a sampling rate of 20 kHz. The recordings were captured without the application of high-pass or low-pass filters and were subsequently saved and stored as axon binary files for further analysis.

3.5 Data Processing

Signals were converted from analogue to digital and vice versa to acquire signals and to provide commands. A computer was used to control and monitor the experimental parameters, to store data and to perform data analysis. Recordings with leakage current exceeding 200 pA were excluded. Each chimeric protein was measured on at least two distinct days to ensure reliability. Baseline control measurements, including RFP, PCOL, and PCO, were systematically conducted on each day of data acquisition.

Current density was calculated by normalizing peak current amplitude to cell capacitance, an indicator for cell size and surface, both of which critically determine current amplitude. All data were acquired to Excel (Microsoft) or STATA 16 (StataCorp) for subsequent analysis. Figures were created using Prism6 (GraphPad) for statistical graphs and IgorPro for representative current traces, previously extracted from Clampfit 10.1. Graphical refinements of figures were performed using Illustrator CS6 (Adobe).

Results are presented as mean $\pm$ standard error of the mean (SEM). They represent the means of n different measurements on different HEK cells. Differences between paired values were expected to be a consistent measure of the effect of the transfected protein. Significance was tested with an unpaired t-test. A p-value of less than 0.05 was considered statistically significant.

3.6 Use of Large Language Models

OpenAI's large language model ChatGPT (available at <https://chat.openai.com>) was used as a language refinement tool. The model was used to rephrase sentences and to improve grammatical structure of already written sections. All ideas, analyses, arguments, and conclusions presented remain the sole work of the author.

3.7 Ethical Clearance

Given the nature of the present study, which exclusively involved the use of established cell lines without the involvement of human participants, animals, or the use of personal data, no ethical clearance was required.

The research team ensured that all procedures were performed in accordance with the regulations of the RWTH Aachen University concerning research not involving human or animal subjects.

4 Results

To identify the structural elements responsible for the functional differences between PCOL and its homolog PCO, a series of chimeric proteins was generated. Chimeric proteins are constructs composed of defined segments from two related proteins, allowing the functional contribution of specific domains to be tested. By systematically exchanging domains between PCOL and PCO, this approach may enable precise mapping of regions that influence ASIC1a activity. Chimeric borders of all constructs are listed in **Table S3**.

4.1 The Effect of PCOL and PCO on ASIC1a Current Density

ASIC1a was co-expressed with either RFP, PCOL, or PCO to confirm the previously observed potentiating effect of PCOL and to evaluate PCO as a suitable negative control. Co-expression with RFP served as a baseline control, resulting in a mean current density of 138.9 pA/pF (SEM $\pm$ 16.7 pA/pF). In the presence of PCOL, current density increased significantly to 490.5 pA/pF (SEM $\pm$ 33.3 pA/pF), representing a 3.5-fold enhancement (mean difference = 351.6 pA/pF; 95%CI = 276.2 to 427.1 pA/pF; $p < 0.001$). In contrast, co-expression with PCO yielded a mean current density of 152.7 pA/pF (SEM $\pm$ 16.7 pA/pF), which did not differ significantly from the control (mean difference = 13.8 pA/pF; 95%CI = -33.0 to 60.7 pA/pF; $p = 0.560$). These results are presented in **Figure 8** and confirm the specific effect of PCOL and support the use of PCO as a negative control in subsequent experiments.

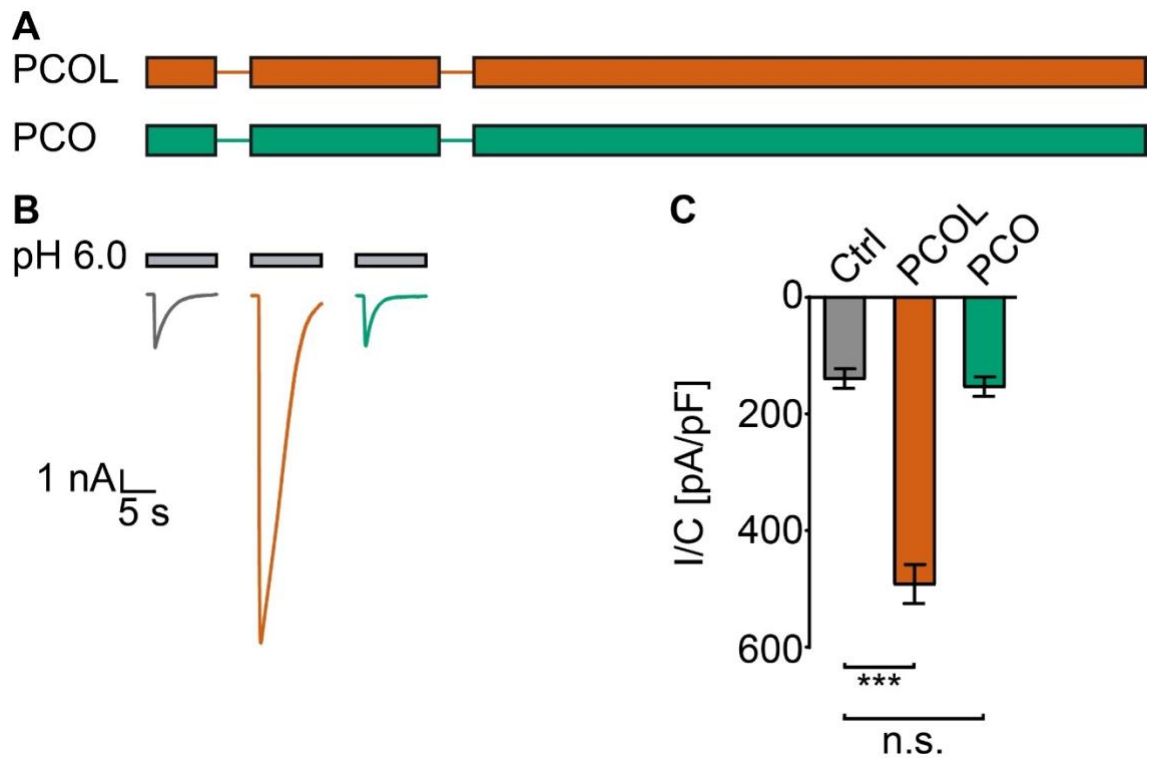


Figure 8: Effects of PCOL and PCO on current density of rASIC1a. Functional co-expression of rASIC1a and RFP (grey), PCOL (orange) or PCO (green) in HEK 293 cells. **A:** Simplified primary structure of PCOL and PCO **B:** Representative current traces. **C:** Statistical analysis of peak current amplitudes (mean ± SEM). Significance levels are: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

In summary, co-expression of ASIC1a with PCOL leads to a 3.5-fold increase in current density upon acidic stimulation, whereas PCO has no significant effect. These results confirm that PCOL specifically enhances ASIC1a activity and support its use as a functional positive modulator, with PCO serving as a suitable negative control.

4.2 Chimeric Investigation of Whole Domains

Based on the described and predicted functional domains of PCO and PCOL a first set of chimeras between PCO and PCOL with chimeric borders between domains was tested.

The N-terminal signal peptide of PCOL was replaced with the respective signal peptide of PCO (*C1*), and the N-terminal signal peptide along with the putative NAD⁺-binding domain of PCOL were replaced with the respective signal peptide and NAD⁺-binding domain of PCO (*C2*). In the presence of *C1* and *C2*, mean current density was increased significantly (**Table 3, Figure 9**).

Subsequently, the counterpart to *C1*, a construct, where the N-terminal signal peptide of PCO was replaced with the respective signal peptide of PCOL (*C3*), and the counterpart to *C2*, a construct, where the N-terminal signal peptide along with the putative NAD⁺-binding domain of PCO were replaced with the respective signal peptide and NAD⁺-binding domain of PCOL (*C4*), were tested. In the presence of *C3* and *C4*, mean current density was not significantly different from control measurements (**Table 3, Figure 9**).

These findings show that neither the signal peptide, nor the NAD⁺-binding domain, but the lyase domain is crucial for the potentiating effect of PCOL on ASIC1a. In the presence of the lyase domain of PCOL a potentiating effect can be observed (*C1* and *C2*), while no such effect can be observed when the lyase domain of PCOL is replaced with the lyase domain of PCO (*C3* and *C4*).

Table 3: Current density in the presence of C1, C2, C3, and C4. Abbreviations: SEM: Standard Error of the Mean, 95%CI: 95% Confidence Interval

Construct (n)	Chimeric border	Current density (mean, SEM)	Difference to control (mean, 95%CI)	Mean potentiation	p-value
C1 (n=8)	PCO E35/ PCOL K28	689.5 pA/pF (± 124.0)	551.6 pA/pF (198.0 to 905.2)	5.0-fold	0.006
C2 (n=4)	PCO A113/ PCOL V106	640.0 pA/pF (± 150.3)	571.3 pA/pF (109.0 to 1033.6)	9.3-fold	0.025
C3 (n=6)	PCOL G27/ PCO K36	169.0 pA/pF (± 59.7)	29.4 pA/pF (-257.9 to 199.1)	0.9-fold	0.778
C4 (n=6)	PCOL E105/ PCO S114	169.5 pA/pF (± 51.9)	-28.9 pA/pF (-245.3 to 187.6)	0.9-fold	0.770

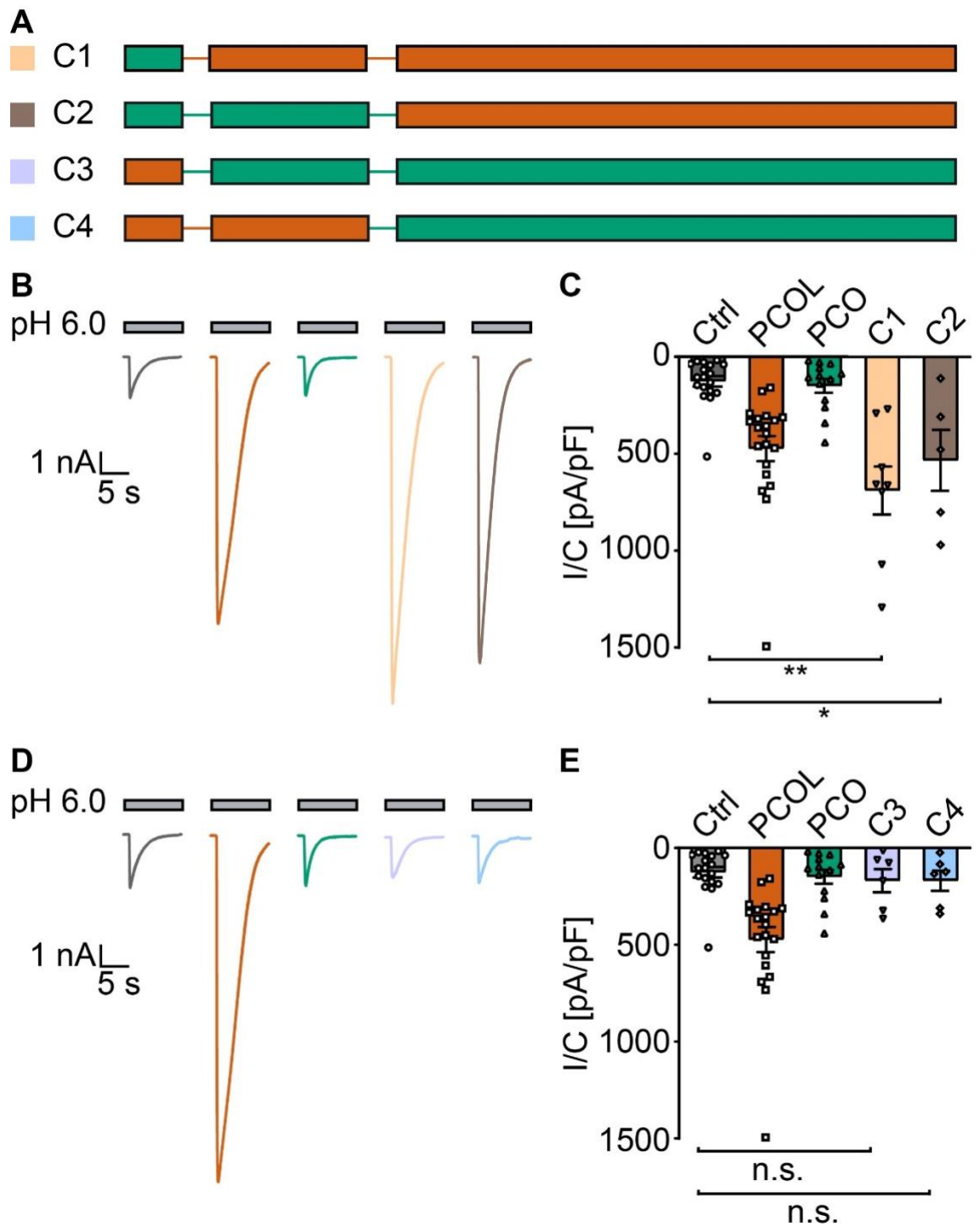


Figure 9: Current density in the presence of control measurements, C1, C2, C3, and C4. A: Simplified primary structure of C1, C2, C3, and C4. PCOL is represented by orange. PCO is represented by green. **B, D:** Representative current traces. **C, E:** Statistical analysis of peak current amplitudes (mean $\pm$ SEM). Significance levels are: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

These results demonstrate that neither the signal peptide nor the NAD⁺-binding domain is sufficient to account for the potentiating effect of PCOL on ASIC1a.

The effect is retained when the lyase domain of PCOL is present, and abolished when it is replaced by the corresponding domain of PCO, thus identifying the lyase domain as critical for function.

4.3 Subdivision of the Lyase Domain

To further narrow down the amino acid sequence that is required for the effect of PCOL on ASIC1a, a series of chimeras with chimeric borders within the lyase domain was tested.

4.3.1 The C-terminal Lyase Region is Functionally Relevant

To generate two complementary chimeras, the domain boundary was placed at the midpoint of the lyase domain. Chimera C5 consists of the N-terminal to mid-lyase region of PCOL, fused to the mid-lyase to C-terminal region of PCO. Conversely, C6 comprises the N-terminal to mid-lyase region of PCO and the mid-lyase to C-terminal region of PCOL. Current densities recorded in the presence of C5 and C6 are presented in **Table 4** and **Figure 10**.

Notably, on the measuring days of C6, control measurements yielded high current densities with a mean current density of 325.0 pA/pF (SEM $\pm$ 134.7) (n=3), resulting in a non-significant difference even between control measurements and PCOL measurements (mean difference = 568.0 pA/pF; 95%CI = -98.1 to 1234.0 pA/pF; p-value = 0.080). Given the striking resemblance of current trace shape between C6 and PCOL (**Figure 10**), and the high current density of the given control measurements we decided to test for significance between C6 and the overall control group (n=63) and observed a significant difference (mean difference = 395.3 pA/pF; 95%CI = 237.3 to 553.3 pA/pF; p-value <0.001), corresponding to a 3.8-fold increase in mean current density.

Table 4: Current density in the presence of C5 and C6 Abbreviations: SEM: Standard Error of the Mean, 95%CI: 95% Confidence Interval

Construct (n)	Chimeric border	Current density (mean, SEM)	Difference to control (mean, 95%CI)	Mean potentiation	p-value
C5 (n=11)	PCOL T286/ PCO G299	280.1 pA/pF (±66.6)	53.4 pA/pF (-200.8 to 307.6)	1.2-fold	0.659
C6 (n=8)	PCO T298/ PCOL D287	534.2 pA/pF (±188.3)	209.2 pA/pF (-529.5 to 947.8)	1.6-fold	0.538

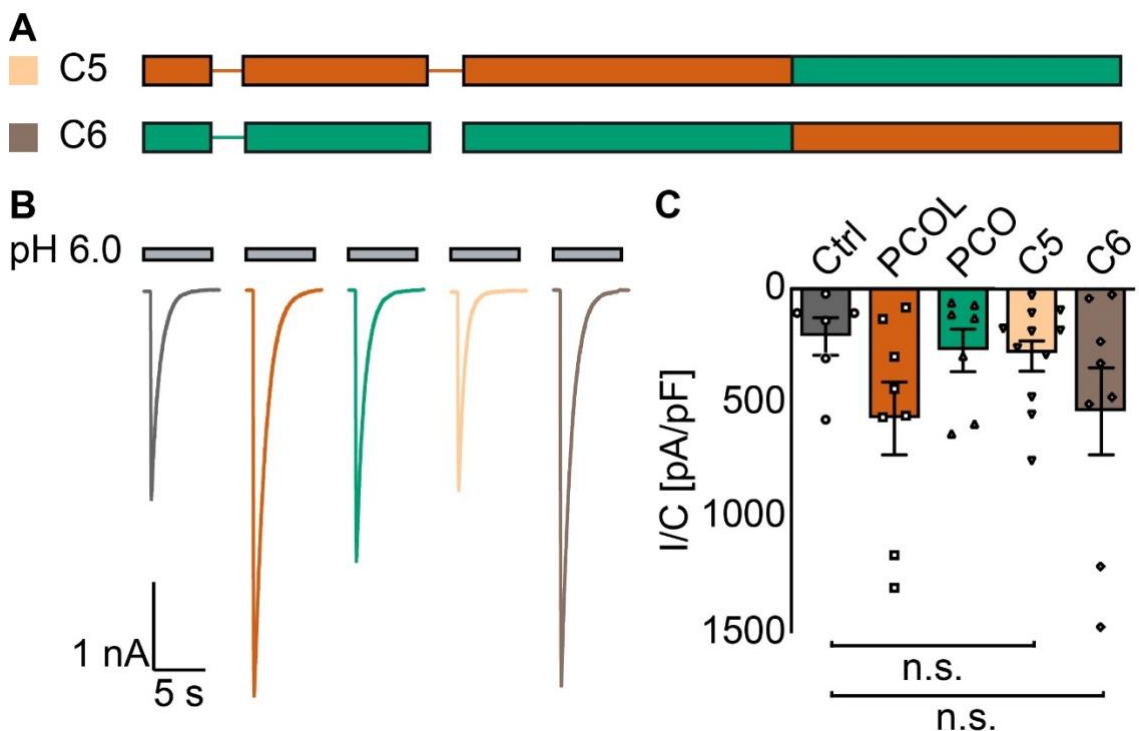


Figure 10: Effect of C5 and C6. **A:** Simplified primary structure of C5 and C6. PCOL is represented by orange. PCO is represented by green. **B:** Representative current traces. **C:** Statistical analysis of peak current amplitudes (mean $\pm$ SEM). Significance levels are: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

These results suggest that the potentiating sequence may be located in the C-terminal half of the lyase domain.

4.3.2 The Last Quarter of the Lyase Domain is Not Sufficient for Potentiation

With a cautious interpretation of C6 as chimera with a potentially potentiating effect on ASIC1a, the chimeric border was subsequently moved further towards the C-terminus. C7 was composed of PCOL DNA of the N-terminus up to the last quarter of the lyase domain, followed by PCO DNA of the last quarter of the lyase domain up to the C-terminus. As a counterpart, C8 was composed of PCO DNA of the N-terminus up to the last quarter of the lyase domain, followed by PCOL DNA of the last quarter of the lyase domain up to the C-terminus.

Neither C7, nor C8 demonstrated a statistically significant potentiation on ASIC1a currents. Results from C7 and C8 are presented in **Table 5** and **Figure 11**.

These results demonstrate that the last quarter of the lyase domain is critical but not sufficient for the potentiating effect of PCOL on ASIC1a currents.

Table 5: Current density in the presence of C7 and C8 Abbreviations: SEM: Standard Error of the Mean, 95%CI: 95% Confidence Interval

Construct (n)	Chimeric border	Current density (mean, SEM)	Difference to control (mean, 95%CI)	Mean potentiation	p- value
C7 (n=11)	PCOL E390/ PCO G400	193.7 pA/pF (±37.6)	-14.9 pA/pF (139.4 to 109.6)	0.9-fold	0.803
C8 (n=11)	PCO D399/ PCOL A391	189.7 pA/pF (±44.2)	-49.7 pA/pF (-212.1 to 112.7)	0.8-fold	0.522

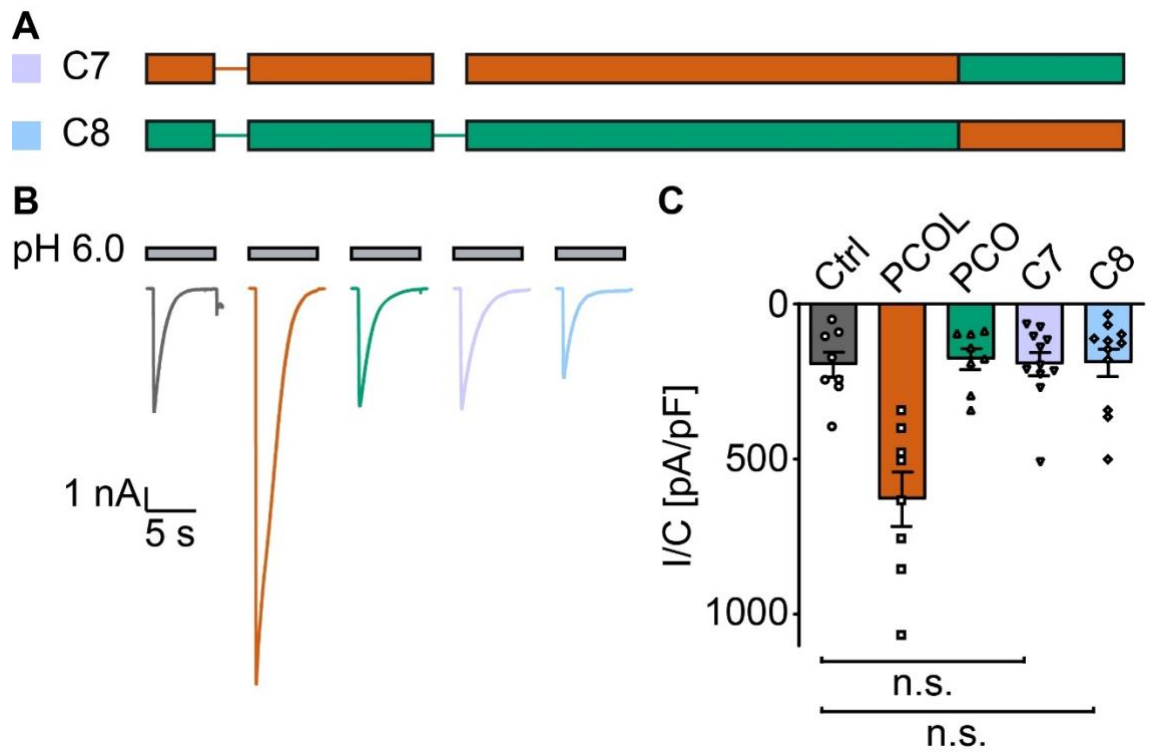


Figure 11: Effect of C7 and C8. **A:** Simplified primary structure of C7 and C8. PCOL is represented by orange. PCO is represented by green. **B:** Representative current traces. **C:** Statistical analysis of peak current amplitudes (mean $\pm$ SEM). Significance levels are: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

The last quarter of the lyase domain alone is not sufficient to induce a potentiating effect, suggesting that at least parts of the responsible sequence lie upstream.

4.3.3 Region E275–A391 is Necessary but Not Sufficient for Function

Considering the results from *C5*, *C6*, *C7*, and *C8* we hypothesized that the decisive amino acid sequence might be located between the chimeric border of *C5* and *C6* - the middle of the lyase domain - and the chimeric border of *C7* and *C8* - the last quarter of the lyase domain. Consequently, *C9*, a chimera that contained the aforementioned sequence of the lyase domain of PCOL as an insertion into PCO, and its counterpart, the chimera *C10*, were created. Neither *C9*, nor *C10* showed a potentiating effect on ASIC1a current density (**Table 6**, **Figure 12**). We concluded that the effect of PCOL on ASIC1a may depend on the region between E275 and A391, as evidenced by a lack of function for *C10*, where this sequence was replaced to the PCO counterpart, ranging from G286 to D399.

Table 6: Current density in the presence of *C9* and *C10*. Abbreviations: SEM: Standard Error of the Mean, 95%CI: 95% Confidence Interval

Construct (n)	Chimeric border	Current density (mean, SEM)	Difference to control (mean, 95%CI)	Mean potentiation	p-value
<i>C9</i> (n=4)	PCO T298/	179.9 pA/pF (±33.3)	38.3 pA/pF (-152.7 to 229.3)	0.8-fold	0.628
	PCOL D287/				
	PCOL E390/				
	PCO G400				
<i>C10</i> (n=6)	PCOL E275/	88.0 pA/pF (±36.4)	-1.4 pA/pF (-135.4 to 132.6)	1.0-fold	0.981
	PCO G286/				
	PCO D399/				
	PCOL A391				

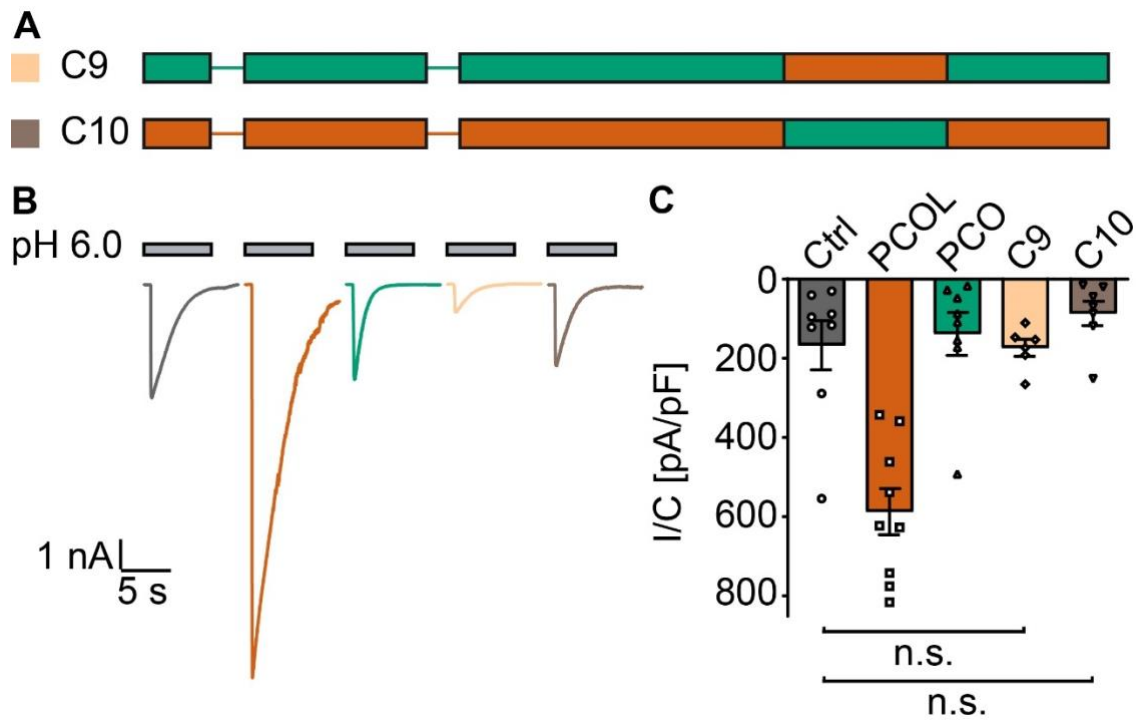


Figure 12: Effect of C9 and C10. **A:** Simplified primary structure of C9 and C10. PCOL is represented by orange. PCO is represented by green. **B:** Representative current traces. **C:** Statistical analysis of peak current amplitudes (mean $\pm$ SEM). Significance levels are: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

The identified region (PCOL E275 to A391) is necessary but not sufficient for function when transplanted into PCO, indicating potential dependencies on flanking regions or structural context.

4.3.4 Stepwise Identification of the N-terminal Border

However, given the lack of current density potentiation in C9 we concluded that further structural elements of PCOL towards the C-terminus may be decisive for the interaction with ASIC1a. Hence, we tested current densities in the presence of a series of chimeric constructs that contained the PCOL sequence from the N-terminus up to the respective chimeric border and the PCO sequence from the respective chimeric border up to the C-terminus. The chimeric border was shifted towards the C-terminus, starting at the chimeric border of C5 (T286 of PCOL and G299 of PCO), until a gain of function was observed at D377 of PCOL and E488 of PCO (**Table 7**, **Figure 13**).

Table 7: Current density in the presence of C11, C12, C13, C14, and C15. Abbreviations:
SEM: Standard Error of the Mean, 95%CI: 95% Confidence Interval

Construct (n)	Chimeric border	Current density (mean, SEM)	Difference to control (mean, 95%CI)	Mean potentiation	p- value
C11 (n=16)	PCOL P320/ PCO P331	124.5 pA/pF (± 20.2)	-8.6 pA/pF (-111.1 to 93.8)	0.9-fold	0.863
C12 (n=8)	PCOL T362/ PCO 373	186.9 pA/pF (± 41.6)	0.5 pA/pF (-187.5 to 188.4)	1.0-fold	0.996
C13 (n=8)	PCOL Y428/ PCO N439	109.7 pA/pF (± 32.5)	-35.5 pA/pF (-154.3 to 83.4)	0.8-fold	0.521
C14 (n=5)	PCOL S454/ PCO C465	270.6 pA/pF (± 29.1)	168.6 pA/pF (-35.8 to 372.9)	2.7-fold	0.096
C15 (n=8)	PCOL D377/ PCO E488	417.9 pA/pF (± 53.3)	321.9 pA/pF (117.5 to 526.3)	4.4-fold	0.006

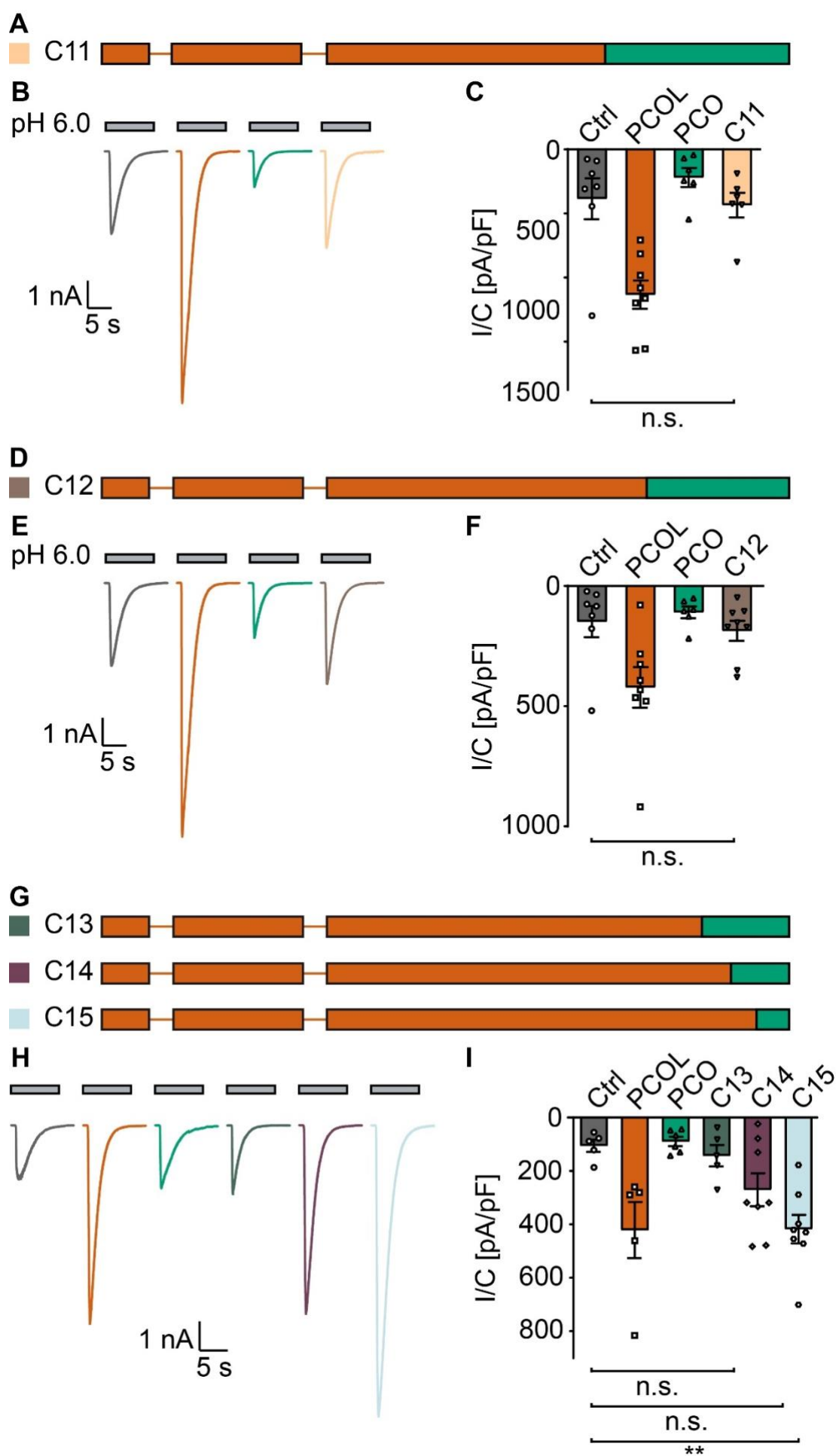


Figure 13: Effect of C11, C12, C13, C14, and C15. **A, D, G:** Simplified primary structure of C11, C12, C13, C14, and C15. PCOL is represented by orange. PCO is represented by green. **B, E, H:** Representative current traces. **C, F, I:** Statistical analysis of peak current amplitudes (mean $\pm$ SEM). Significance levels are: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

In summary, *C11* and *C12* show that introducing only short N-terminal segments of the critical region is not sufficient to reliably induce a potentiating effect on ASIC1a, suggesting that a larger sequence context is required. Further, the modest increase in current density with *C13* and lack of significant effect in *C14* suggest that the threshold for functional gain is only partially met. Finally, *C15* demonstrates that a larger segment of the lyase domain, encompassing approximately residues P321 to D477, is sufficient to confer a strong potentiating effect when introduced into the PCO scaffold.

4.3.5 Stepwise Identification of the C-terminal Border

Neither the counterpart to *C11* (*C16*), nor the counterpart to *C12* (*C17*), where N-terminal parts of the construct were represented by PCO and C-terminal parts by PCOL around similar chimeric borders, caused a potentiation of ASIC1a current density (**Table 8, Figure 14**). Based on the results of *C16*, *C17*, no counterparts to *C13*, *C14*, and *C15* were tested.

Table 8: Current density in the presence of C16 and C17. Abbreviations: SEM: Standard Error of the Mean, 95%CI: 95% Confidence Interval

Construct (n)	Chimeric border	Current density (mean, SEM)	Difference to control (mean, 95%CI)	Mean potentiation	p- value
C16 (n=17)	PCO P330/ PCOL P321	280.0 pA/pF (± 54.8)	122.5 pA/pF (-50.6 to 295.6)	1.8-fold	0.157

C17	PCO T372/	148.9	88.2 pA/pF	2.5-fold	0.149
(n=8)	PCOL	pA/pF	(-38.1 to 214.4)		
	D363	(±32.4)			

While there was no observable potentiating effect of *C16* it appeared as though measured current densities in the presence of *C16* might be dividable into two groups (**Figure 14**). Through an ANOVA we tested whether the two apparent groups can be separated by the mean current density of *C16* (>/<280.0 pA/pF) and yielded an affirmative result (p-value <0.001). The resulting groups of *C16* - *C16 high*, measurements above the mean current density, and *C16 low*, measurements below the mean current density - were then tested individually against the control group. While *C16 low* (n=9) did not significantly differ from control measurements (mean current density 88.2 pA/pF, SEM ±16.8 pA/pF; mean difference -69.3, 95%CI -180.5 to 41.9 pA/pF; p-value = 0.205), *C16 high* (n=8) did differ significantly (mean current density 495.8 pA/pF, SEM ±40.6 pA/pF; mean difference 338.3, 95%CI -199.4 to 477.2 pA/pF; p-value <0.001). In the presence of *C16 high* mean current density was potentiated 3.1-fold.

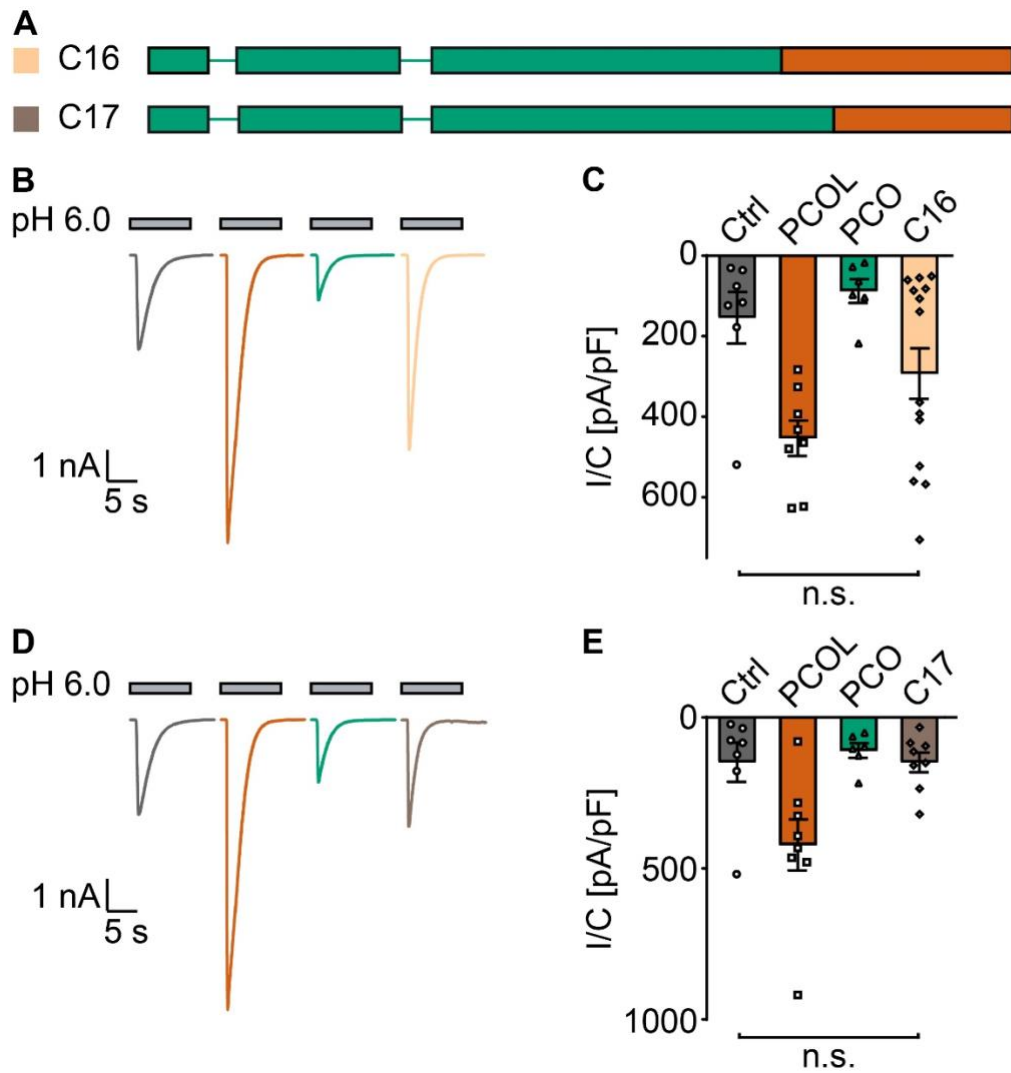


Figure 14: Effect of C16 and C17. **A:** Simplified primary structure of C16 and C17. PCOL is represented by orange. PCO is represented by green. **B, D:** Representative current traces. **C, E:** Statistical analysis of peak current amplitudes (mean $\pm$ SEM). Significance levels are: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

Chimera *C16* produced a variable but occasionally enhanced current density, suggesting a potentially unstable or partial potentiating effect. In contrast, *C17* showed no increase in current. These results indicate that the critical region might lie between the C-terminus and the chimeric border used in *C16*, but is disrupted when the more C-terminal border of *C17* is applied.

4.3.6 Identification of a Minimal Sufficient Region

Based on the findings of *C6* and *C15*, *C18*, a construct that only contains the parts of PCOL that were identified as crucial for the potentiating effect, was tested. In the presence of *C18*, mean current density was 124.6 pA/pF (SEM $\pm$ 17.9 pA/pF) (n=7), which did significantly differ from control measurements (mean difference = 61.6 pA/pF; 95%CI = 2.8 to 120.4 pA/pF; p-value = 0.042), corresponding to a 2.0-fold increase in mean current density (**Figure 15**).

Based on the ambiguous results from *C16* we further tested *C19*, a construct that contains the chimeric borders of both *C16* and *C15*. In the presence of *C19*, mean current density was 147.3 pA/pF (SEM $\pm$ 24.8 pA/pF) (n=8), which did significantly differ from control measurements (mean difference = 84.3 pA/pF; 95%CI = 13.8 to 154.8 pA/pF; p-value = 0.023, **Figure 15**).

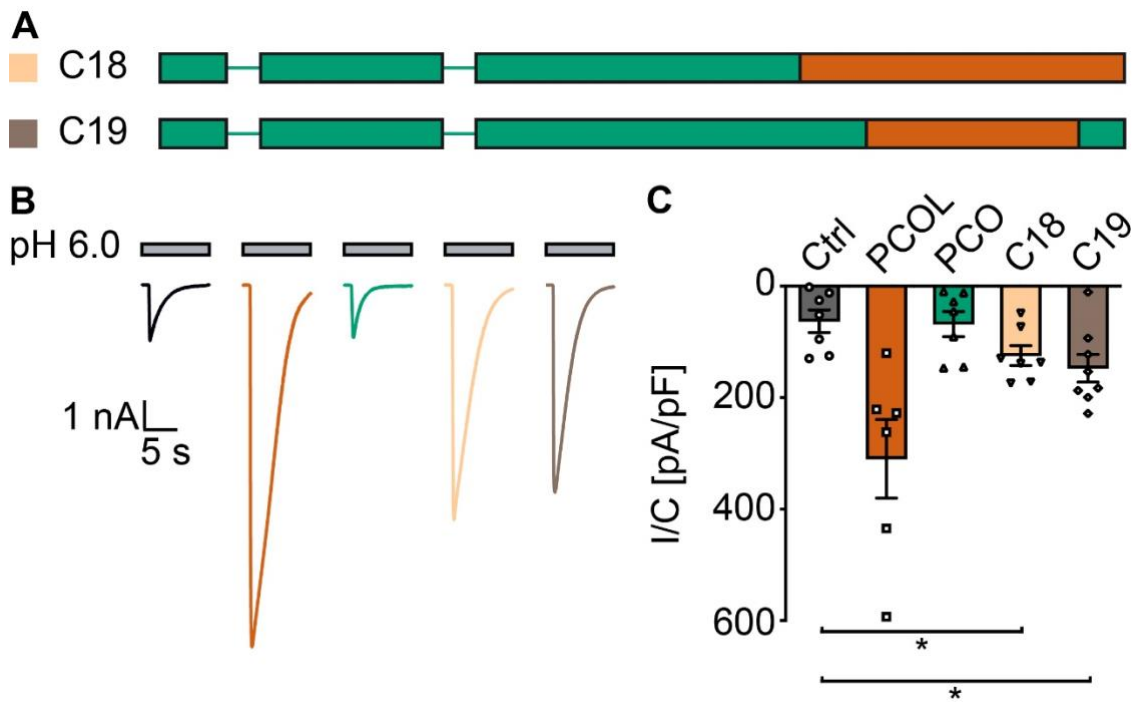


Figure 15: Effect of C18 and C19. **A:** Simplified primary structure of C18 and C19. PCOL is represented by orange. PCO is represented by green. **B:** Representative current traces. **C:** Statistical analysis of peak current amplitudes (mean $\pm$ SEM). Significance levels are: $p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

4.3.7 Analysis of Cell Capacitance as Confounding Factor

To rule out differences in membrane surface area as a confounding factor, cell capacitance was analyzed across all groups. The overall average cell capacitance was 20.1 pF (SD $\pm$ 8.8 pF), with values ranging from 4.5 to 78 pF. When grouped by transfected DNA (e.g., PCOL, PCO, control), capacitance did not significantly differ between groups (ANOVA $p = 0.106$), indicating that differences in current density were not attributable to variations in cell size.

4.3.8 Comparison of Exchanged Sequences

Of the 156 amino acids exchanged in C19, 107 amino acids are mismatches, resulting in a pairwise identity of 31.9% between PCOL and PCO for the exchanged sequence (**Figure 16**). When grouping residues by properties (polar, nonpolar, acidic, basic) and molecular weight of the side chain (small [less than 75 daltons], medium [between 75 and 155 daltons], large [greater than 155

daltons]) 47/107 (43.9%) are identified as clear nonconservative changes (**Table S4**).



Figure 16. Sequence alignment of exchanged amino acids in C19. Differing amino acids are indicated by a red overlay.

On the level of secondary and tertiary structure there was a high level of similarity between the two proteins in the exchanged region (**Figure S3**, **Figure 4**)

Collectively, the results from the progressive subdivision of the lyase domain point to a critical stretch of amino acids between P321 and D477 in PCOL. Exchange of this region with the corresponding sequence from PCO abolishes the potentiating effect, while its insertion into PCO confers partial activity. These findings indicate that this region is both necessary and partially sufficient for the functional interaction between PCOL and ASIC1a.

5 Discussion

5.1 Summary

This study explored the interaction between PCOL and ASIC1a and found that in the presence of PCOL the current amplitude of ASIC1a is potentiated 3.5-fold upon rapid extracellular acidification. This effect was lost, when the amino acids between P321 and D477 were exchanged for the corresponding amino acids of the structurally similar protein PCO, which does not affect the current through ASIC1a. Thus, the exchanged region seems to be critical for the potentiating effect of PCOL. Moreover, an inverse exchange of the AA T298 to G400 in PCO for the AA P321 to D477 from PCOL led to a partial gain of function for PCO further supporting the assumption of this region being not only critical, but also sufficient for partially conducting the differential effect of PCOL and PCO on ASIC1a.

The major finding of this study is the chimeric identification of a region of PCOL and PCO close to the C terminus, where an exchange between the two proteins resulted in a reversal of effects on ASIC1a.

5.2 Comparison to Existing Literature

The present study underscores the role of PCOL in significantly enhancing ASIC1a current density as described previously (Kuspiel et al., 2025), and solidifies the hypothesis that PCOL is a crucial modulatory protein for ASIC1a channels. The observed 3.5-fold potentiation of ASIC1a current density in the presence of PCOL aligns with the previously observed 3- to 3.5-fold potentiation (Kuspiel et al., 2025). In contrast, the closely related protein PCO did not affect ASIC1a current, suggesting that, despite high sequence similarity, the two proteins exert distinct cellular functions.

Lian et al. identified PCYOX1L as one of eight genes associated with high necroptosis scores and severe pathology in Alzheimer's disease, without addressing its functional role (Lian et al., 2024). Clusmann et al. showed that ASIC1a activity can induce necroptosis via RIPK1 activation, and this study demonstrates that PCOL enhances ASIC1a currents; thus, a PCOL-mediated increase in ASIC1a activity may provide a mechanistic link to necroptosis in Alzheimer's disease (Clusmann et al., 2022; Lian et al., 2024).

Other investigations on PCOL concentrated on blood cells that do not express ASICs and are hence less relevant to the present study (He et al., 2023; Petenkova et al., 2023).

Two residues, proposed to constitute the active site of PCO, are known to be involved in the cleavage of farnesylated cysteines (Beigneux et al., 2002; Herrera-Marcos et al., 2018). The equivalent residues in PCOL, Y445 and E457, are present in all tested chimeras that demonstrated a potentiating effect on ASIC1a current density. Moreover, previous experiments have shown that individual replacement of Y445 or E457 in PCOL led to reduced potentiation of ASIC1a current density, and complete loss of potentiation occurred when both residues were mutated (Kuspiel et al., 2025).

Despite the close structural and enzymatic similarity between PCO and PCOL and the conservation of this putative active site, the fact that PCO does not affect ASIC1a current density raises questions about the role of these residues in the interaction (Barone et al., 2024). It remains unclear whether the catalytic activity of PCOL is relevant for the observed potentiation, especially since PCO lacks any

measurable influence. This discrepancy suggests that the interaction between PCOL and ASIC1a involves additional residues or structural elements beyond the conserved active site and that the mechanism of interaction might go beyond the catalytic activity.

Interestingly, other proteins have been identified as chaperones with dual functions, possessing an enzymatic activity independent of their role in protein interactions (Schwenk et al., 2019). This raises the possibility that PCOL could have an additional function that distinguishes it from PCO, possibly explaining the differential effects on ASIC1a.

Additional mutational experiments where PCOL K400A exhibited a partial effect on ASIC1a currents are in line with finding of the present study that the PCOL sequence between P321 to D477 (**Figure 17 A**) is critical for the interaction (Kuspiel et al., 2025). While neither *C13*, *C14*, *C10*, *C17*, nor *C8* elicited potentiated ASIC1a currents despite containing PCOL sequences that included K400, all the chimeras that did evoke a potentiation did contain K400.

Using AlphaFold 3 (Abramson et al., 2024), the three-dimensional structure of the interaction between PCOL and ASIC1a was modelled (**Figure 17 B,C**). The predicted interaction aligns with the findings of this study and suggests that within the sequence from P321 to D477, the region around S370 to A420 of PCOL may be in close proximity to ASIC1a during their interaction.

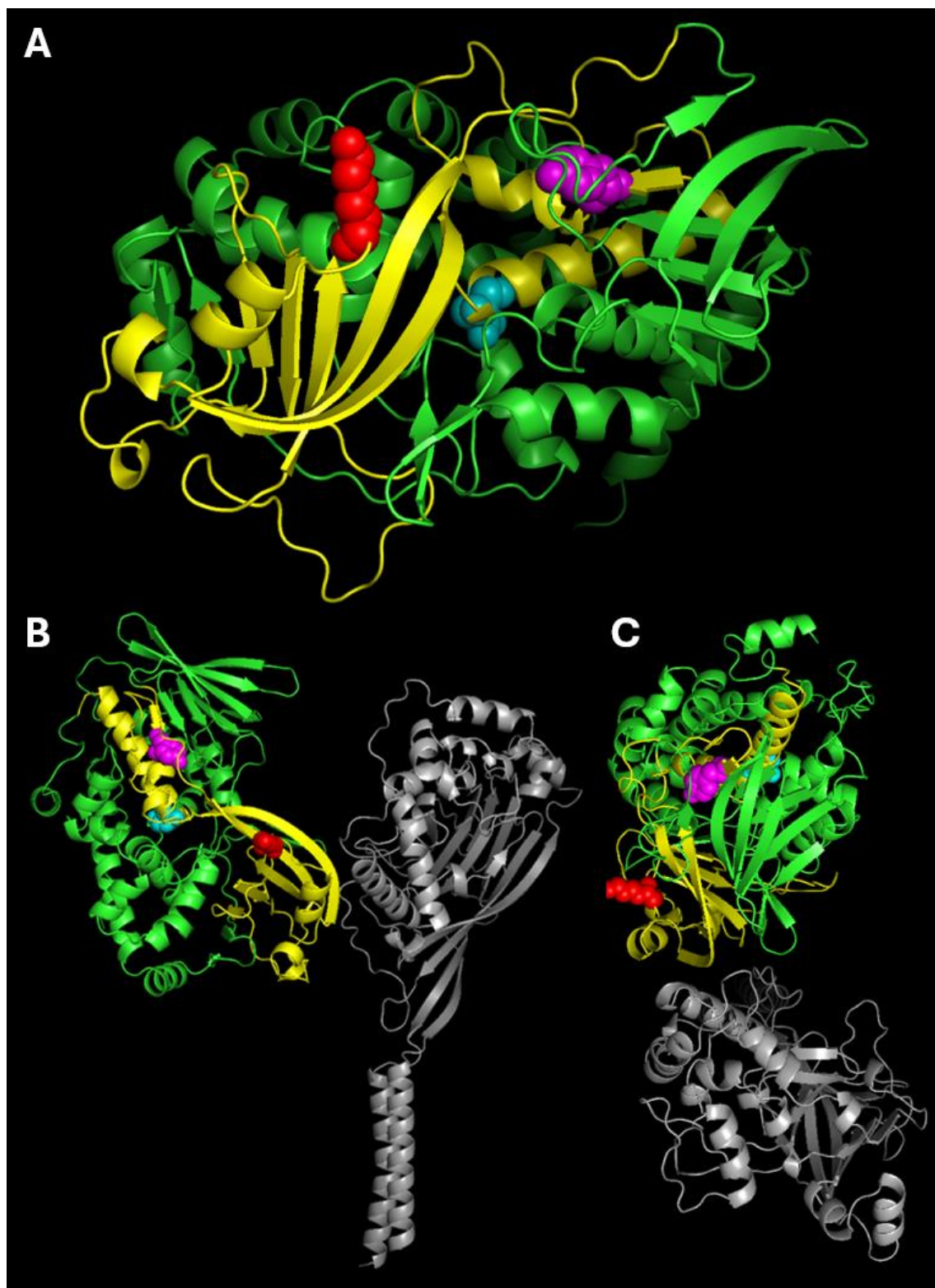


Figure 17. **A:** Critical sequence and residues of PCOL as identified by *C19* and mutational experiments. The critical region as identified by *C19* (marked in yellow) comprises large parts of the antiparallel β -sheet domain, two helices of the aligned α -helix domain, and one small β -sheet of the whirled β -sheet domain. Critical residues are displayed as spheres. K400 is marked in red, Y445 is marked in magenta, and E457 is marked in cyan. Mutational experiments were conducted by Sven Kuspiel (unpublished, personal communication with Sven Kuspiel). **B,C:** Predicted structure of the interaction between PCOL and rASIC1a (in grey). Predictions were performed with AlphaFold 3 (Abramson et al., 2024).

Preliminary competition experiments revealed that the effects of Capsaicin, Clorpromazine, MitTx and Ibuprofen on ASIC1a were limited in the presence of PCOL (Kuspiel et al., 2025), suggesting that they compete for a common binding site on the ASIC1a channel, while neither the effects of Big Dynorphin nor the effects of PcTx on ASIC1a were affected by the presence of PCOL. Considering the known binding and interaction sites of these agents (Baconguis et al., 2014; Borg et al., 2019; Dawson et al., 2012; Leisle et al., 2021; Lynagh et al., 2017; Schmidt et al., 2018) it has been suggested that PCOL interacts with ASIC1a by binding to the lower thumb domain of ASIC1a close to the membrane (see **Figure 2**). Specifically, the T417A mutation caused a complete lack of potentiation (unpublished, personal communication with Sven Kuspiel). While the ASIC1a structures involved in the interaction were not investigated in the present study they are equally important to the interaction site on PCOL and should be further investigated in future studies.

5.3 Limitations

The experiments were conducted using HEK293 cells, a heterologous expression system that resembles the native cellular environment where ASIC1a and PCOL are naturally expressed. However, the initial screen for ASIC1a interaction partners was conducted in mice brains. The HEK293 cell line was selected due to its prevalent use in the laboratory of Stefan Gründer, attributable to its cost effectiveness and high transfection efficiency, particularly with calcium phosphate methods. The choice of HEK293 cells also aimed to maintain consistency with other studies exploring the interaction between PCOL and ASIC1a, thereby facilitating comparative analysis across different experiments.

Within this expression system, ASIC1a and PCOL were overexpressed, potentially leading to skewed biophysical properties. However, control experiments involved overexpressing ASIC1a only, establishing a reliable baseline for comparison.

Although an influence of GFP on the interaction of the proteins cannot be entirely excluded, the applied method of co-expressing GFP separately, rather than fusing it to ASIC1a or PCOL, was chosen to minimize potential interference with their interaction dynamics.

The constructs used were based on the rat versions of the proteins, which may not behave identically to their human counterparts. Consequently, it is essential for future investigations to confirm whether these observed effects are reproducible across different species.

A further limitation of this study is the focus on current density without analysis on how PCOL might affect other biophysical properties of ASIC1a, such as ion selectivity or activation and desensitization kinetics. Further research using different experimental conditions and techniques, including single-channel recordings and ion substitution experiments, is needed to explore these aspects. However, previous biophysical analyses on wild-type constructs have not revealed any changes induced by the presence of PCOL (Kuspiel et al., 2025).

Lastly, it is important to acknowledge that the loss of function in any construct can stem from various causes, such as incorrect folding. Therefore, the presumed absence of interaction represents just one of several potential explanations.

5.4 Outlook

The present study aims to further the understanding of the ASIC interactome and to contribute to the development of novel therapeutic agents that can be deployed in the numerous physiological and pathophysiological conditions involving ASICs and PCOL.

To gain a full understanding of the structural aspects of the interaction between PCOL and ASIC1a further studies are warranted. While X-ray crystallography would allow for a determination of the structure of the proteins at the atomic level, nuclear magnetic resonance spectroscopy would provide insights into the changes in conformation that occur when the proteins interact.

Another area of interest for future studies is the mechanism by which PCOL enhances ASIC1a current density. While it has been suggested that PCOL might increase the surface expression of ASIC1a it remains unclear if it also has other effects on the channel (Kuspiel et al., 2025). Moreover, it remains unclear how an increase in surface expression may be achieved, and further investigations into the effect of PCOL on ASIC1a trafficking and membrane stability are necessary to understand where the interaction happens. One possible mechanism may be the involvement of PCOL as auxiliary protein and chaperone for ASIC1a monomers that influence transportation (Kuspiel et al., 2025). Additionally, future investigations should further research the impact of PCOL on the drug affinity and biophysical properties of ASIC1a.

Another interesting area of research would be the role of PCOL in physiological and pathophysiological processes and conditions that involve ASIC. Questions such as, how the absence or presence of PCOL affects long term potentiation, drug-related behaviour, or infarction volume in ischemic stroke remain unanswered and are critical towards the development of novel therapeutic agents.

Ideally, a full understanding of the interaction can be used to develop targeted drugs that reduce ASIC activity in pathophysiological conditions where overactivation triggers cell death, while maintaining the level of activity that is needed for physiological processes.

6 Conclusion

This study identifies a specific region within the lyase domain of PCOL as both necessary and sufficient for the potentiation of ASIC1a-mediated currents. Through a systematic series of chimeric constructs and electrophysiological measurements, we localized this function-bearing region to the segment spanning residues E275 to A391.

The absence of a potentiating effect in analogous chimeras derived from PCO, despite high sequence homology, highlights the importance of subtle structural and possibly post-translational determinants unique to PCOL. These findings confirm that the functional modulation of ASIC1a by PCOL is not a generic feature of prenylcysteine oxidases but depends on specific structural elements of PCOL.

This work advances our understanding of endogenous ASIC1a modulation and establishes a framework for further investigation into the molecular interface of its interaction with PCOL. Our findings pave the way for resolving the atomic structure of the PCOL–ASIC1a complex and evaluating its suitability as a pharmacological target. Pharmacological modulation of the interaction could offer therapeutic benefit across a spectrum of neurological disorders, ranging from reducing infarct size in ischemic stroke to treating chronic pain conditions.

7 Literature

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8 Supplemental Material

8.1 Supplemental Tables

Table S1: List of primers used for PCR. DNA sequences in uppercase letters are complementary to PCOL. DNA sequences in lowercase letters are complementary to PCO.

Name	Sequence	Complement ary strand	Used for generation of
5'PCOL pBud inf	5' CACGTGGTCGCGGCCGCCCCGCCAT GGCCCCGCGCTGC 3'	sense	PCOL, C3, C4, C5, C7, C8, C11, C13, C15, C16, C17
3'PCOL pBud inf	5' ACCTTCGAAGCGGCCGCTCACAGTTC AGTCTTGACCTTATGG 3'	antisense	PCOL, C1, C2, C6, C8, C9, C12, C14
5'PCO pBud inf	5' cacgtggtcgcggccgccccgccatggggcgcttcgc cgctacg 3'	sense	PCO, C1, C2, C6, C9, C10, C12, C14, C18, C19
3'PCO pBud inf	5' accttcgaagcggccgctcaatccgacagttctgtctta ag 3'	antisense	PCO, C3, C4, C5, C7, C10, C11, C13, C15, C16
5'PCOL- NAD	5' cggggccctccggaaAAAATCGCTGTTATT 3 ,	sense	C1
3'PCO-SP	5' AATAACAGCGATTTTtccggaggggcccg 3'	antisense	C1
5'PCOL- Lyase	5' ctccagtgtgcccgcgtGTGGTGGGAAGGAGC 3'	sense	C2
3'PCO- NAD	5' GCTCCTTCCCACCACagcgggcacactgga g 3'	antisense	C2
5'PCO- NAD	5' GACGCACCGCCGGGgaagatcgcaatagtt g 3'	sense	C3
3'PCOL-SP	5' caactattgcatcttCCCCGGCGGTGCGTC 3'	antisense	C3
5'PCO- LYASE	5' GAGACAGAGGCGAGAGtcaggtggcctggt g 3'	sense	C4
3'PCOL- NAD	5' caccaggccacctaCTCTCGCCTCTGTCT C 3'	antisense	C4
5'PCO Lyase 2	5' CCAAGTGGGGTACGAGACTggatctgaga tcattc 3'	sense	C5
3'PCOL Lyase 1	5' gaatggatctcagatccAGTCTCGTACCCAC TTGG 3'	antisense	C5
5'PCOL Lyase 2	5' gaagtggttataaaacaGACAAGGGCAACA GCTCTG 3'	sense	C6, C10
3'PCO Lyase 1	5' CAGAGCTGTTGCCCTTGTCtgtttataaac cacttc 3'	antisense	C6, C10
PCOL-PCO 3/4-f	5' GAAGCAACCCAGGAGggcatgcatgtgtg g 3'	sense	C7, C10
PCOL-PCO 3/4-r	5' ccacacatgcatgccCTCCTGGGGTTGCTTC 3'	antisense	C7, C10
5'PCOL831 -PCO856-f	5' CTGCACAGCACAGAAggaacccccccaag 3'	sense	C8
3'PCOL831 -PCO856-r	5' cttcgggggggttccTTCTGTGCTGTGCAG 3'	antisense	C8
PCO-PCOL 3/4-f	5' gtccccaccggcagtgatGCAGCAGTGTGG CGAG 3'	sense	C8, C9, C18

PCO-PCOL 3/4-r	5' CTCGCCACACTGCTGCatccactgccggtgg gggac 3'	antisense	C8, C9, C18
5'PCOL966 -PCO991-f	5' CCTTTGAAGGTTTTACTCCAcccatcgagg aattcaatg 3'	sense	C11, C19
3'PCOL966 -PCO991-r	5' cattgaattcctcgatgggTGGAGTAAACCTT CAAAGG 3'	antisense	C11, C19
5'PCO990- PCOL967-f	5' cttccgaaatttgatcccCCCATTGATGACATT CAGGGC 3'	sense	C12, C18
3'PCO990- PCOL967-r	5' GCCCTGAATGTCATCAATGGGgggatca aaatttcggaag 3'	antisense	C12, C18
5'PCOL109 2-	5' CGCCAACATACTCACCACAgatgactcag atatgtttatc 3'	sense	C13, C18
PCO1117-f 3'PCOL109 2-	5' gataaacatatctgagtcacTGTGGTGAGTAT GTTGGCG 3'	antisense	C13, C18
PCO1117-r 5'PCO1116 -	5' gtctgcgattctgtcactGACTTCCCGAGCTTC TTCTG 3'	sense	C14, C19
PCOL1093- f			
3'PCO1116 -	5' CAGAAGAAGCTCGGGAAGTCagtgacaa gaatcgagac 3'	antisense	C14, C19
PCOL1093- r			
5'PCOL129 0-	5' GCCCACCCCCTCTACaatcctcctcagaag 3'	sense	C15
PCO1315-f 3'PCOL129 0-	5' cttctgaggaggattGTAGAGGGGGTGGGC 3'	antisense	C 15
PCO1315-r 5'PCOL136 8-	5' GAGTGGGCGGCCAGCtgcagtgagatgagt g 3'	sense	C16
PCO1393-f 3'PCOL136 8-	5' cactcatctccatgcaGCTGGCCGCCCACTC 3'	antisense	C16
PCO1393-r 3'PCOL143 7-	5' gtaccttcgaagcgccggtcacagttctgtcttaagcttt tcatacagggtcgtcctgatcgatcatgtcctcGTCCTGG TACCAGCGGTTATAAG 3'	antisense	C17, C18, C19
PCO1462- r_inf			

Table S2: Primers for post-PCR sequencing. List of sequencing primers that were used to verify successful DNA construction via Sanger sequencing.

Primer name	sequence	direction
pCR3.1-BGHrev	TAG AAG GCA CAG TCG AGG	reverse (3'→5')
EF1	TTTCGCCCTAACTTCGTGAT/ AAA ATT TCT CTT TCA GGT GTC GTG AAA CGT GGG TCG CGG CCGC	forward (5'→3')

Table S3: Overview of chimeric borders for C1 - C19.

Construct	Chimeric border or borders
C1	PCO E35 / PCOL K28
C2	PCO A113 / PCOL V106
C3	PCOL G27 / PCO K36
C4	PCOL E105 / PCO S114
C5	PCOL T286 / PCO G299

C6	PCO T298 / PCOL D287
C7	PCOL E390 / PCO G400
C8	PCO D399 / PCOL A391
C9	PCO T298 / PCOL D287; PCOL E390 / PCO G400
C10	PCOL E275 / PCO G286, PCO D399 / PCOL A391
C11	PCOL P320 / PCO P331
C12	PCOL T362 / PCO 373
C13	PCOL Y428 / PCO N439
C14	PCOL S454 / PCO C465
C15	PCOL D377 / PCO E488
C16	PCO P330 / PCOL P321
C17	PCO T372 / PCOL D363
C18	PCO T298 / PCOL D287; PCOL D377 / PCO E488
C19	PCO P330 / PCOL P321; PCOL D377 / PCO E488

Table S4: Non-conservative changes between PCOL and PCO in the critical region as identified through C19. In C19 157 amino acids of PCO were exchanged for 156 amino acids of PCOL. Out of these, 47 were clear non-conservative changes. To identify nonconservative changes amino acids were grouped by properties (polar, nonpolar, acidic, basic) and molecular weight of the side chain (small [less than 75 daltons], medium [between 75 and 155 daltons], large [greater than 155 daltons]).

PCOL AA (Position)	Properties AA in PCOL	PCO AA (Position)	Properties AA in PCO
G327	Nonpolar, Small	D337	Acidic, Medium
S328	Polar, Small	P338	Nonpolar, Medium
Y345	Polar, Large	L355	Nonpolar, Medium
F348	Nonpolar, Large	S358	Polar, Small
P349	Nonpolar, Medium	R359	Basic, Large
D350	Acidic, Medium	H360	Basic, Large
P351	Nonpolar, Medium	K361	Basic, Large
K352	Basic, Large	D362	Acidic, Medium
N358	Polar, Medium	A368	Nonpolar, Small
F364	Nonpolar, Large	D374	Acidic, Medium
P365	Nonpolar, Medium	S375	Polar, Small
S366	Polar, Small	D376	Acidic, Medium
L371	Nonpolar, Medium	S381	Polar, Small
V377	Nonpolar, Medium	S387	Polar, Small
I379	Nonpolar, Medium	S389	Polar, Small
S380	Polar, Small	H390	Basic, Large
A381	Nonpolar, Small	K391	Basic, Large
S382	Polar, Small	E392	Acidic, Large
R384	Basic, Large	P394	Nonpolar, Medium
R385	Basic, Large	P395	Nonpolar, Medium
K386	Basic, Large	P396	Nonpolar, Medium
Q387	Polar, Medium	A397	Nonpolar, Small
E390	Acidic, Large	G400	Nonpolar, Small
A392	Nonpolar, Small	H402	Basic, Large
Q397	Polar, Medium	F407	Nonpolar, Large
P399	Nonpolar, Medium	K409	Basic, Large
K400	Basic, Large	D410	Acidic, Medium
F403	Nonpolar, Large	T413	Polar, Medium
T405	Polar, Medium	E415	Acidic, Large
E406	Acidic, Large	Q416	Polar, Medium
K408	Basic, Large	S418	Polar, Small
T409	Polar, Medium	K419	Basic, Large
R412	Basic, Large	L422	Nonpolar, Medium
Y415	Polar, Large	D425	Acidic, Medium
T419	Polar, Medium	R429	Basic, Large
A420	Nonpolar, Small	K430	Basic, Large
E421	Acidic, Large	P431	Nonpolar, Medium
L427	Nonpolar, Medium	Y437	Polar, Large
G429	Nonpolar, Small	N439	Polar, Medium
S430	Polar, Small	P440	Nonpolar, Medium
R431	Basic, Large	P441	Nonpolar, Medium
R432	Basic, Large	Q442	Polar, Medium
T433	Polar, Medium	K443	Basic, Large
K436	Basic, Large	S446	Polar, Small
Q442	Polar, Medium	R452	Basic, Large
N472	Polar, Medium	H482	Basic, Large
Q476	Polar, Medium	G486	Nonpolar, Small

8.2 Supplemental Figures

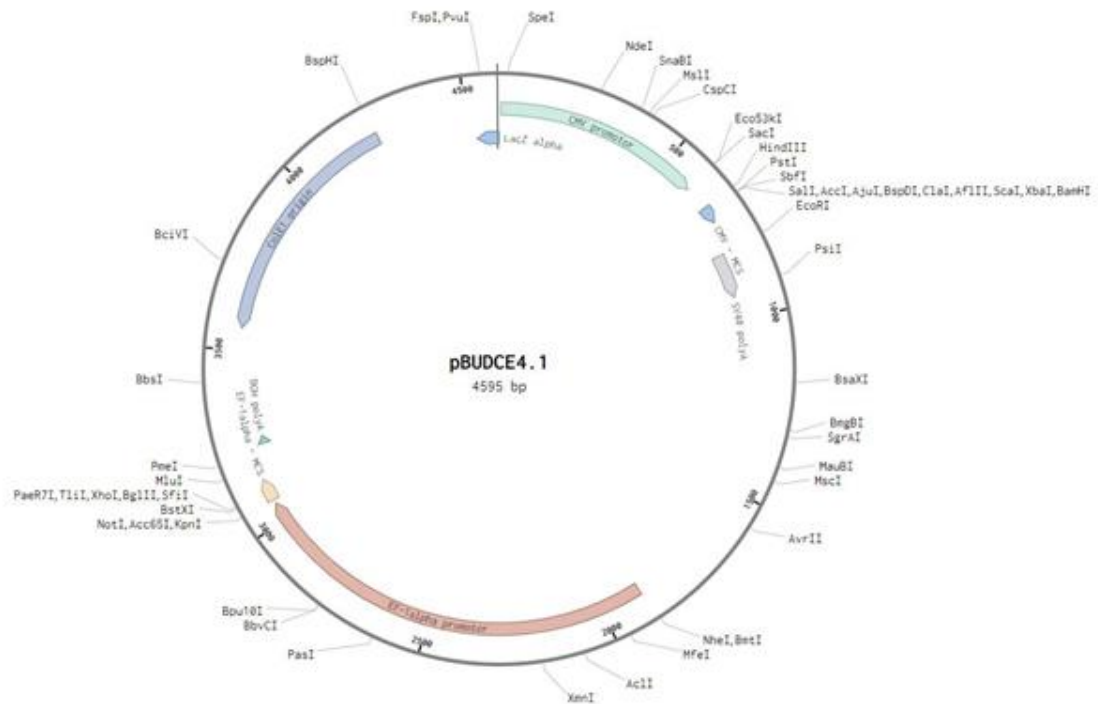


Figure S1: Vector map of the pBUDCE4.1 plasmid. The map was generated by Dr. Sven Kuspiel using Benchling with automatic annotation of standard vector features. Restriction sites for commonly used commercial restriction enzymes were manually added. pBUDCE4.1 is designed for the independent expression of two transgenes. It contains a CMV promoter upstream of the first multiple cloning site (MCS1), followed by a Zeocin resistance cassette under the control of the EM7 promoter. The second expression cassette consists of an EF1 α promoter driving the second multiple cloning site (MCS2), located downstream of an SV40 early promoter. The plasmid backbone includes a ColE1 origin of replication for propagation in *E. coli*.

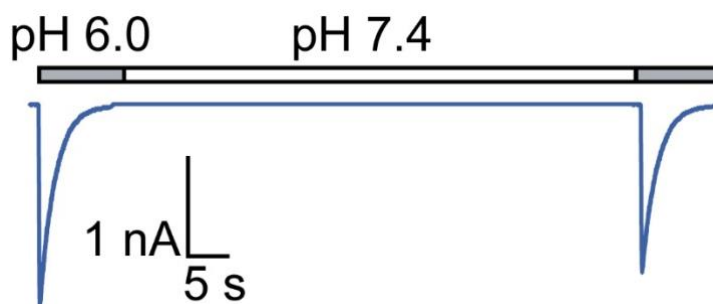
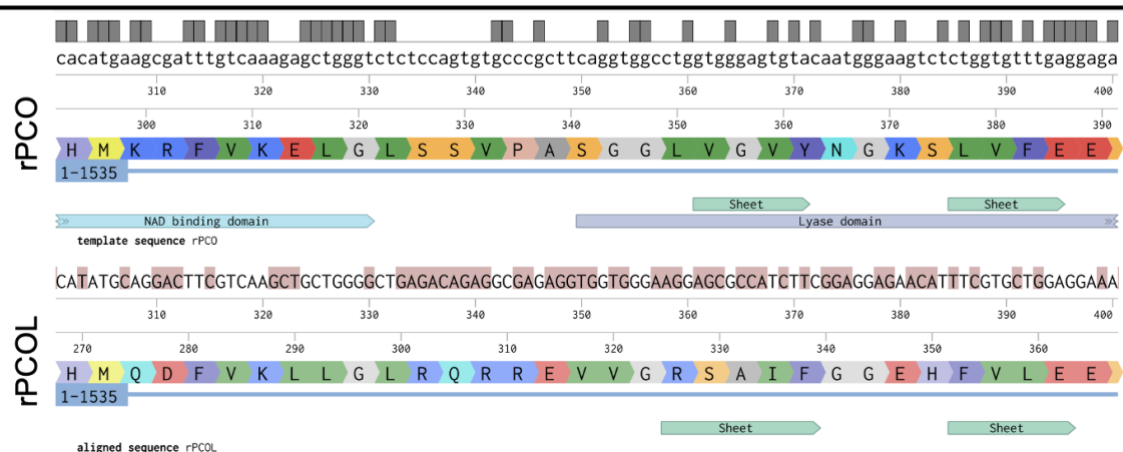
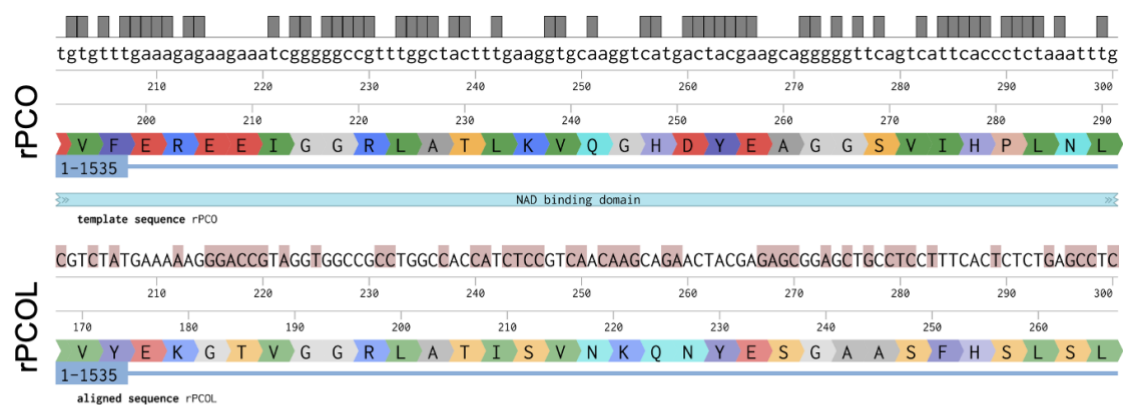
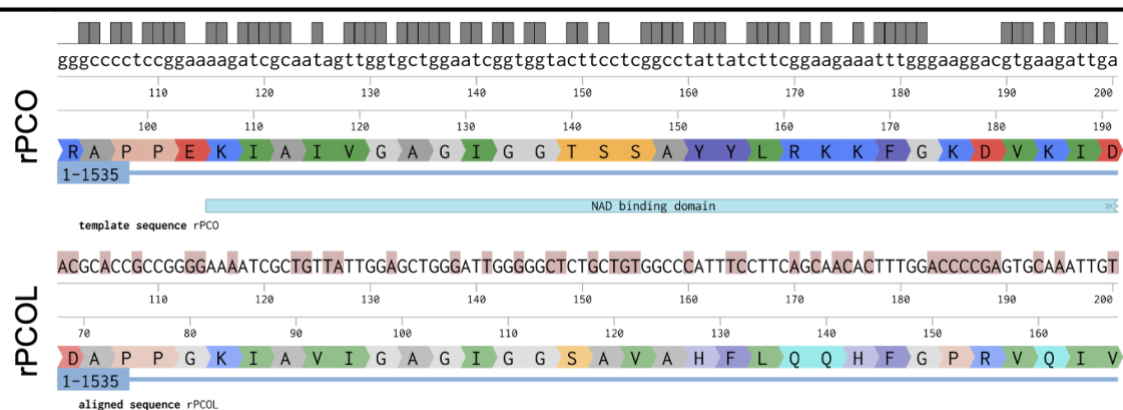
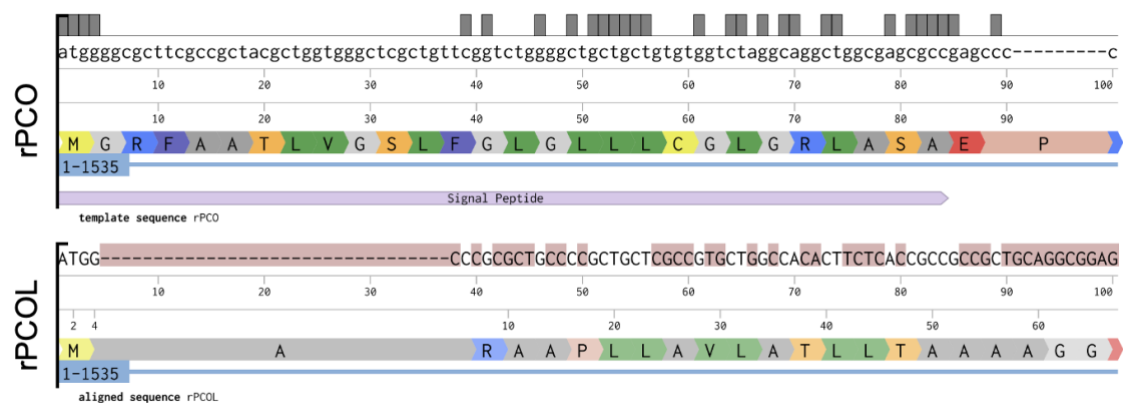
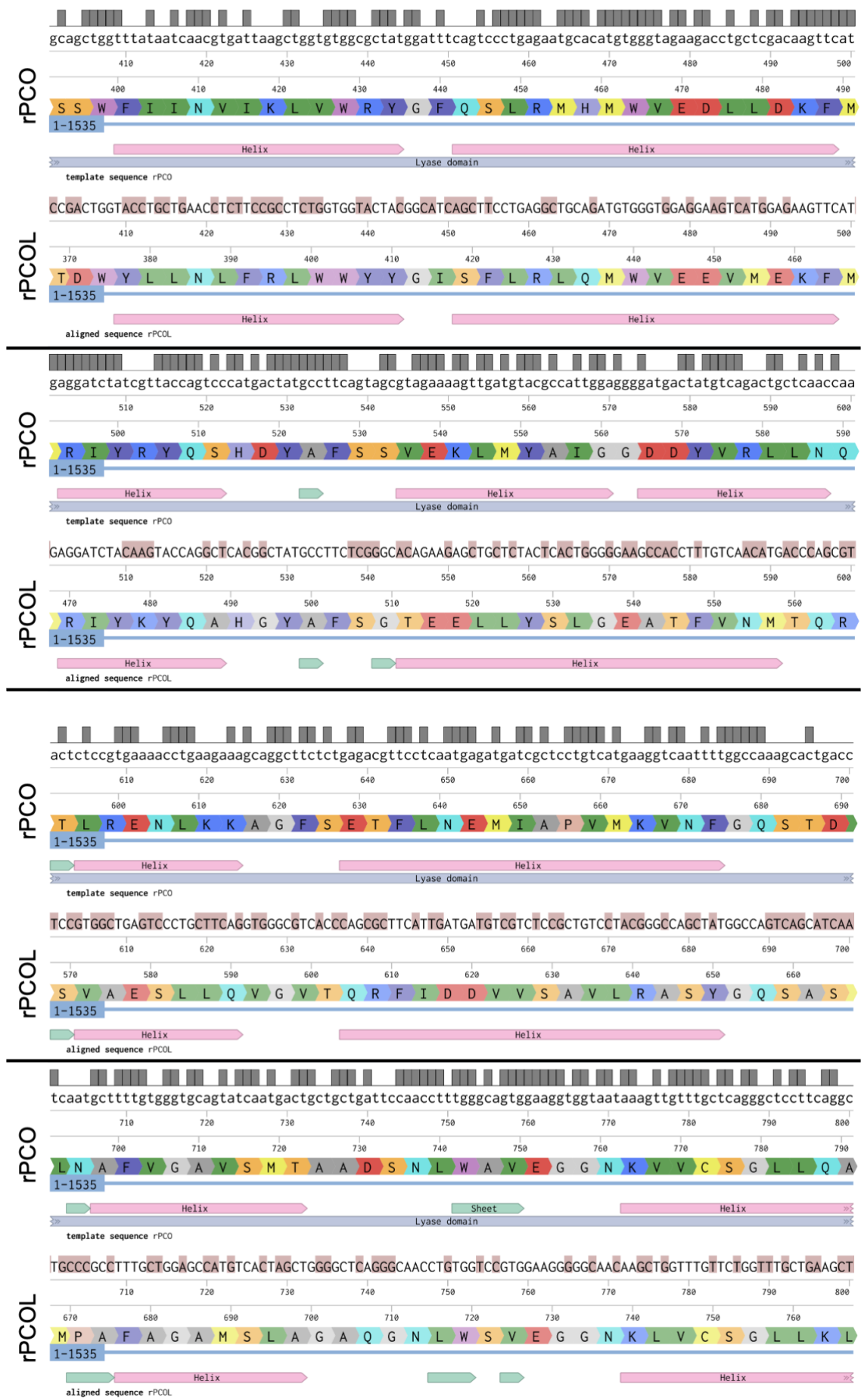
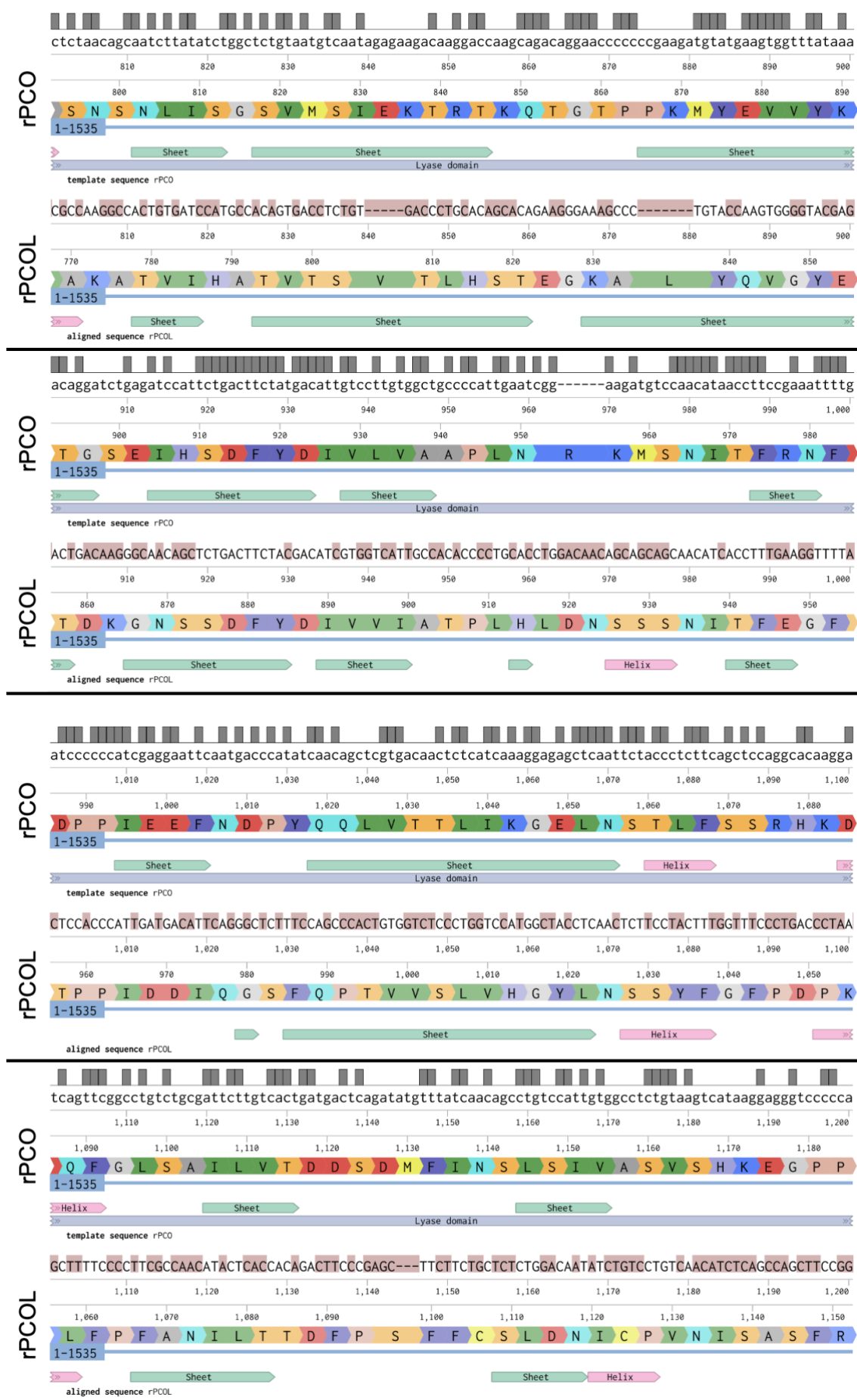


Figure S2: Protocol for stimulating cells. After 10 sec of perfusion of the standard bath solution ASICs were stimulated by rapidly decreasing the extracellular pH by perfusing with the stimulation solution for 10 sec. After another 60 sec of incubation at pH 7.4 the second stimulation was performed. As detailed in the methods section the second peak was considered for measurements.







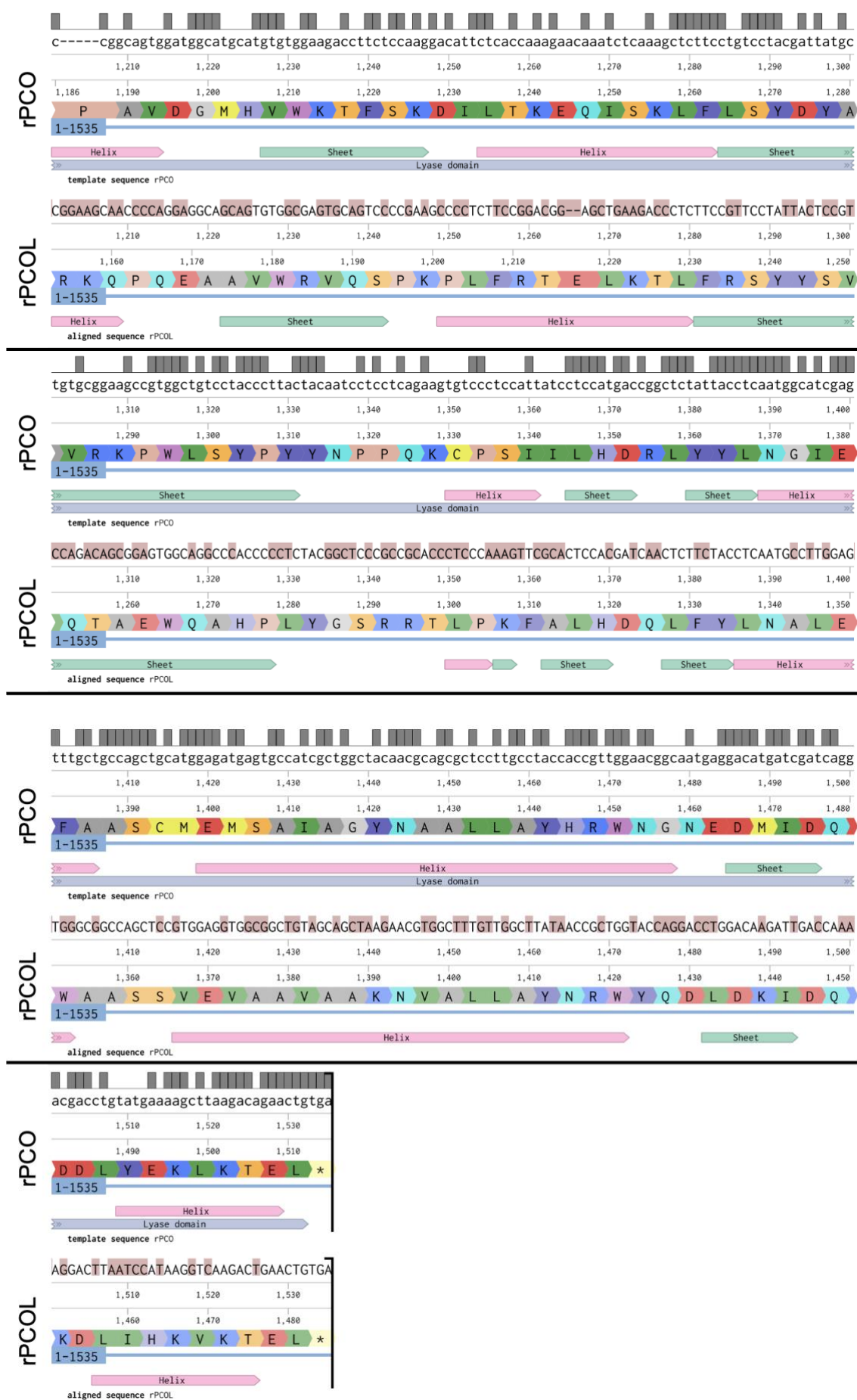


Figure S3: PCOL and PCO show a high level of sequential homology. Alignment of bases and amino acids of rPCOL and rPCO (both from *rattus norvegicus*). Alignment was performed in Benchling with MAFFT v7 (Kato & Standley, 2013). PSI – Blast search restricted to *rattus norvegicus* revealed PCO as protein with the highest level of sequence homology. Secondary structural elements, such as α -helices (pink) and β -sheet structures (green) are indicated as predicted by AlphaFold. Further, known functional domains of PCO are indicated below the amino acid sequence. These are a N-terminal signal peptide (~30 AA), a NAD(P) binding domain (~50 AA) and a large prenylcysteine oxidase/lyase domain (~300 AA) (Herrera-Marcos et al., 2018).

9 Publication list

“Assessing ChatGPT's ability to emulate human reviewers in scientific research: A descriptive and qualitative approach” Aiman Suleiman, [...] *Nikolai Ratajczak*, et al.; veröffentlicht in “Computer Methods and Programs in Biomedicine“, doi: 10.1016/j.cmpb.2024.108313

“Increased Postoperative Opioid Consumption in the Presence of Coadministration of 5-Hydroxytryptamine Type 3 Antagonists with Acetaminophen: A Hospital Registry Study” *Nikolai Ratajczak*, [...] Maximilian S Schäfer; veröffentlicht in “Anesthesiology“; doi: 10.1097/ALN.0000000000005033

“Acetaminophen 5-HT3 Antagonist Interaction: Reply” *Nikolai Ratajczak*, [...] Maximilian S Schaefer; veröffentlicht in “Anesthesiology“; doi: 10.1097/ALN.0000000000005350

“Intraoperative Opioid Waste and Association of Intraoperative Opioid Dose with Postoperative Adverse Outcomes: A Hospital Registry Study” Simone Redaelli, [...] *Nikolai Ratajczak*, et al.; veröffentlicht in “Pain and Therapy “; doi: 10.1007/s40122-023-00574-2

10 Acknowledgements

I would like to express my sincere thanks to PD Dr. Dominik Wiemuth and Prof. Dr. Stefan Gründer who introduced me to this interesting topic, gave me a warm welcome to the study group and actively supported when- and wherever necessary.

Moreover, I am especially grateful to my direct instructor Dr. Sven Kuspiel. His patience and specifically his constructive feedback were very motivating, helpful, and supportive.

I thank A. Oslender-Bujotzek for expert technical assistance in molecular biology.

I am also thankful for the bureaucratic support of Birgit Franzen and to all other lab members that were a great help in many situations and created a pleasant working environment.

11 Erklärung zur Datenaufbewahrung

Erklärung § 5 Abs. 1 zur Datenaufbewahrung

Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten im Institut für Physiologie des Universitätsklinikums Aachen bei meinem Betreuer, PD Dominik Wiemuth, hinterlegt sind.

12 Erklärung über den Eigenanteil

Eidesstattliche Erklärung gemäß § 5 Abs. (1) und § 11 Abs. (3) 12. der Promotionsordnung

Hiermit erkläre ich, Nikolai Lukasz Ratajczak, an Eides statt, dass ich folgende in der von mir selbstständig erstellten Dissertation „Modulatory Influence of PCOL on ASIC1a: Electrophysiological Investigation of Key Functional Domains“ dargestellten Ergebnisse erhoben habe. Bei der Durchführung der Arbeit hatte ich folgende Hilfestellungen, die in der Danksagung angegeben sind.

	Doktorand , Nikolai Lukasz Ratajczak	Prakt. Betreuer , Dr. Sven Kuspiel	Assistentin , Adrienne Oslender- Bujotzek	Doktorvater , PD Dominik Wiemuth	Large Languag e Model, ChatGPT	Summ e (%)
Studienüberwachung		50		50		100
Studiendesign/Konzeption	25	30		45		100
Datenauswertung	100					100
Durchführung der restlichen Experimente	85		15			100
Statistische Auswertung	80	10		10		100
Bereitstellung von Materialien				100		100
Interpretation der Datenauswertung	60	20		20		100
Durchführung der molekularbiologischen Laborarbeit	5		95			100
Durchführung der Patch- Clamp Messungen	95	5				100
Durchführung der Arbeit in der Zellkultur	95	5				100
Schreiben der Monographie	100					100
Korrektur der Monographie	88	5		5	2	100

Eidesstattliche Erklärung gemäß § 5 Abs. (1) und § 11 Abs. (3) 12. der Promotionsordnung

Unterschrift des Doktoranden, Nikolai Lukasz Ratajczak

Als Betreuer der obigen Dissertation bestätige ich die Angaben von Nikolai Lukasz Ratajczak

Unterschrift des Doktorvaters, Dr. rer. nat. PD Dominik Wiemuth

13 Curriculum Vitae

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AUSBILDUNG

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Dean's List Stipendiat (Top 3 % der Studierenden) mit interdisziplinärem Netzwerk und ideeller Förderung aufgrund herausragender Studienleistungen (Staatsexamina: M1 „sehr gut“, M2 „gut“, M3 „sehr gut“). Research Ambassador Program: Stipendium für einen Forschungsaufenthalt an der Harvard Medical School.

Praktisches Jahr

Universitätsmedizin Göttingen, Innere Medizin

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University of Cape Town, Victoria Hospital Wynberg, Chirurgie Kapstadt, ZA (05/24-09/24)

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Hôpital Sainte Musse, Notaufnahme und Kardiologie

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Aachen, GER (08/19)

Bischöfliche Maria-Montessori-Gesamtschule

Krefeld, GER

Abitur, Abschlussnote 1,0 mit ministerpräsidentialer Bestenehrung

09/17

BERUFSERFAHRUNG

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Pflegehilfskraft

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Pflegerische Tätigkeiten, Anamnese- und Befunderhebung, Einblicke in Schockraum-Management.

Luisenhospital Aachen, Notaufnahme und Kassenärztlicher Notdienst Aachen

Aachen, GER

Studentische Hilfskraft

01/19-01/21

Administrative Tätigkeiten, Anamnese- und Befunderhebung, Triagieren, Mitversorgung, Assistenz bei Notfall-Gastro- und Koloskopien außerhalb der Dienstzeiten.

RWTH Uniklinikum Aachen, Lehre

Aachen, GER

Tutor und Mentor

10/18-09/24

Durchführung von Lehrveranstaltungen in den Instituten für Chemie, Anatomie und Physiologie, mit besonderem Fokus auf das Skills Lab der Klinik für Anästhesie und Intensivmedizin. Entwicklung und Konzeption neuer Kurse und Leitung des studentischen Teams insbesondere im Bereich Sonografie.

FORSCHUNG

Harvard Medical School, Beth Israel Deaconess Medical Center Boston, USA
Austausch- und Forschungsstudent am Center for Anesthesia Research Excellence 10/22-11/23

Retrospektive Datenanalyse zu perioperativen Komplikationen unter Anwendung statistischer Modelle mittels STATA und R. Finanzielle Förderung durch ein Research Student Stipendium des BIDMC.

RWTH Uniklinikum Aachen, Institut für Physiologie Aachen, GER
Promotionsstudent 09/21-heute

Untersuchung des Interaktionspartners eines Ionenkanals durch in vitro Expression chimärer Proteine und elektrophysiologische Charakterisierung mittels Patch-Clamp Technik.

AUSZEICHNUNGEN, STIPENDIEN

be.boosted e.V. Berlin, GER
Fellow 10/23-04/24

Sechsmonatiges Leadership-Programm in Rhetorik, Verhandlung und Gruppendynamik, mit Stipendium für Reisekosten.

Studienstiftung des deutschen Volkes GER
Stipendiat 11/19-heute

Leistungsabhängige ideelle und finanzielle Förderung. Teilnahme an zahlreichen Seminaren, Akademien, Sprachkursen (Französisch, Arabisch) und einem Lebenswissenschaftlichen Kolleg, sowie Förderung mehrerer Auslandsaufenthalte.

Deutsche Gesellschaft für innere Medizin Wiesbaden, GER
Reisestipendium zur Kongressteilnahme 04/22 & 04/24

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Förderung nicht vereinbar mit Förderung durch die Studienstiftung. 11/19

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Student Network for Open Science Berlin, GER
Co-Organisator, Teamleitung 06/24-heute
Mitorganisation des „reprodICUbility“ summer school und Hackathons. Team Lead für 6 Teilnehmende.

be.boosted e.V. Berlin, GER
Trainer, Senior Trainer 05/24-heute

Konzipierung und Durchführung eines Führungstrainings für BerufseinsteigerInnen mit unterschiedlichen Hintergründen. Inhaltserstellung und -vermittlung, Kooperation mit Partnern und Logistikorganisation.

German American Conference Boston, USA
Co-Organisator, Team Digitale Logistik 04/23-11/23

Co-Organisator der größten studentischen transatlantischen Konferenz. Erstellen und Pflegen der Konferenz-Website im Team für digitale Logistik, Organisation eines Workshops zu „Design-Thinking-Strategy“.

Taiwan Do Akademie Krefeld, GER
Ehrenamtlicher Kursusleiter für Kung-Fu Kinder- und Erwachsenentraining 2012-2017
Leitung von Kursen nach zweijähriger Ausbildung mit Inhalten zu Soziologie, Kommunikation und Sportgesundheit.