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Structure and molecular mechanism of TRPC5 receptor activation

Struktura a molekulární mechanizmy aktivace TRPC5 receptoru

Bachelor's thesis
Supervisor: Mgr. Lucie Zímová, Ph.D.

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Poďakovanie patrí mojej školiteľke Mgr. Lucie Zímovej, Ph.D., najmä za cenné rady a nekonečnú trpezlivosť.

Prehlásenie

Prehlasujem, že som záverečnú prácu spracoval samostatne pod vedením školitelky Mgr. Lucie Zímovej, Ph.D a že som uviedol všetky použité informačné zdroje a literatúru. Táto práca ani jej podstatná časť nebola predložená k získaniu iného alebo rovnakého akademického titulu.

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Abstract

The Transient Receptor Potential Canonical 5 (TRPC5) receptor is a non-selective calcium permeable ion channel that functions as a polymodal cellular sensor. TRPC5 is highly expressed in the kidney and the nervous system, localized at the plasma membrane, where it contributes to calcium homeostasis. Abnormal expression and/or function of TRPC5 is known to contribute to conditions like depression, anxiety and progressive kidney disease. Several studies demonstrated the involvement of TRPC5 in the noxious cold detection and in the development of cold allodynia in neuropathic diseases. Our understanding of the activation mechanisms of this receptor has significantly grown in recent years due to the discovery of selective agonists and specific inhibitors and also due to success in revealing the three-dimensional structure of the TRPC5 channel. This thesis summarizes the contemporary knowledge of TRPC5 function, structure and activation mechanisms and points to the physiological importance of this channel.

Key words: ion channels, protein structures, calcium ions, mechanisms of activation, TRPC5

Abstrakt

Iónový kanál Transient Receptor Potential Canonical 5 (TRPC5) je polymodálnym bunkovým senzorom priepustným pre vápenaté ióny. Je vo vysokej miere exprimovaný na plazmatickej membráne nervových a obličkových buniek, kde prispieva ku vápnikovej homeostáze. Abnormálna expresia a funkcia TRPC5 kanálu prispieva ku poruchám ako depresia, úzkostná porucha a progresívne ochorenie obličiek. Existujú štúdie dokazujúce účasť TRPC5 v detekcii bolestivého chladu a v rozvoji chladovej alodýnie pri neuropatických ochoreniach. Naše porozumenie aktivačným mechanizmom tohoto receptoru sa značne zvýšilo na základe objavení selektívnych agonistov a špecifických inhibítorov, tak isto ako na úspešnom odhalení trojrozmernej štruktúry kanálu TRPC5. Táto práca sumarizuje aktuálne poznatky ohľadom funkcie, štruktúre a aktivačných mechanizmov TRPC5 a ukazuje na fyziologickú dôležitosť tohoto kanálu.

Kľúčové slová: iónové kanály, proteínové štruktúry, vápenaté ionty, mechanizmy aktivácie, TRPC5

Contents

List of Abbreviations

ARD – Ankyrin repeat domain

CaBP1 – Ca^{2+}-binding protein 1

CaM – Calmodulin

CBII – Second calmodulin-binding site

CFA – Complete Freund's Adjuvant

CIRB – Calmodulin and IP_3 Receptor Binding

CMZ – Clemizole

Cryo-EM – Cryogenic electron microscopy

DAG – 1,2-diacylglycerol

DTT – Dithiothreitol

GPCR – G protein-coupled receptor

GSH – Glutathione

GSSG – Oxidized glutathione

HLH – Helix-loop-helix region

hTRPC5 – Human TRPC5 channel

I-V – Current-voltage relationship

IP_3 – Inositol triphosphate

LFW motif – L575, F576, W577 amino acids on TRPC5 subunit

mTRPC5 – Mouse TRPC5 channel

NCS-1 – Neuronal Ca^{2+} sensor 1

NHERF – Na^+/H^+ exchanger regulatory factor

PCR – Polymerase Chain Reaction

PDB – Protein Data Bank

PIP_2 – Phosphatidylinositol-4,5-bisphosphate

PKC – Protein kinase C

PLC – Phospholipase C

TMD – Transmembrane domain

TNF-α – Tumor Necrosis Factor alpha

TRPA – Transient Receptor Potential Ankyrin channel

TRPC – Transient Receptor Potential Canonical channel

TRPM – Transient Receptor Potential Melastatin channel

TRPML – Transient Receptor Potential Mucolipin channel

TRPP – Transient Receptor Potential Polycystin channel

TRPV – Transient Receptor Potential Vanilloid channel

VSLD – Voltage sensing-like domain

WT – Wild-type

1. Introduction

The superfamily of Transient Receptor Potential (TRP) channels is an extensive group of transmembrane proteins. TRP channels are known for their importance in nociception and for their function in transporting cations across the cell membrane. More precisely, 28 TRP mammalian channels have already been characterized and they are distributed into six subfamilies according to sequence homology: TRPA (ankyrin), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPC (canonical) channels (Venkatachalam & Montell, 2007). With rare exceptions, TRP channels are Ca^{2+}-permeable non-selective cation channels found predominantly on the plasma membrane of almost every cell type (Nilius, 2007). They consist of six-segment transmembrane polypeptide subunits that are symmetrically arranged into the homo/hetero-tetrameric structure that forms a central pore and therefore makes a functional ion channel (Cao, 2020).

The uniqueness of TRP channels resides in their polymodality. A polymodal receptor can be activated by different gating stimuli and can act as a molecular integrator of these signals (Nilius, 2007). For example, a TRP receptor can be activated by temperature in a specific range, mechanically, and/or by a chemical compound. Signals produced by TRP receptor activity on a peripheral ending of a nociceptive neuron are transmitted through the spinal cord dorsal horn to the brain, creating the sensation of pain or acute discomfort (Julius, 2013).

One of the most studied TRP channel members are those that enable transduction of thermal stimuli in primary nociceptive neurons. They are known as ThermoTRP and belong to four different TRP subfamilies: TRPV, TRPM, TRPA and TRPC (Garcia-Avila & Islas, 2019). A thermal activation and specific chemical activations of several TRP receptors also play great role in flavours and taste (Fig. 1) (Ishimaru & Matsunami, 2009; Julius, 2013).

This thesis aims at summarizing recent advancements in understanding of TRPC5 channel structure and regulation. The first TRPC5 structure became available in 2019 (Duan et al., 2019). Several other high-resolution structures in different conformations followed last and this year (Song et al., 2021; Wright et al., 2020). They capture TRPC5 in complexes with inhibitors and lipids and enable previously impossible interpretations of observations from accumulated functional studies. Furthermore, for a long time, the researchers in the TRPC field lacked a specific agonist and the TRPC5 currents were predominately elicited indirectly through an unknown mechanism, by activating G-proteins and their downstream signal transduction pathways. At last in 2015, the TRPC4 and TRPC5 channels were identified as

cellular targets of the natural compound Englerin A that demonstrated unique ability to selectively and potently inhibit renal cancer cell growth by elevating Ca^{2+} influx and Ca^{2+} cell overload (Akbulut et al., 2015). Moreover, in the few last years, evidence has arisen about the significance of TRPC5 channel in cellular and organismal context and about TRPC5 involvement in pathophysiology of arthritis or Huntington's disease (Alawi et al., 2017; Hong et al., 2015b).

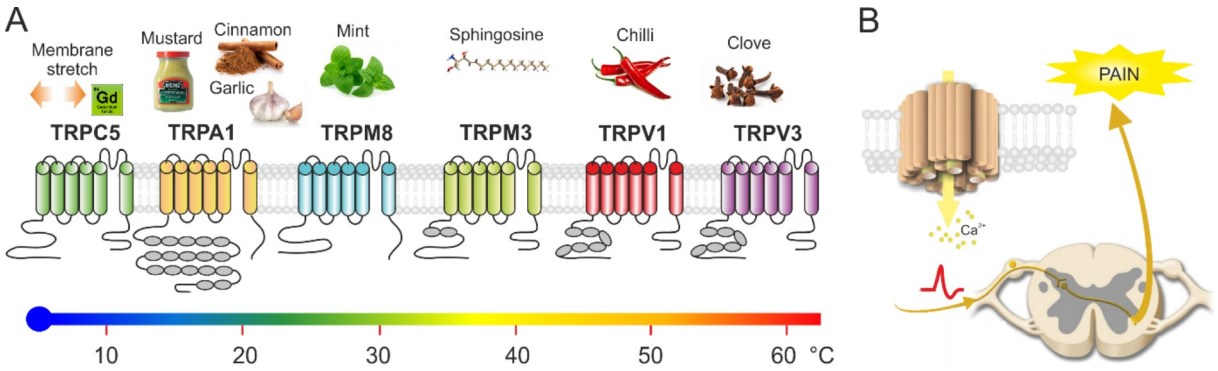

Figure 1: Activation of thermosensitive TRP channels by temperature in primary afferent sensory neurons. (A) Schematic representation of ThermoTRP range of activation temperatures with indicated chemical agonists. (B) TRPA1 activation on peripheral nerve endings resulting in pain signaling. (Adapted from Barvikova 2018).

2. TRPC Channels

Mammalian canonical transient receptor potential (TRPC) channels are non-selective Ca^{2+} permeable cation channels (Wang et al., 2020). Members of the TRPC subfamily are particularly involved in neuronal development, kidney diseases and vasorelaxation (Hall et al., 2020; Kochukov et al., 2013; Phelan et al., 2013; Riccio et al., 2009). To date, seven proteins from the TRPC family have been characterized based on their sequence homology with prototypical Drosophila TRP. Both Drosophila and mammalian TRPC channels share an ability to be activated downstream of receptors that signal through phospholipase C (PLC) (Trebak et al. 2003). Mammalian TRPC channels are widely expressed in numerous cell types of many different tissues, displaying a wide diversity in expression patterns and functions (Wang et al., 2020). Based on their ability to be activated by multiple factors, it is believed that they are used as integrators of physiological and environmental stimuli (Clapham, 2003). TRPC proteins are divided into the two subgroups, TRPC1/4/5 and TRPC3/6/7, based on their amino acid sequences and functional similarities. The TRPC1 does not form a homomeric functional channel like the rest of TRPCs do. Instead, the TRPC1 acts as a regulatory subunit in heteromeric channels containing TRPC4 or TRPC5 (Strubing et al., 2001) or in some other TRP subtypes (Sours-Brothers et al., 2009; Strubing et al., 2001). Members of the TRPC3/6/7 subgroup share 75% identity and are likely to assemble as hetero-tetrameric channels (Hong et al., 2015a). The TRPC2 is a pseudogene in primates (Vannier et al., 1999).

Potentiation of TRPC channels is caused by a G protein-coupled receptor or tyrosine kinase receptor-mediated activation of a phospholipase C (PLC) that cleaves plasma membrane associated with phosphatidylinositol-4,5-bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG), inositol triphosphate (IP_3), and to an oxonium ion (Clapham, 2003; Schaefer et al., 2000).

While it has been known for 20 years that TRPC3/6/7 can be directly activated by the second messenger DAG (Hofmann et al., 1999; Okada et al., 1999), the TRPC4/5 were considered to be DAG insensitive. Only recently has it been shown that the TRPC4/5 DAG sensitivity is extensively regulated by interaction with the adapter proteins Na^+/H^+ exchanger regulatory factors (NHERF) (Storch et al., 2017).

The TRPC4 and 5 are closely related and they share a 65% sequence identity (Clapham et al., 2001). The most characterized similarity is a PDZ binding motif of the VTTRL amino acid sequence at the end of the C-terminus that is missing in the TRPC3/6/7 (Tang et al., 2000). The PDZ domain enables an interaction with NHERF 1 and NHERF 2 which act as a crosslink

with the actin cytoskeleton (Lee-Kwon et al., 2005; Obukhov, Nowycky, 2004). PIP_2 depletion causes C-terminal conformational change in TRPC4/5 and a dissociation of the NHERF proteins. This dissociation enables a conformation that is accessible for the cleavage product DAG, and therefore creates a DAG sensitive TRPC4/5 channel (Mederos & Schnitzler et al., 2018).

Selective agonists of TRPC4/5 such as Englerin A (Akbulut et al., 2015), antagonists Pico 145 (Rubaiy et al., 2017) and ML204 (Miller et al., 2011) are considered to be important pharmacological tools. The second important tool lies in revealing the atomic structure of these proteins. The recent mouse TRPC5 (Duan et al., 2019) and human TRPC5 (Wright et al., 2020) structures, obtained by cryogenic electron microscopy (Cryo-EM), provide a molecular scaffold to understand the channel function. Furthermore, another study by Song et al. (2021) capturing human TRPC5 in complexes with two structurally distinct inhibitors, the xanthine HC-070 and clemizole, was published this year.

3. Structure of TRPC5 Ion Channel

TRPC5 channels share a general transmembrane topology with voltage-gated calcium, potassium, and sodium channels. Similarly, TRPC5 is tetrameric and each subunit contains six transmembrane helices arranged in domain swapped fashion (Fig. 2) (Duan et al., 2019).

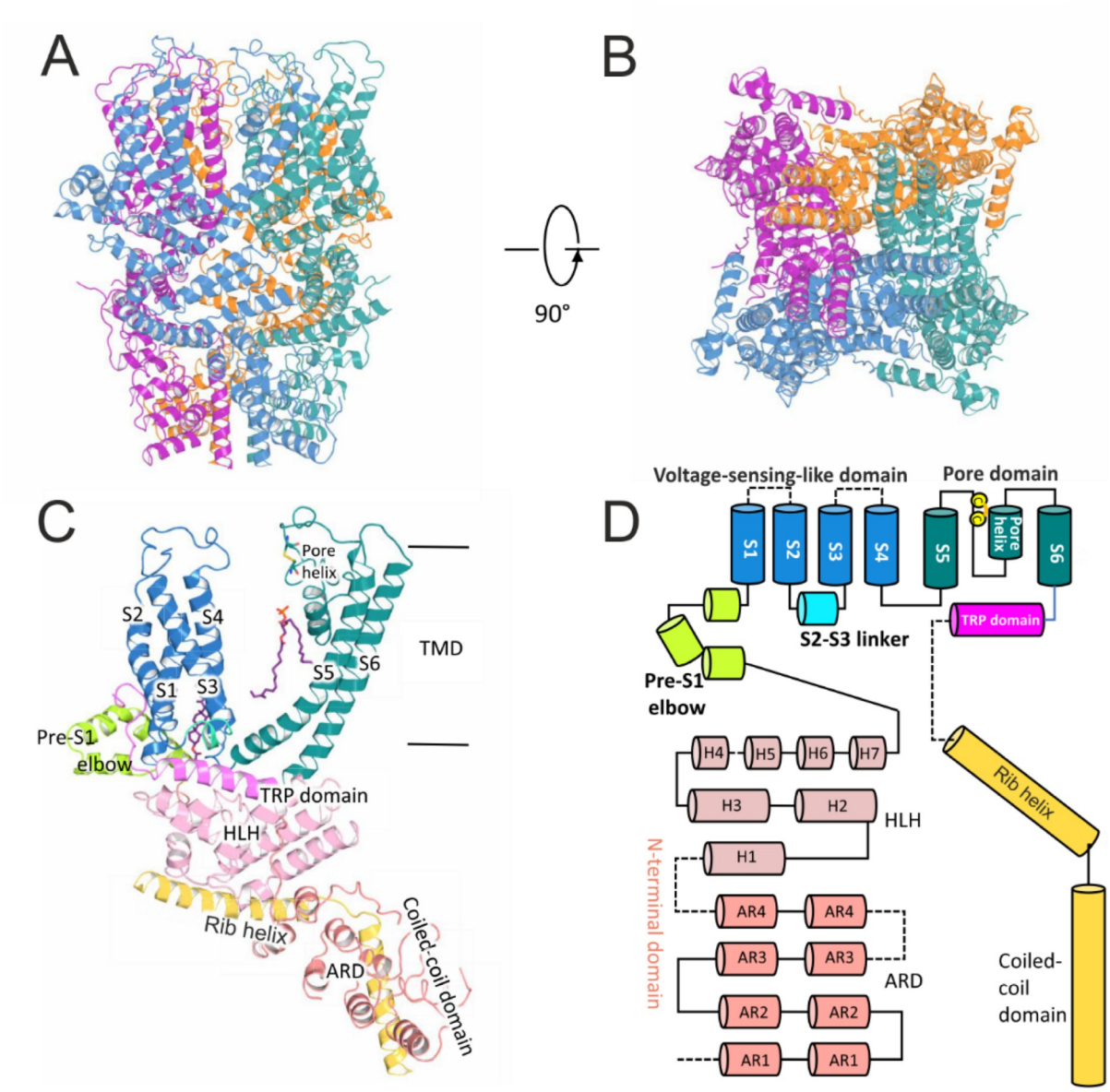

Figure 2: Structure of mouse TRPC5. (A) Ribbon model of mouse TRPC5 (mTRPC5) with each monomer displayed by a different color (Protein Databank, PDB, ID: 6AEI). (B) Top view of a ribbon model of mTRPC5. (C) Ribbon diagram of a single subunit of mTRPC5 depicting structural details. TMD – transmembrane domain, HLH – helix-loop-helix region, ARD – ankyrin repeat domain. (D) Linear diagram of mTRPC5 subunit is color-coded, same as the ribbon diagram in (C). (Adapted from Duan et al. 2019).

Moreover, TRPC5 has a long N-terminal domain that folds into four ankyrin repeats, an a HLH (helix-loop-helix) region of seven α helices and a pre-S1-elbow domain. The first four transmembrane helices (S1-S4) are folded into a voltage sensing-like domain (VSLD) and the two subsequent helices (S5 and S6) form the pore domain. The pore domain includes a re-entrant pore loop, which lines the ion permeation pathway. The transmembrane part is followed by a C-terminus containing the TRP domain, a rib (connecting) helix and the helix contributing to a coiled-coil domain. The rest of the C-terminal structure (approximately 210 residues) has not yet been resolved by any structural study available (Song et al., 2021; Wright et al., 2020).

Similar to other TRP channels, TRPC5 has a negatively charged extracellular opening that attracts positively charged ions, a selectivity filter with the narrowest part defined by a glycine at position 581 that constricts the upper gate of the pore (Fig. 3A). Lumen of the pore below the selectivity filter is mainly hydrophobic and leads to the lower gate formed by conservated residues I621, N625 and Q629 (Duan et al., 2019, Song et al., 2021).

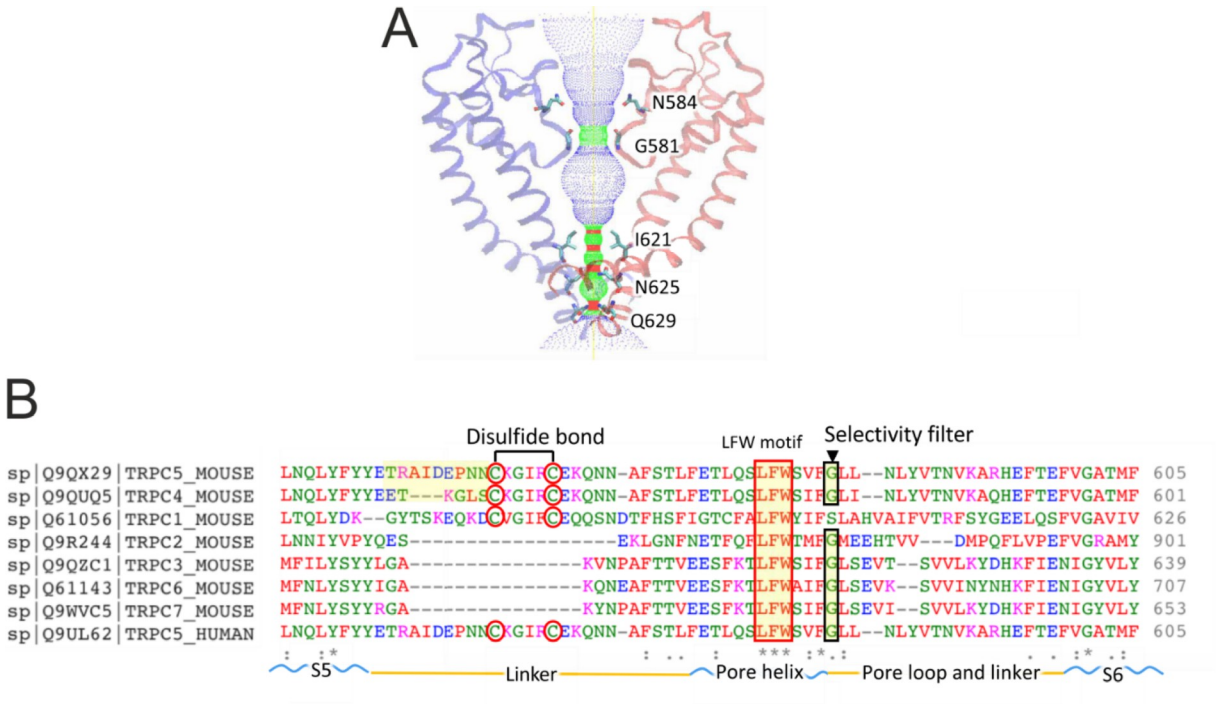

Figure 3: TRPC5 ion conduction pathway. (A) Side view of the mTRPC5 channel pore region in a non-conductive conformation with key amino acids labeled. (B) Sequences of mouse and human TRPC5 channels aligned to other TRPC channels (Clustal Omega) capturing the section between S5 and S6 helixes. The two cysteines forming disulfide bonds, a conserved LFW motif, and the selectivity filter are labeled. (Adapted from Duan et al. 2019)

A less conserved feature among the TRPC channels are the extracellularly exposed C553 and C558 that stabilize complicated structures formed by long pore loops (Hong et al., 2015a). These residues form a disulfide bond that is characteristic for TRPC1,4 and 5 (Fig. 2D and Fig. 3B). The disulfide bond seems to be important for gating as the reducing agent dithiothreitol (DTT) activates a wild-type TRPC5 channel. Mutations of a single or both cysteines to alanine lead to an inactive channel. (Duan et al., 2019)

The key part of the pore domain is a region called LFW motif (L575, F576, W577). This region, which is highly conserved among TRPCs, stabilizes the pore by hydrophobic interactions between adjacent subunits (Fig. 3B). LFW mutated to AAA results in a correctly folded channel that is not functional (Strübing et al., 2003).

3.1 TRPC5 structure compared to other TRP channels

The transmembrane domain is the most evolutionarily conserved region of TRP channels. Indeed, the comparison of TRPC5 transmembrane domain to the transmembrane domains of previously solved TRPC3 (Tang et al., 2018), TRPC4 (Duan et al., 2018a) and TRPC6 (Tang et al., 2018) channels structures demonstrates a relatively high spatial conservation. The overlay of the TRPC channels reveals a great similarity within the VSLD except the S3 helix. Interestingly, the S3 of the TRPC3 and TRPC6 channels are four helical turns longer than S3 of the TRPC4 and TRPC5 channels, indicating the ability of extracellular interactions. Unlike the TRPC3 and TRPC6 channels, the TRPC4 and TRPC5 channels possess long pore loops with a pair of cysteines (C553 and C558) that confer to redox sensitivity. An analogous extracellular disulfide bond was also described for TRPM4 (Duan et al., 2018c) or TRPM7 (Duan et al., 2018b) though without closer description of their impact on channels activity.

Several intracellular features are preserved in all TRPC channels. At N-terminus, there are 4 ARD and pre-S1 elbow. At C-terminus, there is TRP helix and rib helix, both running parallel to the membrane bilayer and vertical coiled-coil domain helix. Distinct to the TRPM, TRPV or TRPA channels whose TRP helix and the S6 show continuous alpha helical structure (Liao et al., 2013; Paulsen et al., 2015; Winkler et al., 2017), the TRP helix of TRPC5 (and other resolved TRPCs) is disengaged from the S6 (Duan et al., 2018a, 2019; Tang et al., 2018). The TRP helix of TRPC5 channel forms almost right angle to the S6. This conformation allows the TRP helix to approach the S4-S5 linker and a tight coupling. Moreover, the C-terminus of the TRP helix is in close contact with membrane lipids indicating possible involvement of TRP

helix–lipid interaction in TRPC5 channel regulation (Duan et al. 2019, Wright et al., 2020, Song et al. 2021).

3.2 Lipid/Pico145 Binding Site

On the TRPC5 channel structure by Wright et al. (2020), the LFW motif participates in binding the antagonist Pico145. In apo structures of TRPC4/5 channels (Duan et al., 2019), there is an apparent density corresponding to a lipid at the site of contact between monomers (Fig. 4A). Interestingly, in the presence of Pico145, the density at the mentioned site was consistent with the shape and size of Pico145, and modelling Pico145 into this density gave a perfect fit (Fig. 4B, C). The authors propose a mechanism of Pico145 action where the antagonist binds to a conserved lipid binding site between TRPC5 subunits, displaces bound phospholipids and affects pore helices (Wright et al., 2020).

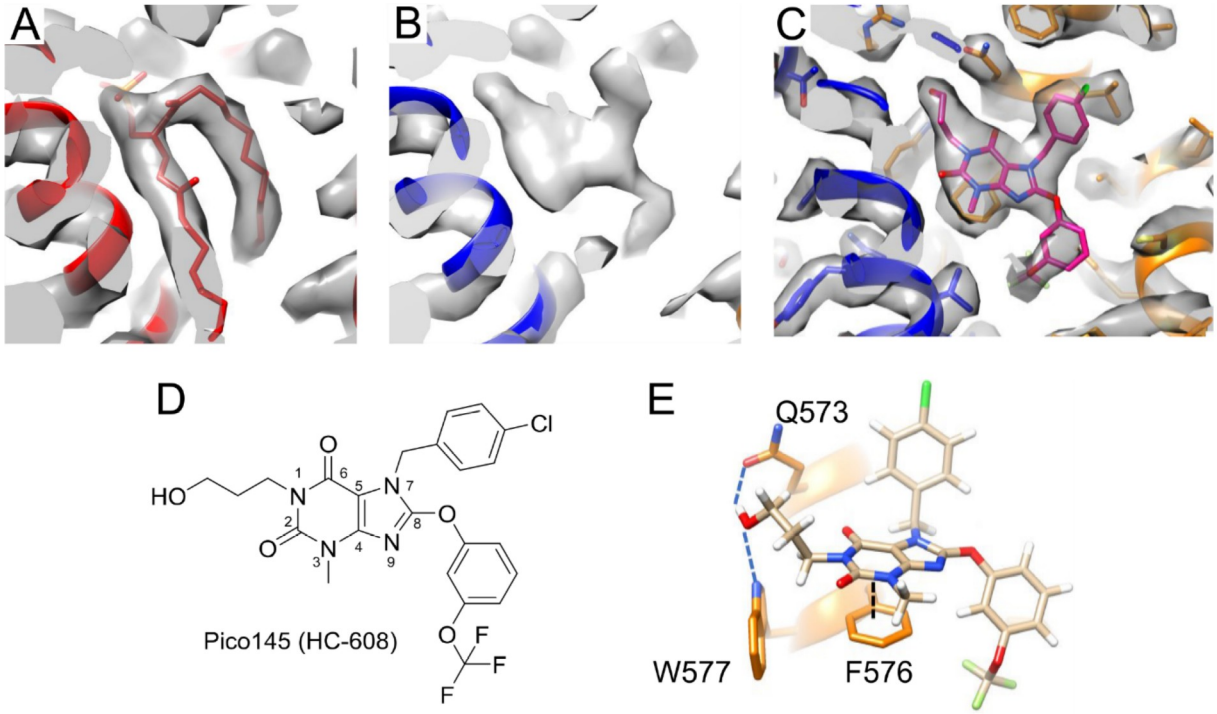

Figure 4: Lipid/Pico145 binding site of TRPC5 (A) Density observed in apo structure of mTRPC5 (PDB 6AEI) that was modelled as a phospholipid. (B) Density at the same site in the presence of Pico145 (PDB 6YSN) (C) Pico145 molecule (magenta) fitted into density from (B). The binding site spans two monomers (shown in orange and blue). (D) Molecule of Pico145. (E) Pico145 hydrogen bonds to Q573 and W577 (dashed blue lines) and π-stacks with F576 (dashed black line). (Adapted from Wright et al. 2020)

Also, Wright et al. (2020) showed that mutations of F576 and W577 in the LFW motif result in lower sensitivity to agonist Englerin A. This suggests that this lipid/Pico145 binding site may also be a site of interaction with Englerin A. However, a functional study that preceded the first resolved TRPC5 structure identified residues responsible for Englerin A interaction to be K554, H594, and E598, located approximately 10 Å apart from the LWF motif (Jeong et al., 2019).

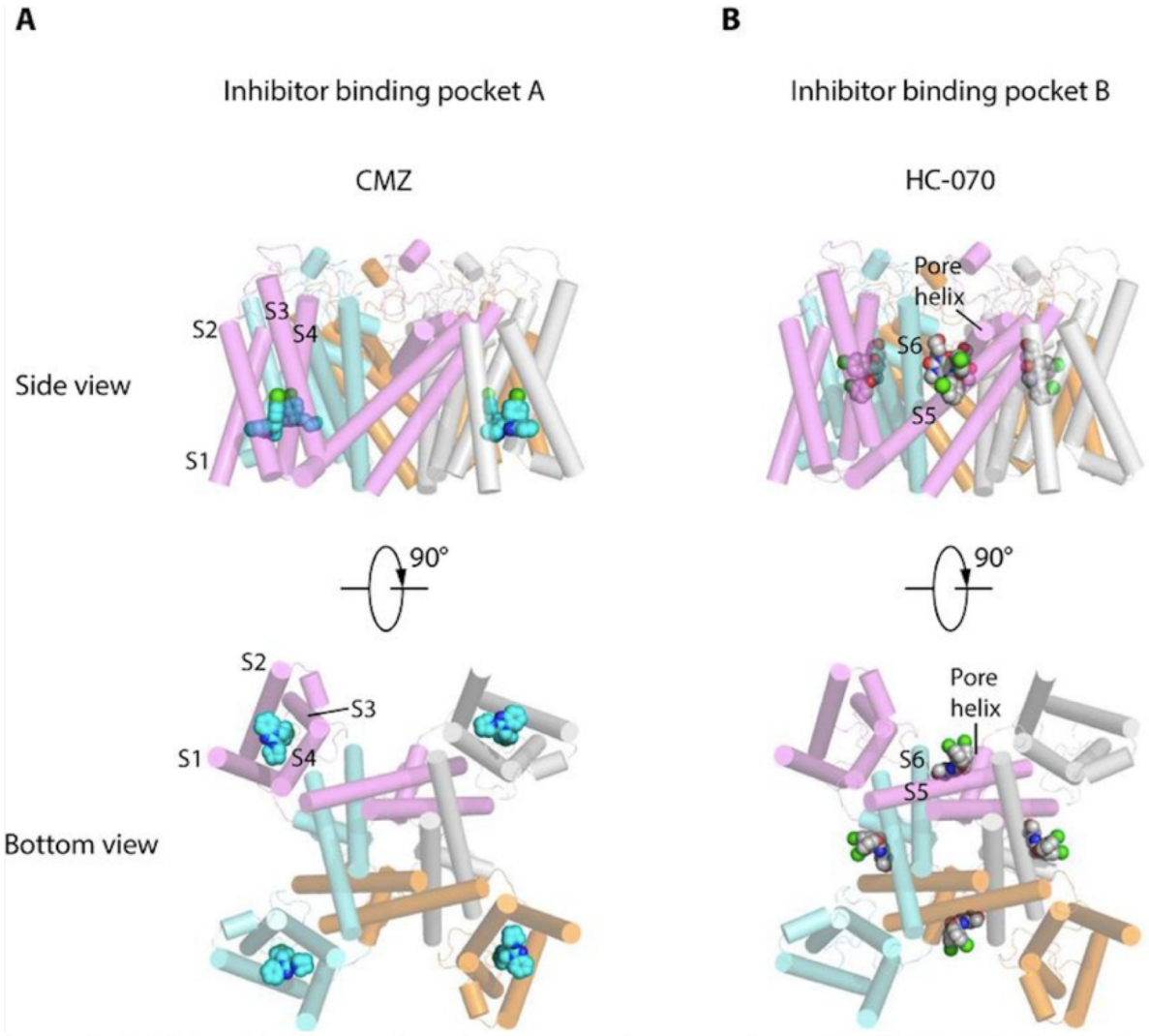

Figure 5: Inhibitor-binding pockets in transmembrane region of TRPC5. (A) Clemizole-binding pocket inside VSLD is shown in side view and bottom view. (B) Lipid/Pico145 binding site with bound HC-070. (Adapted from Song et al. 2021)

This year, Song et al. reported that lipid/Pico145 binding site also accommodates the molecule of HC-070 (Fig. 5B), which is another potent TRPC5 inhibitor structurally similar to Pico145 (Song et al., 2021). Moreover, on their second cryo-EM map of hTRPC5 in a complex with other inhibitor clemizole (CMZ), they observed at the lipid/Pico145 site a density highly resembling DAG. Authors showed that purified protein sample contained significantly higher

level of DAG than the purification buffer control and concluded that their results identified a putative DAG binding site (Fig. 6) (Song et al., 2021).

3.3 Putative Cholesterol-Binding Site

Apart from the lipid/Pico145 binding site formed by the LFW motif, Duan et al. (2019) and Song et al. (2021) described lipid binding sites sandwiched by S1, S4 from one subunit and S5 from an adjacent subunit (Fig. 6). The cryo-EM densities at these sites corresponded to cholesterol-mimicking molecules (cholesteryl hemisuccinate) used for cryo-EM sample preparation. Previously similar densities of the putative cholesterol-mimicking molecules were observed in corresponding positions, e.g., in TRPM4, TRPM8, TRPC4 and TRPC6 (Autzen et al., 2018; Duan et al., 2018a, 2018c; Tang et al., 2018). Duan et al. (2019) showed by single point mutagenesis that disruption of this lipid binding site causes loss of receptor-operated activation in mTRPC5.

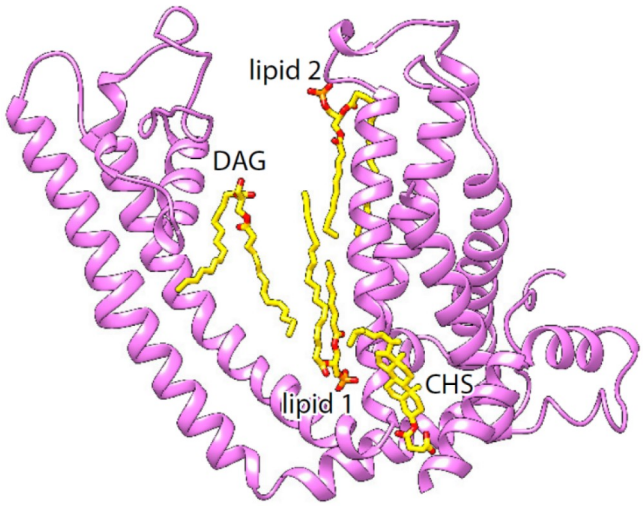

Figure 6: Positions of lipids modelled to nonprotein densities in transmembrane domain of CMZ-bound hTRPC5 structure (PDB 7D4P). Position of DAG corresponds to binding into lipid/Pico145 binding pocket. CHS, cholesteryl hemisuccinate. (Adapted from Song et al. 2021)

3.4 Clemizole-Binding Site

A next crucial inhibitor-binding pocket in TRPC5 was revealed with clemizole (CMZ) (Fig. 5A). CMZ has distinctly different size, shape and chemical properties than Pico145 and it has just recently been localized inside of the intracellular cavity of VSLD. Its binding is mediated by F414 forming a π-π stacking interaction with the chlorophenyl ring of CMZ. Side

chains of Y374, M442, Y446 and P659 contact CMZ through hydrophobic interactions. Residues related to CMZ binding are highly conserved between TRPC4 and TRPC5, which suggests the same binding pocket in both channels. (Song et al. 2021)

3.5 Calcium-Binding Site

Putative cation density was observed inside the intracellular VSLD cavity in both CMZ and HC-070 maps. This putative cation was in close proximity to CMZ in CMZ-bound TRPC5 (Song et al. 2021), coordinated by E418, E421 on S2 and N436, D439 on S3. The identity and function of this cation was not assigned by the authors, but several clues point towards calcium. TRPC5 is strictly regulated by Ca^{2+}, so this region could be a potential conserved Ca^{2+} binding site as was already shown for several TRP ion channels such as TRPM8 (Yin et al., 2019) or TRPA1 (Zhao et al., 2020; Zimova et al., 2018). Moreover, the ion inside of the VSLD cavity in ligand-bound and APO structures of the TRPC4 channel has already been identified as Ca^{2+} by Vinayagam et al. (2020). This calcium ion was not only coordinated by polypeptide chain residues, but also by oxo group of the ligand which could mean that ligands (for example, inhibitors) stabilize putative calcium ion (Vinayagam et al. 2020).

3.6 Zinc-Binding Site

	Starting Residue			H172				C176		C178			C181		Final residue
TRPC5	170	R	P	H	Q	I	R	C	N	C	V	E	C	V	182
TRPC4	170	R	P	H	E	V	R	C	N	C	V	E	C	V	182
TRPC1	187	K	P	H	A	V	G	C	E	C	T	L	C	S	199
TRPC3	261	R	P	H	D	Y	F	C	K	C	G	D	C	M	273
TRPC7	192	R	P	H	D	Y	F	C	K	C	N	E	C	T	204
TRPC6	247	R	P	H	D	Y	F	C	K	C	N	D	C	N	259

Figure 7: Table of amino acid sequences of TRPC channel subfamily with highlighted conserved residues involved in Zn^{2+} binding. (Adapted from Wright et al, 2020)

In cryo-EM structure of TRPC5 by Wright et al. (2020) there has been identified a partially disordered cation density at a site located between ankyrin repeat domain and HLH region at the N-terminus. The sequence alignment revealed that the residues identified to be involved in cation binding (H172, C178 and C181) are highly conserved across the TRPC subfamily (Fig. 7) (Wright et al., 2020). A PDB motif query search revealed a structurally similar motif

that coordinates a Zn^{2+} ion, and furthermore, it was recently shown that intracellular application of Zn^{2+} at micromolar concentration can open neuronal TRPC5 channels by unknown mechanism (Park et al., 2019). The authors were unable to unambiguously identify the density as Zn^{2+} because of a lower local resolution and speculated that their observation can mean that this region is flexible and/or has fewer than four zinc-binding sites occupied at one time (Wright et al., 2020). However, Song et al. (2021) mutated mentioned cysteines residues coordinating the putative zinc ion and achieved higher resolution of this region. Mutations did not affect calcium activation and therefore suggest a different regulatory role instead of ion channel gating (Song et al., 2021).

Almost all information about the structures of TRPC channels so far is obtained from cryo-EM structures determined in closed channel conformations. This presents a problem because physiologically activated TRPC channels may exhibit different atomic arrangement in crucial regions of the protein. TRPC5 is no exception and upcoming effort is directed at solving a high-resolution atomic structure of the TRPC channels in an open conformation. (Chen et al. 2020)

4. Mechanisms of TRPC5 Activation

4.1 Voltage-Dependent Activation

TRPC5 channel has multiple different activation mechanisms. As mentioned above, TRPC5 is voltage dependent and can be activated by membrane depolarization. The current-voltage relationship (I-V) of homomeric TRPC5 (but also TRPC4) has a unique characteristic shape often called doubly rectifying shape (Strubing et al., 2001). Beginning from negative potentials, channel activity increases with membrane depolarization, peaks around -50 mV, then declines to a minimum around 20 mV before increasing at very positive potentials. This behavior can be interpreted by a conductance curve (G-V curve) of TRPC5 (Fig. 8). Conductance curve is N-shaped, ideal to trigger action potential discharge without interfering with the shape and amplitude of the action potential. This is important in electrogenesis as TRPC5 is predominantly expressed in neurons. (Zholos, 2014)

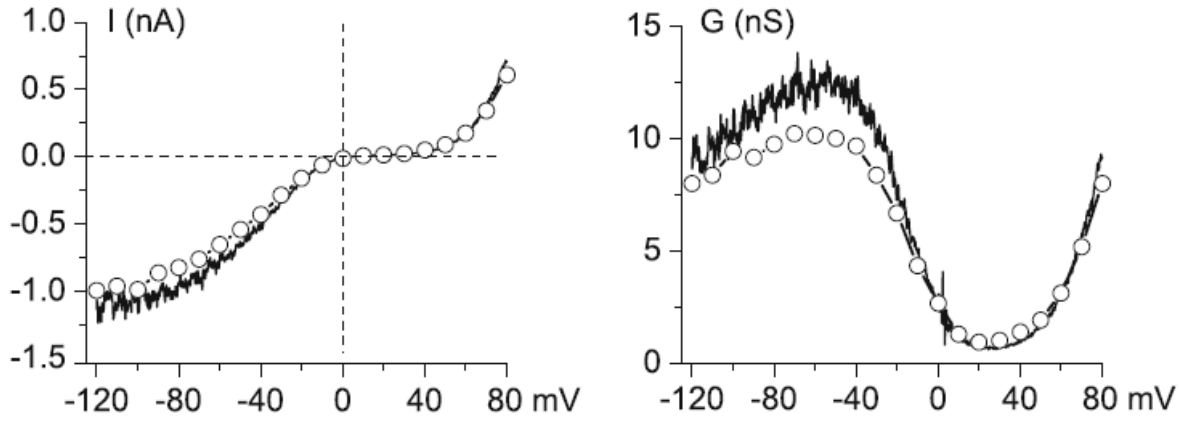

Figure 8: I-V (left) and G-V (right) curves of TRPC5 channel obtained by patch clamp showing typical double rectifying (I-V) shape and N-shaped G-V. (Adapted from Zholos et al. 2014)

However, the characteristic shape of I-V inwardly-rectifying curve is not obtained from heteromeric TRPC1/5. Instead, the curve is outwardly-rectifying and the role of the channel changes from membrane depolarizer (TRPC5) to membrane stabilizer (TRPC1/5) (Kim et al., 2019). The doubly rectifying shape is caused by intracellular magnesium (Mg^{2+}) that is blocking the channel at depolarized potentials by binding aspartate residue D633 that lines an inner vestibule of the channel. Mutation of D633 to asparagine (corresponding residue in human TRPC1) abolishes the inward-rectification feature of the homomeric TRPC5 channel (Obukhov and Nowycky 2005).

4.2 Potentiation by Trivalent Cations

TRPC5 channel can also be reversibly potentiated by trivalent cations La^{3+} and Gd^{3+}, which is interesting as these ions are usually inhibitors for the other cation channels. The TRPC5 lanthanide-binding site is extracellular, between E543 and E595 (Jung et al., 2003). However, E543 and E595 are oriented in such way that cation binding is only possible when the channel is partially open and binding sites are accessible. That means that bound Gd^{3+} and La^{3+} can increase activity, not activate a closed channel on its own (Duan et al., 2019).

4.3 G-Protein Mediated Potentiation

As mentioned earlier, G-protein coupled receptors, heteromeric G-proteins and G-protein signal transduction pathways are important and characteristic activation mechanisms of TRPC channels. The most intensively studied has been the Gq-protein pathway and its implication to TRPC5 channel activation (Fig. 9). The pathway is complex, and one component immediately affects the others' components, which have to be considered: Gαq, Gβγ, PLC-β, PIP_2, IP_3, DAG, and PKC (Kim et al., 2020).

Mammalian TRPC5 channels are transiently stimulated by the phospholipase C (PLC) that is activated by an extracellular ligand binding to G protein-coupled receptor (GPCR)-$G_{q/11}$ (X Chen et al., 2017). Activation of PLC leads to hydrolysis of PIP_2 and formation of IP_3 and DAG. IP_3 binds to a receptor on endoplasmic reticulum that triggers release of intracellularly stored Ca^{2+} while DAG activates protein kinase C (PKC). PKC causes desensitization of the channel by phosphorylating T972 from the PDZ binding motif at the end of the C-terminus (Storch et al., 2017).

Interestingly, non-hydrolyzed PIP_2 associated with the TRPC5 promotes channel activation (Trebak et al. 2009). Recently, a scenario has been proposed where DAG plays a dual role as it can first strengthen PIP_2-channel interactions promoting channel activation, and second, promote the TRPC5 channel inhibition through PKC. Research also suggests that PIP_2 depletion contributes to TRPC5 current inhibition after PLC-mediated activation and provides evidence that channel-PIP_2 interactions are important for maintenance of TRPC5 channel activity (Ningoo et al., 2021).

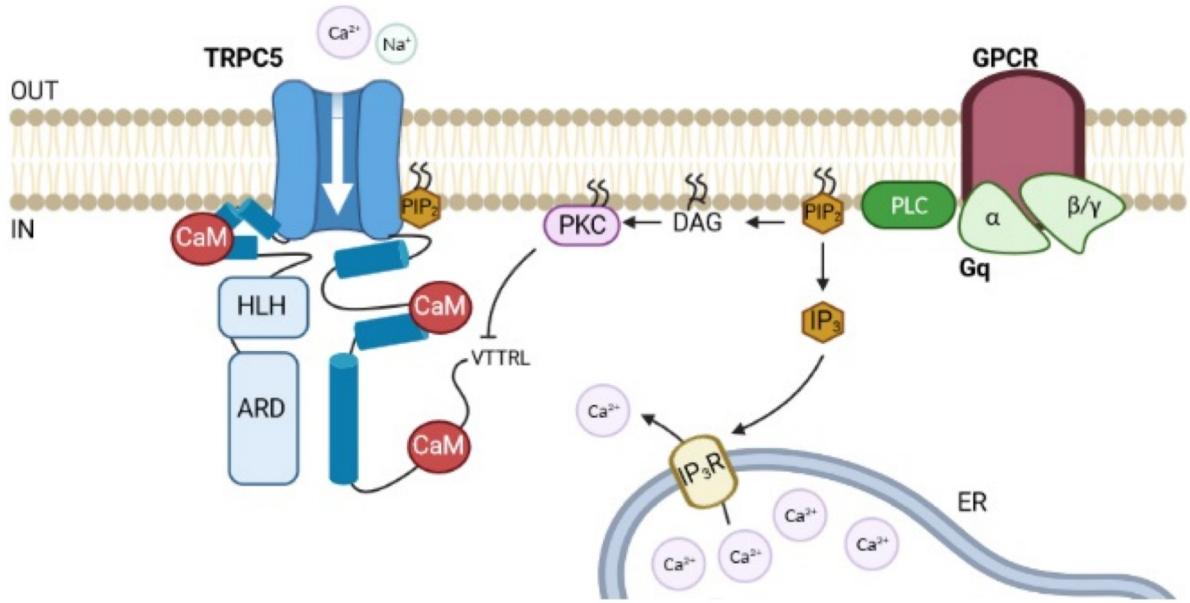

Figure 9: G-protein signaling pathway involved in activation of TRPC5 channel: The G-protein coupled receptor (GPCR) binds a ligand and activates Gq protein. Gqα stimulates phospholipase C (PLC) that catalyzes hydrolysis of phosphatidylinositol4,5-bisphosphate (PIP_2) into second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 activates its receptor IP_3R resulting in Ca^{2+} release from endoplasmic reticulum (ER). DAG activates protein kinase C (PKC) that phosphorylates T972 at the VTTRL motif at the end of C-terminus causing desensitization of the TRPC5 channel. Non-hydrolysed PIP_2 directly interacts with TRPC5 and is required for maintaining its activity. Parts of this pathway are able to directly stimulate TRPC5 activity, namely: Gqα, DAG and Ca^{2+}. Schematic location of calmodulin (CaM) binding sites at the C and N-terminus are shown as red circles. (Created in BioRender.com)

In later studies, there was proposed a G-protein activation mechanism independent of the Gq-PLC pathway. This claim was tested on Gαq-QL mutation, which disrupts GTPase activity of the protein, hence Gαq remains constitutively active (Myeong et al., 2015). A problem with this mutation was that even if Gαq could directly activate the channel, the overall effect would be negative since all of the PIP_2 necessary to maintain the TRPC activity would have been depleted. This problem was solved by creating a $G\alpha q^{QL/LA}$ mutant that has constitutive activity due to the QL mutation but cannot interact with PLC-ß. Further experiments have shown that $G\alpha q^{QL/LA}$ mutated protein could directly activate heteromeric TRPC1/5, proving Gq-PLC independent G-protein activation of the TRPC5 channel. Results were further verified by co-immunoprecipitation (Kim et al., 2019).

4.4 Potentiation by Calcium Ions

TRPC5 is tightly regulated by internal and external calcium. For the TRPC5 channel to be activated and to produce a current, there has to be at least 10 nM intracellular Ca^{2+} concentration (Okada et al., 1998). If the intracellular calcium is raised to high levels (10 μM), the channel exhibits high level of activation (Schaefer et al., 2000). Even when the intracellular levels of calcium are slightly higher (around 200 nM), the Ca^{2+} slowly induces TRPC5 activation without any other receptor stimulation (Shi et al., 2012; Zeng et al., 2004). Thus, Ca^{2+} plays both a permissive and potentiating role for TRPC5 activity inside of the cell. If the calcium is added extracellularly, TRPC5 exhibits a dramatic increase of ATP-induced current, indicating high dependence of TRPC5 activity on the presence of extracellular Ca^{2+} (Okada et al., 1998). Previous studies suggested that La^{3+} and Ca^{2+} potentiate TRPC5 by similar extracellular mechanisms (Jung et al., 2003). Another possibility is that Ca^{2+} can activate TRPC5 channel from the inside upon entering the cell through the channel, which has been proved to be right. In the experiment, the potentiation was prevented by buffering Ca^{2+}, but was promoted in a voltage-dependent manner at higher concentrations of calcium ions (Blair et al., 2009).

Duan et al. (2019) found cation-binding site in VSLD on S2-S3 linker coordinated by hydrogen bonding with several highly conserved residues (including E418, E421, N436 and D439). They constructed a mutated channel where three negatively charged residues were neutralized (E418Q-E421Q-D439N) and in path-clamp experiments observed that the mutated channel was no longer activated by Gd^{3+} while the Englerin A activation was preserved. On the other hand, the disturbance of this negatively charged motif by introduction of positively charged arginine (N436R) resulted in loss of potentiation to both activators tested (Duan et al., 2019). In a follow-up study, it was shown that the triple mutant cannot be activated by extracellular calcium anymore, suggesting the essential role of this site for calcium activation (Song et at. 2021). Further investigation is needed to evaluate if Ca^{2+} directly binds to this site to regulate TRPC5 activity as was shown for TRPA1 channel (Zhao et al., 2020; Zimova et al., 2018).

4.5 Regulation by Ca^{2+} Sensing Proteins

TRPC5 is also tightly regulated by calmodulin (CaM). There are three putative calmodulin-binding sites in TRPC5 (Fig. 9). One is located on the N-terminal tail just before the transmembrane region and two more are on the C-terminal tail. The first one is called CIRB

(Calmodulin and IP_3 Receptor Binding) and can be found in all TRPC isoforms (Tang et al., 2001). The second calmodulin-binding site (CBII) in the C-terminus have been studied most extensively (Ordaz et al., 2005). Affinity of Ca^{2+} for CaM binding to CBII is significantly (more than 10 times) higher than that to the CIRB. Therefore, CBII is crucial for Ca^{2+}/CaM-dependent facilitation of agonist-induced activation of TRPC5 (Ordaz et al., 2005).

Last year, a structure of TRPC4 with CaM (Vinayagam et al. 2020) was published. It elucidated the details of how CaM binds to the rib helix of the channel and revealed that this interaction enables ordering a previously disordered region, fixing the channel in closed conformation (Vinayagam et al., 2020). Due to a high homology of this (CIRB) region, a similar interaction could be expected with TRPC5.

Furthermore, TRPC5 has been shown to be regulated with the additional two Ca^{2+}-sensing proteins: Ca^{2+}-binding protein 1 (CaBP1) and neuronal Ca^{2+} sensor 1 (NCS-1). The CaBP1 negatively regulates TRPC5 by binding to the C-terminus of the TRPC5 channel, inhibiting intracellular Ca^{2+} activation (Kinoshita-Kawada et al., 2005). The NCS-1 also binds to the C-terminal end of a monomer and plays an important permissive role in almost all modes of TRPC5 activation (by agonists, store depletion, elevated concentration of Ca^{2+} and lanthanides) (Hui et al., 2006). In conclusion, TRPC5 is balanced by positive (CaM and NCS-1) and negative (CaBP1) effects from Ca^{2+}-sensing proteins.

4.6 Activation by Physical Factors

Although TRPC5 is constitutively active at ambient temperature in heterologous expression systems, its activity increases as the temperature drops below 37 °C, peaking at around 25 °C at negative membrane potentials. The cold-induced current was constant at a range of physiologically relevant potentials (-40 mV to -80 mV) and was not significantly inactivated by persistent cold temperatures, unlike other TRP channels. The experiment with TRPC5 knockout mice showed no significant temperature-related behavioral change (Zimmermann et al., 2011). The role of TRPC5 as a cold sensor was confirmed 10 years later in odontoblasts (see below in chapter 5.2).

It has been shown that TRPC5 is involved in mammalian osmo-mechanosensory transduction. TRPC5 can be activated by hypoosmotic and pressure-induced membrane stretch. This activation is independent of PLC signaling pathway, although experiments confirmed that moderate levels of the plasma membrane PIP_2 are required (Gomis et al., 2008).

5. Physiological Relevance

5.1 TRPC5 Knockout in Mice

Gene knockout is a method of inactivating a particular gene (TRPC5 gene in this case). It is used to study an actual impact on physiology and/or behavior of a living organism (mice) caused by an absence of a functional protein. In the case of TRPC5, the ablation of its gene was done through homologous recombination using targeting construct to delete the exon 5 genomic region that encodes amino acids 412-459. The deletion of the exon 5 region was catalyzed by Cre-recombinase and later confirmed by PCR (Liu et al., 2003).

TRPC5 knockout mice did not show any abnormalities in weight, neurological reflexes, basic motor functions and sensorimotor responses (Riccio et al., 2009). However, TRPC5 knockout mice were notably less anxious in response to innately aversive stimuli compared to wild-type mice. This was caused by altered glutamatergic synaptic transmission in the amygdala, more precisely by reduced Group I metabotropic glutamate receptor (mGluR) and cholecystokinin$_2$ (CCK_2) responses (Riccio et al., 2009). Moreover, TRPC5 contributes to conditioned fear. TRPC5 knockout mice also showed reduction in pilocarpine-induced seizures and seizure-induced neuronal death in the hippocampus (Phelan et al. 2013).

5.2 Cold Pain Signaling in Teeth

The TRPC5 channel is cold sensitive in heterologous expression systems (trigeminal and dorsal root ganglion neurons and the superficial laminae of the spinal dorsal horn) which is typical for pain and temperature sensing receptors (Zimmermann et al., 2011). However, no cold sensing function has been ascribed to TRPC5 until very recently.

Teeth, unlike other tissue, become extremely cold sensitive when inflamed. Odontoblasts, which are cells beneath tooth enamel incorporated into dentin just above dental pulp, are crucial in cold signaling transduction in teeth. In healthy teeth, TRPC5 is a cold sensor and with the TRPA1 are sufficient for cold sensing. When a tooth becomes inflamed, TRPC5 becomes increasingly expressed in sensory axons but another cell type seems to be a direct site of TRPC5 cold transduction – odontoblasts. TRPC5 also provides a mechanism for prolonged cold sensing by relative sensitivity to intracellular Ca^{2+} concentration and lack of desensitization (Bernal et al., 2021).

Moreover, in addition to being an odontoblast cold sensor, TRPC5 may signal prolonged pain and act as a sensor of oxidative stress during an inflammation (Yamamoto et al., 2010). This could mean that TRPC5 is an effective target for further pharmaceutical research focused on treating dentin hypersensitivity and inflammatory tooth pain (Bernal et al., 2021).

5.3 TRPC Channel Contribution to Arthritis Pain

Latest evidence suggests that both TRPC5 and the heteromeric TRPC5/1 are functionally expressed in fibroblast-like synoviocytes obtained from patients with rheumatoid arthritis (Xu et al., 2008). Rheumatoid arthritis is a long-term progressive autoimmune disease-causing inflammation of the synovium in peripheral joints, leading to the cartilage degradation, joint involvement and loss of function.

Lately, it has been demonstrated in vivo that TRPC5 have a protective function in arthritis development. In the study by Alawi et al. (2017), arthritis was induced in TRPC5 KO and WT mice by injection of Complete Freund's Adjuvant (CFA) into the hind knee joint. Early response cytokine Tumor Necrosis Factor alpha (TNF-α) promotes the inflammatory responses in autoimmune diseases like rheumatoid arthritis. The pro-inflammatory cytokines are crucial to the pathogenesis of rheumatoid arthritis, leading the series of events including the recruitment and activation of inflammatory cells and propagation of autoimmune joint destruction (Konttinen et al., 1999). TNF-α receptor is capable of structural interaction with TRPC5. Although the mechanism is not fully understood, it is known that TRPC5, TRPC4 and TNF-α receptor are parts of a signaling complex, the activation of which leads to increased loading of endoplasmic reticulum calcium stores due to increased store-operated calcium entry (Mace et al., 2010). In CFA-induced arthritis KO mice, the expression of TNF-α was considerably elevated and may have caused increased hyperalgesia observed in KO mice. Deletion of TRPC5 also resulted in neutrophil-driven inflammation 14 days after induction of arthritis, indicating protective role of TRPC5 which has been localized in the synovium alongside the neutrophils (Alawi et al., 2017).

In summary, evidence indicates that activation of endogenous TRPC5 acts as a protector against inflammatory stress and may be a next target for new therapeutic strategies for rheumatoid arthritis (Alawi et al., 2017).

5.4 TPRC5 Contribution to Striatal Neuron Loss in Huntington's Disease

Huntington's disease is a neurodegenerative disease characterized by progressive loss of neurons caused by neuronal cell death associated with oxidative stress (Bredesen et al., 2006).

Due to its high energy demand and oxygen consumption, the brain is vulnerable to oxidative stress and needs various antioxidants. Glutathione (GSH) is the most abundant antioxidant in mammalian cells. During oxidative stress, GHS is converted to oxidized glutathione (GSSG). Cellular redox potential is maintained while GSSG can be restored to GSH by GSSG reductase. GSSG glutathionylates cysteines in target proteins as a form of redox sensing and response. Cytosolic oxidation by GSSG activates TRPC5 channels by glutathionylation, most likely on the C176 and C178. Glutathionylation of TRPC5 potently activates the channel, resulting in a sustained increase of Ca^{2+} concentration and consequent cell toxicity and neurodegeneration as the key event in pathogenesis of Huntington's disease. (Hong et al., 2015b).

5.5 Amygdaloid TRPC5 Channels in Neuropathic Pain Behavior

Amygdala has an important role in primary emotions, it is particularly involved in processing and regulation of emotional aspects of pain by descending control of spinal pain-relay neurons (LeDoux, 2007; Neugebauer et al., 2009). Amygdaloid TRPC4 and TRPC5 channels, that were shown to be in control of innate fear, are important for peripherally induced neuropathic pain behavior (Riccio et al., 2009).

Effect of TRPC4 and TRPC5 channels on affective pain sensitivity was experimentally tested on neuropathic rats. Neuropathy was induced to anesthetized animals by small incision to the left limb. Microinjections of the TRPC4 and TRPC5 channel blocker ML-204 into the amygdala of rats reduced the sensory and the affective pain sensitivity in neuropathic animals. However, it failed to attenuate mechanical hypersensitivity in the control group of healthy animals. This finding suggests that in chronic painful neuropathy occurs an alternation in function of amygdala. In this pathophysiological condition, the TRPC4 and TRPC5 channel have a significant pain facilitatory effect. Current evidence suggests that TRPC4 and TRPC5 channels might play a role in comorbidity between affect and chronic pain and could provide therapeutic targets for affective disorder-associated chronic neuropathic pain conditions. (Wei et al., 2015)

6. Conclusion

Although pain is vital for dangerous environmental stimuli detection, chronic pain does not serve the same physiologically protecting purpose and still burdens millions of people around the world. In the last two decades, important advances have been made in understanding the ion channels involved in pain perception and TRP channels have become a promising target for intensive research in this direction. This thesis focuses on one channel of the TRP ion channel family that has been intensively studied in relation to its essential role in the kidneys, but the interest of this thesis is mainly aimed at its role in the nervous system and pain perception.

In this thesis, I summarized contemporary knowledge about the TRPC5 channel focusing mainly on cryo-EM structures released in the last two years and recent functional studies that succeeded in discovering potent TRPC5 agonists and inhibitors. The published structures revealed important properties and binding sites crucial for the study of the molecular and physiological mechanisms in a potential TRPC5-aimed pharmaceutical application. For instance, a TRPC5 channel inhibitor called GFB-887 is currently undergoing phase II of clinical trial (clinicaltrials.gov, Identifier: NCT04387448) study to evaluate the safety and tolerability in patients with ongoing diabetic nephropathy.

Currently there are 21 known unique proteins that interact with human TRPC5 (gpsprot.org, April 2021). In addition to those discussed here, related TRPC channels and NHERF proteins that belong among TRPC5 interacting partners, there are also proteins involved in governing Ca^{2+} levels in endoplasmic reticulum. There is also an evidence that thermoTRPs exists as a component of a higher order supramolecular complexes that have an ability to change composition and/or dynamics based on the current state of a tissue e.g., inflammation. These alterations may play an important role in channels activity and/or nociceptor excitability (Fernández-Carvajal et al., 2011). If and how TRPC5 channel is engaged in such supramolecular complexes is not known and represent an attractive target for further research.

Pain management is persistently an important area of medical need. Learning how to activate, inhibit or modulate TRP channels activity certainly would be beneficial for discovering alternative pathways leading to pain relief. Unfortunately, analgesics targeting TRP have not been successful yet due to unacceptable side effects (Bamps et al., 2021). However, TRP channels still hold a great potential for creating safe, effective and non-addictive pain relief medications in the future.

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