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Structural and Functional Study on Transient Receptor Potential Vanilloid 1 (TRPV1) and Ankyrin Receptor (TRPA1) Channels

Ph.D. thesis

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Nove Hrady, 2010

Acknowledgements

All praise to almighty Allah, the creator, the dominant, the most supreme, the self-existing and all sustaining, whose grace and mercy enabled me to accomplish this piece of work.

I wish to express my deep sense of gratitude to Assoc. Prof. Rüdiger H. Ettrich and Dr. Viktorie Vlachova for hiring me as their research student and giving me freedom to explore and discover new insights into structure and function of TRPs channels. Their learned guidance and useful training enable me to fight against future challenges in all fields of life. Their skillful advices, sincere co-operation, and learned guidance, competency, supervisory, intellectual and sympathetic attitude through out the course of this research enabled me to complete my work. I learned a lot from them and they have been a very kind to me and for them no acknowledge could ever adequately express my obligation.

I feel a great pleasure and honor to express my heartful gratitude to Prof. Vassilios Sarafis for being greatly profited by his marvelous guidance, keen interest & amiable attitude during my present research. I would like to thanks to my seniors Ladislav Vyklický (late) and Jan Tesinger for supporting me over the years with their kind discussion on molecular biology and ion channel physiology. They had been an inspiration for me and their professional support was very helpful for me to accomplish my work

I am thankful to all the persons of Institute of Systems Biology and Ecology, Nove Hrady and Institute of Phsyiology Prague, and but some names deserve special mention, Dalibor Stys, Eva Csefalvay, Jaroslava Kohoutová, Kamila Blafkova, Babak Minofar, Morteza Khabiri, Anamika Mishera, Ali Rashek, Jr. Ladislav Vyklický, Jan Benedikt, Ivan Dittert, Karolina Novakova-Tousova, Klara Ivankova-Susankova, Lucie Sura, Magda Kuntosova, for their scientific and moral support.

My acknowledgement could never adequately express obligation to my affectionate mother, brothers and sisters for their love, encouragement and prayers for my success, who always wished to see me glittering on skies of success.

Dedications

To my parents and family members

You were always there
to help me
You were always there
to guide me
You were always there
to laugh with me
You were always there
to cry with me
But most important
you were always there
to love me
and I want to assure you that
I am always here
to love you

Susan Polis Schutz

Samad A., 2010 Structural and functional study on Transient receptor potential Vanilloid 1 (TRPV1) and Ankyrin receptor (TRPA1) channels. PhD. Thesis, in English - 121 p., Faculty of Biological Sciences, University of South Bohemia, České Budějovice.

Annotation:

Investigations of structural and functional relationships of rat transient receptor potential cation channel, subfamily V, member 1 (TRPV1), also known as the capsaicin receptor, and human transient receptor potential cation channel, subfamily A, member 1, also known as TRPA1, are presented. Capsaicin induced Ca^{2+} -dependent desensitization of rat TRPV1 channel is studied and lead to the identification of key amino acid residues in the C-terminal domain of TRPV1 interacting with the membrane phospholipid PIP_2 and an intradomain interaction that controls the open and desensitized state of the TRPV1 channel.

Further the molecular basis of agonist AITC- and voltage-dependent gating on TRPA1 is explained. Hereby, residue P949 located near the center of the sixth transmembrane spanning helix (S6) is structurally required for normal functioning of the receptor and the distal bi-glycine G958XXXG962 motif controls its activation/deactivation properties. Furthermore, the gating region is extended towards the cytoplasmic part of the channel, putatively located near the inner mouth of the channel pore. A following series of experiments lead to the identification of a limited number of residues that appear important for allosteric regulation of the channel by chemical and voltage stimuli (K969, R975, K989, K1009, K1046, K1071, K1092 and K1099). In addition, three charge-neutralizing 'gain-of-function' mutants (R975A, K988A, and K989A) which exhibited higher sensitivity to depolarizing voltages were characterized, indicating that these residues are directly involved in voltage-dependent modulation of TRPA1.

Financial support:

This work was supported by the Czech Science Foundation, 305/06/0319, 303/07/0915, the Research Project Fund of the AS CR, AV0Z50110509, AV0Z60870520, European Commission FP6 Specific Targeted Project "PHOTOLYSIS" LSHM-CT-2007-037765, and by the Ministry of Education, Youth and Sports of the Czech Republic, 1M0517, LC06010, MSM6007665808 and LC554.

Declaration:

I hereby declare that I did all work, summarized in this thesis, on my own or in collaboration with the coauthors of manuscripts under supervision of Assoc. Prof. RNDr. Rüdiger H. Ettrich and Dr. Viktorie Vlachova, using only the cited literature.

České Budějovice, September 29, 2010 Abdul Samad

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1. Biological Significance of Transient Receptor Potential Channels

Transient receptor potential (TRP) channels are the most recently discovered members of the ligand-gated ion channel superfamily of receptors. These structurally related channels are expressed in many tissues where they subserve a wide range of functions throughout the body, most notably signal transduction (Clapham, 2003; Moran et al., 2004; Montell, 2005; Nilius, 2007; Nilius et al., 2007). Several TRP channels are vital polymodal cellular sensors (i.e. thermo, chemo, and mechanosensors) in both excitable and non-excitable cells (Ramsey et al., 2006; Colburn et al., 2007; Kim and Baraniuk, 2007; Venkatachalam and Montell, 2007; Benton, 2008; Rosenzweig et al., 2008; Mizuno, 2008; Shimizu et al., 2008; Kwon et al., 2008). Based on sequence homology, the mammalian TRP superfamily can be subdivided into six subfamilies (Fig 1): TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPA (Ankyrin), TRPP (Polycystin), TRPML (Mucolipin) (Venkatachalam and Montell, 2007).

Cumulative reports demonstrate the role of TRP channels in the sensing of wide diversity of acute noxious physical, chemical and mechanical stimuli which render into nociceptive, neuropathic, and psychological pain (Levine and Alessandri, 2007). Indeed, it is very much clear now that changes in TRP channel expression and function turns into hypersensitivity and pain disorders in mammals (Cortright et al., 2007; Dray, 2008; Brignell et al., 2008).

1.1 Hot, cold and painful

A subset of TRP channels (TRPV1, TRPV2, TRPV3, TRPV4, TRPM8, and TRPA1) known as the ''Thermo TRPs'' are reportedly involved in sensation of distinct temperature thresholds. TRPV1 and TRPV2 are sensors for uncomfortably warm (>43˚C) and very hot (>52˚C) temperature respectively, whereas TRPV3 (>30–39˚C) and TRPV4 (~25–34˚C) contribute to the perception of moderate temperature. TRPM8 (< 28˚C) appears to function in our perception of cool temperature, and TRPA1 (< 17 ˚C) may be a cold sensor (Venkatachalam and Montell, 2007; Bandell et al., 2007).

A shared feature of mammalian thermo TRPs (TRPV1, TRPM8 and TRPA1) is their expression in sensory neurons and they respond to chemical stimuli as well. This property was initially found for the vanilloid receptor TRPV1, a channel which is activated by capsaicin.

This pungent compound can elicit the same sensation as thermal heat, while TRPM8, which is activated by menthol and icilin, can elicit the cooling sensation. Later on, cold activated receptor ANKTM1 (TRPA1) was cloned that can be activated by pungent or cooling chemicals such as allyl isothiocyanate (AITC) or menthol (Story et al., 2003).

Activation of thermo TRPs in sensory neurons by noxious thermal, chemical and mechanical stimuli initiates sensation of pain and these neurons are termed as nociceptors. TRPV1 and TRPA1 expression in nociceptors make them a hot target to understand their regulatory mechanisms for the treatment of pain (Conway, 2008; Cortright and Szallasi, 2009). We are just at the very beginning to understand the role of these receptors in native cell because of our limited knowledge about their structure and function which is an immediate requirement of the present time.

1. 2 Biological importance of TRPV1

TRPV1 originally cloned by functional screening of rat dorsal root ganglion (DRG) complementary DNA (cDNA) library with capsaicin. It has a diverse tissue distribution. Its highest expression level is in sensory neurons (Sanchez et al., 2004). However, TRPV1 is predominately expressed in Aδ and C peptidergic neurons that express substance P (SP), calcitonin gene related peptide (CGRP), nerve growth factor (NGF) receptor tyrosine kinase A (TrkA) and in isolectin B4 (IB4) positive, nonpeptidergic neurons in rats (Tominaga et al., 1998). Peptidergic neurons are important in the development of neurogenic pain and inflammation while nonpeptidergic neurons play a critical role in chronic pain development. TRPV1 is also expressed in nonneuronal tissues, *i.e.* keratinocytes of the epidermis, smooth muscles and bladder urothelium, glial cells, liver, and polymorphonuclear granulocytes, mast cells and macrophages (Nilius et al., 2007). An altered expression and function in these tissues can lead to functional bowl disease, inflammatory bowl disease, osteoarthritis, arthritis, bladder disease, cystitis, asthma, breast cancer, schizophrenia, myasthenic syndrome, and allodynia migraine and pain in general (Chen et al., 2008; Vardanyan et al., 2008; Yang et al., 2008).

TRPV1 is a polymodal cellular sensor of nociceptive afferent neurons activated by heat (noxious range > 43 ^{o}C), voltage, extracellular protons, capsaicin, resiniferatoxin (RTX), camphor, piperine, allicin, olvanil and endogenous fatty acids (*e.g.* palmitoyl ethanolamide, 12 hydroperoxyeicosatetraenoic acid (12 HPETE), N arachidonoyl dopamine NADA) (Cortright et al., 2007; Holzer, 2008). Moreover, TRPV1 is considered vital in mediating heat

hypersensitivity during inflammation through inflammatory mediators i.e. NGF, prostaglandins, bradykinin (BK), serotonin, ATP, protein kinases (PKA, PKC), lipoxygenase products, and adenosine (Pingle et al., 2007).

Cultured sensory neurons from TRPV1 knock out mice showed the loss of capsaicin, resiniferatoxin, proton, and thermal (42°C– 52°C) sensitivity *in vitro*. Behavioral responses to capsaicin were also absent in transgenic mice. In contrast, these mice showed normal physiological and behavioral responses to noxious mechanical stimuli, and, moreover, these mice did not develop thermal hyperalgesia during inflammation (Caterina et al., 2000; Davis et al., 2000). However TRPV1 knock out mice retain responsiveness to high threshold noxious heat which shows that some of the alternative thermoreceptors have since been identified (Caterina et al., 1999).

New reports on the role of TRPV1 in pain and other systematic diseases are continuously emerging, however the most striking property of TRPV1 receptor is the Ca^{2+} dependent desensitization which keeps cells safe from Ca^{2+} overload. The precise mechanism of this phenomenon can be best answered by the availability of high resolution structural data.

1. 3 Biological importance of TRPA1

TRPA1 (formerly ANKTM1) is the most recently discovered member of the sensory TRP channel family, originally cloned as a cold (≤ 17 °C) activated channel from noxious cold sensitive but menthol insensitive sensory neurons where it is coexpressed with TRPV1 (Story et al., 2003). Later on, the cold sensitivity of TRPA1 was proved controversial and menthol was found to exert a bimodal effect (Bautista et al., 2006; Karashima et al., 2007, Zurborg et al., 2007). However the most recent reports keep TRPA1 in the list of cold sensitive TRP channels (Sawada et al., 2007; Fajardo et al, 2008; Karashima et al., 2009).

TRPA1 mRNA and protein expression has been found in the peripheral ganglia that contain nociceptive neurons and in the mechanosensitive epithelia of the inner ear as well. TRPA1 expressing peripheral neurons coexpres NGF receptor TrkA, CGRP, SP, peripherin and TRPV1 (Diogenes et al., 2007). In summary, TRPA1 is expressed by C fiber nociceptors and not by Aδ fiber neurons in neonatal rats, adult rats and mice and involved in thermosensation and pain. TRPA1 expressing neurons show up to 97% overlap with expression of TRPV1, but not with TRPM8 (Kobayashi et al., 2005; Nagata et al., 2005; Bautista et al., 2006; Nilius et al., 2007; Du et al., 2007; Ji et al., 2008; Nassenstein et al., 2008).

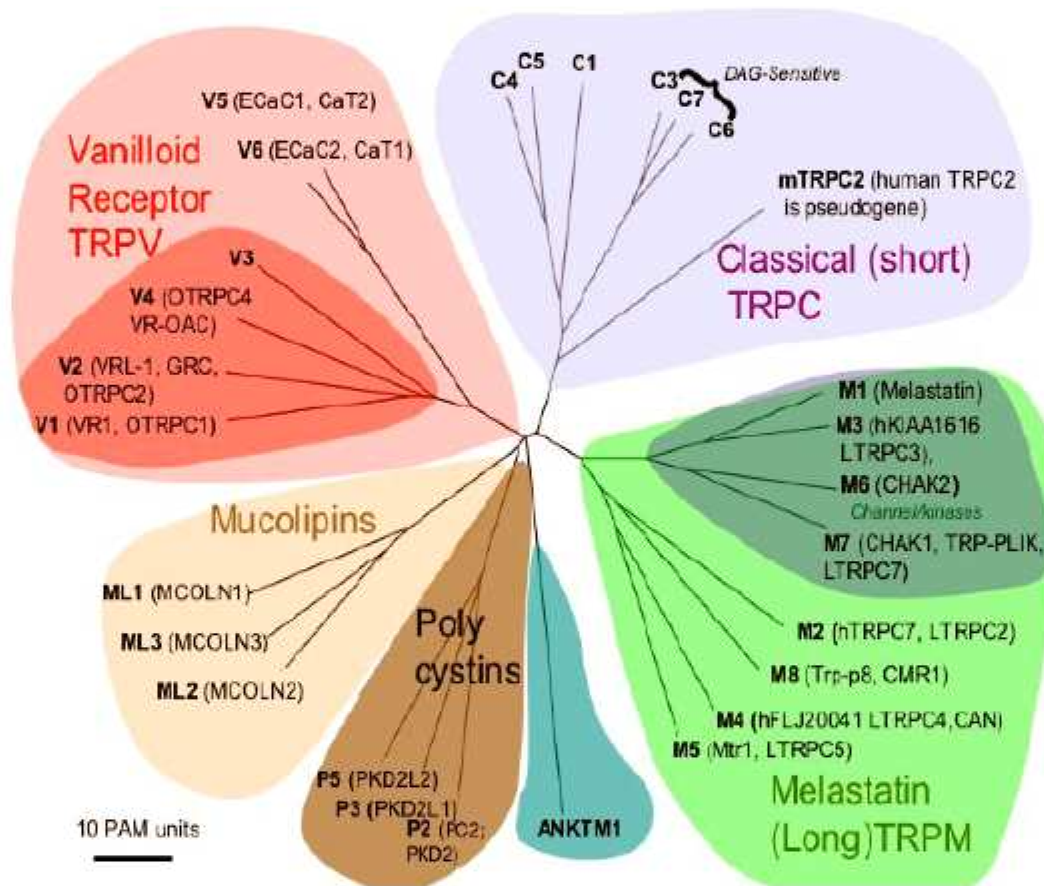

Fig. 1: Mammalian TRP family tree. The evolutionary distance is shown by the total branch lengths in point accepted mutations (PAM) units, which is the mean number of substitutions per 100 residues (Clapham, 2003).

In nociceptors, TRPA1 clearly acts as a receptor for pain producing chemicals and neurons from TRPA1 deficient mice were completely insensitive to AITC and allicin, H_2O_2, alkenyl aldehydes and 15-deoxy-Δ12, 14-prostaglandin J2 (15dPGJ2) (Bandell et al., 2004; Bautista et al., 2006; McNamara et al., 2007; Namer et al., 2008; Andersson et al., 2008, Andre et al., 2008; Bessac et al., 2008). AITC and allicin are thiol reactive exogenous ligands and are rarely in contact with most TRPA1 expressing nociceptive neurons. However, similar thiols reactive compounds are produced during inflammation and can activate the TRPA1 receptor in nociceptors (Hyslop et al., 1995; Sprong et al., 1997; Chen et al., 1999; Gao et al., 2003; Uchida 2003; Namer et al., 2008; Andersson et al., 2008; Cruz-Orengo et al., 2008; Takahashi et al., 2008).

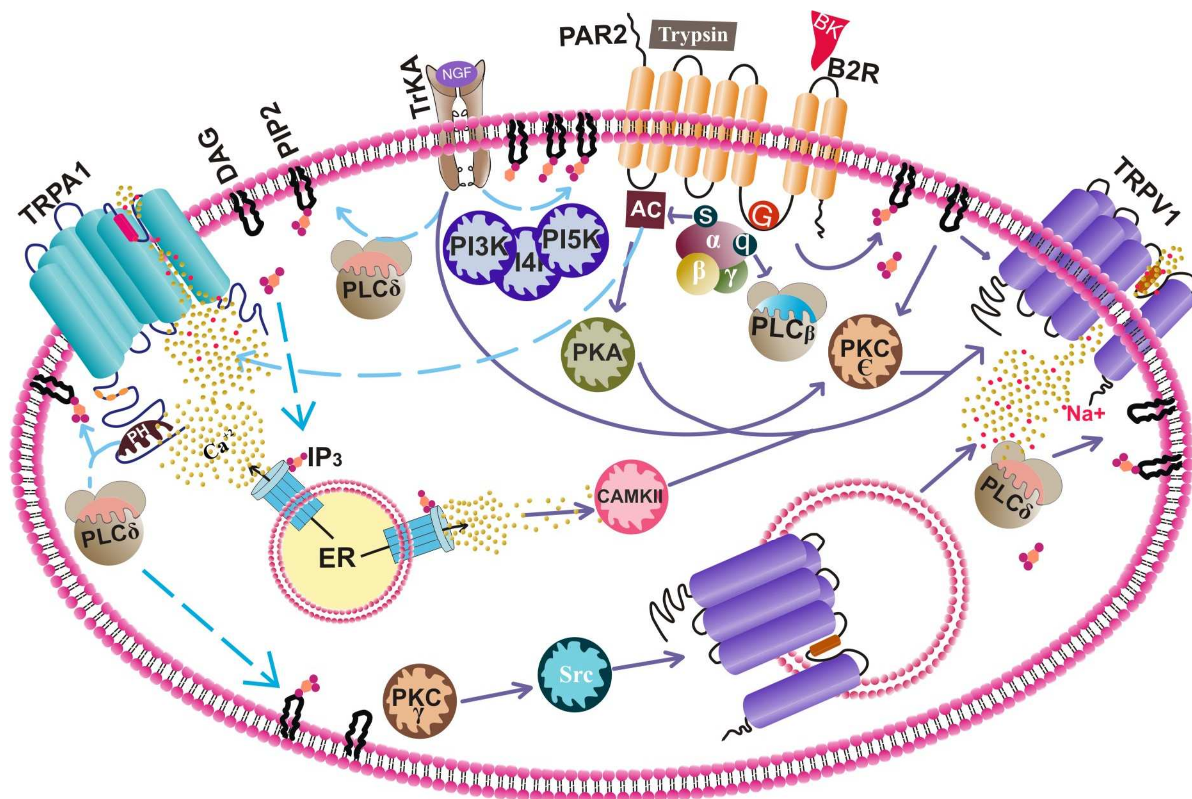

Fig. 2: Receptor mediated activation of vanilloid (TRPV1) and ankyrin (TRPA1) receptor channel in sensory neurons. Proinflammatory compounds (bradykinin, trypsin, nerve growth factor) bind to either G-protein-coupled receptors (GPCR) i.e bradykinin receptor (B2R), protease activated receptor 2 (PAR2) or tyrosine-kinase receptors (TrkA) to activate phospholipase C (PLC), protein kinases A (PKA) and C (PKC), Ca^{2+}-calmodulin-dependent kinase II (CAMKII) and PI3 kinase (PI3K) which, in turn, activate/sensitize (+) or desensitize (−) TRPV1/TRPA1 channels to physical stimuli, and also increase Ca^{2+} release from the endoplasmic reticulum (ER). Increase in the concentration of intracellular Ca^{2+} activates PKC, CAMKII and TRPA1. This scheme illustrates TRPV1/TRPA1 channels may not only act as ligand-gated ion channels but may also increase neuron excitability through the activation of intracellular signaling pathways. Solid lines represent processes engaging TRPV1 while dotted line represent processes engaging TRPA1.

TRPA1 can be also activated by acetaldehyde, an intermediate substance of ethanol metabolism (Bang et al., 2007), and thymol, a major component of thyme and oregano used in oral care (Lee et al., 2008), and evoke sensation of pain and cooling respectively.

Bradykinin (BK), a structurally unrelated proalgesic and proinflammatory agent produced endogenously, activates the TRPA1 in nociceptive neurons (Bautista et al., 2006). An application of BK to HEK293 cells coexpressed with bradykinin receptor (B2R) and TRPA1 potentiates the AITC and cinnamaldehyde evoked TRPA1 responses. The functional interaction between B2R and TRPA1 in the DRG occurs through downstream signaling of cAMP-PKA and phospholipase C (PLC) (Wang et al., 2008). Further evidence in support of BK evoked TRPA1 activation is the fact that TRPA1-/- mice did not develop pain hyperalgesia after exposure to BK (Bandell et al., 2004; Bautista et al., 2006).

TRPA1 is a polymodal cellular sensor, and, in addition to chemical and cold stimuli, it can transduce mechanical stimuli as well. TRPA1 knockout mice demonstrated deficits in sensing mechanical stimuli, suggesting a role for TRPA1 in somatic mechanosensation (Kwan et al., 2006). A recent report reveals that TRPA1 mediates an osmotically activated ion channel that further supports a role for TRPA1 in mechanosensation (Zhang et al., 2008).

2. General Structural Aspects of Transient Receptor Potential (TRP) Channels

All TRP channels are complexes formed by four receptors – each receptor has a "six-putative transmembrane spanning region. A hydrophobic stretch between the fifth (S5) and sixth (S6) segments is believed to form the cation permeable pore. Hydropathy analysis predicts a seventh N-terminus transmembrane (TM) segments in some TRP channels (TRPC, TRPM, TRPP, TRPML), however experimental results reveal that first hydrophobic domain of TRPC3 is located on intracellular side and a general consensus is on six TM topology of TRP channels (for a review see Ramsey et al., 2006). Although no complete crystal structure of any TRP member has yet been published, in consistence with voltage gated Kv channels and recent reports on TRPV1, TRPM2 and TRPC3 structure through electron cryomicroscopy corroborate TRP channels as tetramers and both amino (NH_2) and carboxyl (COOH) termini are located on the cytoplasmic side of the membrane (Mio et al., 2005, 2007; Maruyama et al*.,* 2007; Moiseenkova-Bell et al., 2008).

Various important sequence elements and domains (Fig. 2) have been identified in cytoplasmic N- and C-terminal domains of TRP receptors. Functionally, the most important are the 6 transmembrane segments comprising the pore loop region between the S5 and S6 segments. Knowledge about the molecular architecture of the channel and its modulation through cytoplasmic domains is crucial in determining the physiological implications of any ion channel. In TRP superfamily TRPV, TRPC, and TRPM members are particularly focus for structure/function relationship and characterization of the pore domain.

2.1.1 Pore region of TRPV

The mammalian TRPV subfamily contains six members: TRPV1-V6. Based on their permeation properties TRPV1/2/3/4 are called non-selective cation (P_{Ca}/P_{Na} ~10) channels while TRPV5/V6 are highly selective Ca^{2+} (P_{Ca}/P_{Na} > 100) permeable channels (Venkatachalam and Montell, 2007).

The pore region GM(L)GD in TRPV1/2/3/4 channels shows a significant sequence homology with GYGD signature sequence in the pore of bacterial potassium (KcsA) channels, a crystal structure of which has been solved at 2 Å resolution (Doyle et al., 1998; Voets et al., 2002).

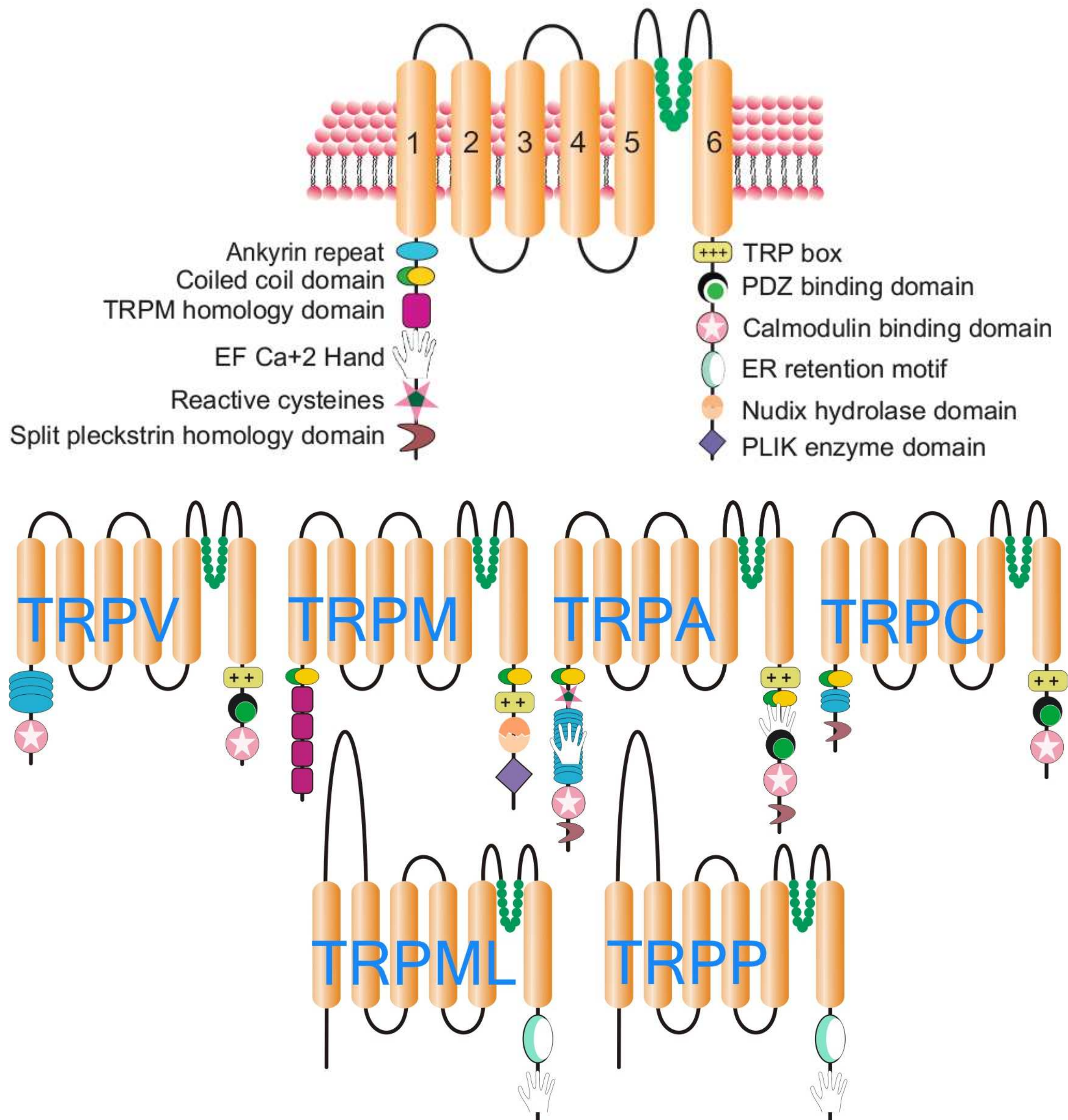

Fig. 3: Membrane topology of mammalian TRPs channel. TRPs are 6 transmembrane tetramer channels with amino and carboxyl termini on the cytoplasmic side. A hydrophobic pore-loop between 5th and 6th transmembrane region serves as selectivity filter for cations. TRP channels are non selective cation ($P_{Ca}/P_{Na} \sim 10$) channels except monovalent TRPM4 and TRPM5 and Ca^{2+} selective TRPV5 and TRPV6. Based on sequence homology several structural domains have been identified in TRP superfamily which is shown in the Figure. An individual mammalian sub-family as a unit of known putative structural domain is also shown.

Neutralization of a negative charge residue in the signature sequence of TRPV1 (D546) and TRPV4 (D682) reduces Ca^{2+} and Mg^{2+} permeability and decreases the sensitivity of the pore blocker ruthenium red in TRPV1 and TRPV4. Additionally, in TRPV4, mutation of neighboring residue D672 further reduces the selectivity for divalent cations and changes the relative permeability for monovalent cations (García-Martínez et al., 2000; Voets et al., 2002).

Charged residues in the selectivity filter of TRPV5/V6 also have dominant influence on the permeation properties of the channels. Neutralizing the aspartate (D542/D541) residue in TRPV5/V6 result in the loss of Ca^{2+} permeation, Ca^{2+} dependent current decay, and block by extracellular Mg^{2+} or Cd^{2+}, whereas the permeation of monovalent cations remains unchanged. A more detailed experiment in a region preceding (D542/D541) through substituted cysteine accessibility method (SCAM) indicates that these residues form a pore helix, a structure similar to KcsA crystal structure (Owsianik et al., 2006a). In a predicted structure of TRPV5/TRPV6 channel, a pore helix is followed by a selectivity filter with a diameter approximately 5.4 Å at its narrowest point as measured by the excluded volume theory (Voets et al., 2004) whereas TRPV1 has larger pore diameter 10.1 Å (Chung et al., 2008).

2.1.2 Pore region of TRPC

Pore region of TRPC channels is different from TRPV channels in the sense that S5-P-S6 regions of TRPC do not show a significant sequence homology to the KcsA channel; however P loop region seems to form a pore helix, a structure similar to the KcsA and TRPV5/V6 channels (Owsianik et al., 2006b).

Neutralization of all seven negative charged residues in the region between TM5 and TM6 of TRPC1 results in decreased Ca^{2+} but intact Na^{+} currents through TRPC1 channel, and shift in the reversal potential. This data indicates that the pore-forming region of TRPC1 is also localized between TM5 and TM6. In a similar approach neutralizing 3 of 5 negative charges in the loop between TM5 and TM6 of TRPC5 outcomes in a loss of La^{3+} potentiation while charge neutralization in the center of the pore does not influence the channel properties. Mutations of D633 which is situated intracellularly between the end of TM6 and the TRP box to either noncharged or positively charged residues display markedly reduced inward currents and decreased voltage-dependent Mg^{2+} block. Analogous to the glutamate residues identified in TRPC5, the negatively charged residues that determine pore properties of TRPC1 seem to be located in the distal parts of the putative pore entrance (Jung et al., 2003; Liu et al., 2003; Obukhov and Nowycky, 2005). In conclusion, negatively charged residues in the exterior of the pore region in TRPC1/C5 control permeation properties (Owsianik et al., 2006b).

2.1.3 Pore region of TRPM

The mammalian TRPM subfamily consists of eight members that share ~20% homology to TRPC channels and can be classified into three main groups: TRPM1-3, TRPM4-5, and TRPM6-7, while TRPM2 and TRPM8 are structurally distinct channels. TRPM permeability characteristics are highly variable from Ca^{2+} impermeable (TRPM4 and TRPM5) to highly Ca^{2+} and Mg^{2+} permeable e.g TRPM6, TRPM7 and the specific splice variants of TRPM3 (Nilius, 2007).

The pore forming regions (S5-P-S6) of all TRPM members are highly conserved and show limited homology to the pore regions of KcsA and TRPVs channels. Structural characterization shows that it consists of pore helix and a conserved aspartate residue (corresponding to the GM(L)GD motif in TRPV1-4 channels) seems to form the selectivity filter. Swapping of the proposed selectivity filter of TRPM4 (**981**EDMDVA**986**) with TRPV6 (**538**TIIDGP**543**) results in a functional channel with combined properties of TRPM4 and TRPV6 (Nilius et al., 2005a). Charge neutralization of E981 by alanine abolishes TRPM4 affinity to block by spermine, where as mutations to the other negative charges in the region were ineffective. This indicates strongly that E981 is placed in the inner part of the pore where it is accessible to intracellular spermine. Mutating the adjacent aspartates D982 and D984 strongly affected the rundown and voltage dependence of the channel. Substituting Q977 with negatively charges glutamate a corresponding reside in divalent cation permeable TRPM, converted the channel with moderate Ca^{2+} permeability. These mutations study indicates that the putative pore region contains critical determinants of the pore properties of TRPM4.(Owsianik et al., 2006a)

2.2 Cytoplasmic specific motifs in TRP superfamily

The following are the currently known particular cytoplasmic specific motifs in the TRP family.

2.2.1 TRP domain

A TRP domain is a highly conserved region of 25 amino acid sequence located just C-terminal to the S6 segment and initially defined in TRPC subfamily (Clapham et al., 2001; Minke and Cook, 2002; Montell et al., 2002). TRP domain includes two groups of

specific amino acids that are referred as TRP box. They are highly conserved in TRPC subfamily, while in TRPM and TRPV it is partially conserved (Gracia-Sanz et al., 2004).

2.2.2 Ankyrin repeat Domain/ TRPM Homology domain

The ankyrin repeats (ANK) are 33 residue motifs consisting of two alpha helices separated by loops (Sedgwick and Smerdon, 1999). Multiple N-terminal ANK motif is present in group 1 TRPs except TRPM and considered as a putative structural signature. The number of ANK repeats varies between different members of the same subfamily. TRPCs contain three to four ANK motifs and TRPV1 has 6 ANK motifs, while 14-15 in TRPA1 and 29 in TRPNs. TRPM channels harbor a characteristic TRPM homology region in their N-terminal region instead of ANK repeat motif and their function is not known yet (Clapham, 2003; Schindl and Romanin, 2007; Phelps and Gaudet, 2007).

2.2.3 Coiled-coil domain

A supercoil structural motif that mediates protein oligomerization is termed as coiled-coil domain. It consists of two to five twisted amphipathic α helices. It is characterized as heptad with the occurrence of apolar residues in the first and fourth position while polar residues at the fifth and seventh position (Burkhard et al., 2001).

TRPM channels contain N- and C-terminal coiled-coil domain. The TRPM C-terminal coiled- coil domain is highly conserved region of 88-120 amino acids distal to the S6 segment. In a recent report (Fujiwara and Minor, 2008), a crystal structure of the TRPM7 C-terminal coiled-coil domain comprises of four fold antiparallel coiled-coil architecture that bears unique features relative to other antiparallel coiled-coils. In TRPC1, the N-terminal coiled-coil domain is required for homo tetramerization (Engelke et al., 2002; Schindl and Romanin, 2007).

2.2.4 Calmodulin (CaM) binding domain (CaMBD)

Calmodulin (CaM) is an ubiquitous, intracellular calcium receptor and has a prominent role in controlling the activity of numerous protein targets in crucial physiological processes by binding to calmodulin binding domain (CaMBD). Calmodulin (CaM) controls many Ca^{2+}-dependent cellular processes and is an important modulator of various types of ion channels. Solution structure of the CaMBD reveals two separate flexible amphipathic helices

connected by a highly flexible type II β turn (Yamauchi et al., 2003). While crystallographic studies of MARCKS (myristoylated alanine-rich C-kinase substrate) calmodulin (CaM) binding domain in complex with Ca^{2+} CaM assumes that domain has flexible conformation that entirely depends on the environment (Sham et al., 2008). No crystal structure of CaMBD of TRP channel has been available yet.

TRPL, a close homologue of drosophila TRP, is the first candidate identified in TRP family, modulated by calmodulin. It has two C-terminus CaMBDs and can interact with CaM in a Ca^{2+} dependent and independent manners (Phillips et al., 1992). In TRPC1, calmodulin and inositol 4, 5-triphosphate competes for the same binding site referred as CaM/IP3R binding region (CIBR). Calmodulin has inhibitory effect while IP_3R can activate the channel by displacing calmodulin. Later on two C-terminal CaMBD were identified in TRPC1 and TRPC4 (Zhang et al., 2001; Trost et al., 2001).

Calmodulin also regulates various TRPV channels. TRPV1/V5/V6 contain CaMBD in their N and C-terminal tails simultaneously. Calmodulin plays a key role in TRPV1 desensitization by binding to ANK repeats in Ca^{2+} dependent manner while it binds to C-terminus in a Ca^{2+} independent manner (Numazaki et al., 2003). Studies on TRPM4 show the presence of five calmodulin binding sites in its N and C-terminal tail (Nilius et al., 2005b).

2.2.5 PDZ binding domain

PDZ domains help anchor transmembrane proteins to the cytoskeleton and hold together signaling complexes. PDZ containing scaffolds assemble specific proteins into large molecular complexes at defined locations in the cell. They are specialized in binding to their target proteins at their extreme C-termini (Kim and Sheng, 2004). A crystal structure of PDZ domains of disc large tumor suppressor protein (DIgA) showed a hydrophobic binding pocket consisting of a five-stranded antiparallel β barrel flanked by three alpha helices (Morais Cabral et al., 1996).

In TRP channels, PDZ binding domain is identified in TRPC subfamilies. In TRPC4/C5 channels, the PDZ binding motif comprises of sequence VTTRL in their C-terminus. It has been found that deleting the PDZ binding domain in TRPC4 strongly reduces its surface expression. No obvious PDZ domain is identified in TRPV5 but this channel interacts with PDZ domain of NHERF2 through their C–terminus for targeting to the plasma membrane (Tang et al., 2000; Mery et al., 2002).

2.2.6 Receptor intrinsic enzymatic activity

A characteristics feature of TRPM family is the intrinsic enzyme in the C-terminus i.e. Nudix hydrolase domain and PLIK (Phospholipase C interacting kinase) enzyme domain. TRPM2 contains Nudix hydrolase domain (NUDT9) in the C-terminus that that serve as ADP ribose mediating gating domain (Perraud et al., 2003). TRPM6 and TRPM7 harbors alpha kinase domain in their C-terminus (Clapham, 2003). Studies in this direction shows that intracellular ATP regulates TRPM6 channel activity via its alpha kinase domain independently of alpha kinase activity (Thebault et al., 2008)

2.2.7 EF hand

The EF hand is a helix-loop-helix structural domain found in a large family of calcium-binding proteins. The EF-hand motif contains a helix-loop-helix topology, in which the Ca^{2+} ions are coordinated by ligands within the loop. It consists of two alpha helices positioned roughly perpendicular to one another and linked by a short loop region (usually about 12 amino acids) that usually binds calcium ions. The six residues involved in the binding are in positions 1, 3, 5, 7, 9 and 12; these residues are denoted by X, Y, Z, -Y, -X and -Z. The invariant Glu or Asp at position 12 provides two oxygens for liganding Ca^{+2} (bidentate ligand).

The calcium ion is bound by both protein backbone atoms and by amino acid side chains, specifically those of the acidic amino acid residues, aspartate and glutamate. These residues are negatively charged and make a charge-interaction with the positively charged calcium ion. Five of the loop residues bind calcium and thus have a strong preference for oxygen-containing side chains, especially aspartate and glutamate. The sixth residue in the loop is necessarily glycine due to the conformational requirements of the backbone. The remaining residues are typically hydrophobic and form a hydrophobic core that binds the stabilizes the two helices (Ban et al., 1994; Lewit-Bently and Rety, 2000; Nelson et al., 2002; Zhou et al., 2006).

The EF hand motif is among the most common in animal cells and more than 1000 have been identified from their unique sequence signatures. They participate in modulation of Ca^{2+} signals and direct transduction of the ionic signal into downstream biochemical events (Nelson et al., 2002). TRPA1 seems to have three putative EF hand motifs in its N-terminus and C-terminus (Karashima et al., 2008) while TRPP and TRPML have one in their C-terminus (Voets et al., 2005).

2.2.8 Split pleckstrin homology domain

Pleckstrin homology (PH) domain is a structural protein module of approximately 100 amino acids mediating cellular signaling, cytoskeletal organization, regulation of intracellular membrane transport and modification of membrane phospholipids (Maffucci and Falasca, 2001). Split PH domain represents a unique class of PH domains that have been implicated in interactions with complementary partial PH domains. These domains exist as independent structural units alone and two halves of the split PH domain can fold together to form an intact PH domain. Split PH domains are found in various proteins, including phospholipase C (PLC-γ1), syntophins, Rho-kinases, myosin X, the Vps36 subunit of the yeast ESCRT-II and phosphoinositol 3(PI3)-kinase enhancer, TRPC, TRPM4, TRPM5 (Yan et al., 2005; Teo et al., 2006; Haslam et al., 1993; Mayer et al., 1993).

PLC- enzyme consists of two ends of SH2SH2SH3 supramodule are flanked by the split halves of a PH domain. The reassembled split PH domain selectively binds inositol-1,4,5-triphosphate, a target molecule of the parent PLC-γ PH domain.. In the structure of PLC-γ PH domain in complex with IP3 the inositide ring binds to the cleft and makes extensive hydrogen bonds with the positively charge residues around the loops. PH domain from PLC-γ1 is among the few exceptions in the family of PH domains for its stereospecific and high affinity binding of PIP_2 (Ferguson et al., 1995; Lemmon and Ferguson, 2000)

There is growing evidence about TRP (*e.g* TRPV1, TRPM8, TRPM4, TRPM5, TRPC3) channel regulation by PIP_2 and this interaction is made by specific PIP_2 binding sites in N- and C- terminus of the TRPs channel (Rohacs and Nilius, 2007). There are two specific PIP_2 binding sites, namely TRP box and Pleckstrin homology domain identified in the cytosolic region of TRP channel which can occur together or as an individual (Rohacs, 2006; Qin, 2007).

There are two regions of consensus sequence (R^{1136} $ARDKR^{1150}$) and $R^{1147}LKR^{1150}$ for a PH domain in the C-terminus of TRPM4 in addition to TRP box. Mutagenesis studies identify first PH domain is most favorable for PIP_2 binding while positive charge residues in TRP box and the second PH domain may contribute to the whole PIP_2 binding site (Nilius et al., 2006). In TRPC3, PIP_2 interaction is via split pleckstrin homology domain which involves amino terminal portion (40-46 amino acid) of TRPC3 and sequence of PLC-γ1 that includes carboxy terminal half of a split PH domain (PH-c) (Lemmon, 2005; van Rossum et al., 2005).

3. Structure of TRP channels

Currently no structural information for any full length TRP channel is available at the atomic level due to inherent difficulties in overexpression, purification, functional characterization and crystallization of membrane proteins. In alternative approaches, progress still goes on toward better understanding of TRP channel structure by cryo-electron microscopy, X-ray crystallization of cloned cytoplasmic domain and large scale mutagenesis combined with electrophysiology along with homology modeling together with molecular simulation studies. Homology modeling predicts structures based on sequence homology with known structures. Principle behind it is that if two proteins share a high enough sequence similarity, they are likely to have very similar three-dimensional structures. If one of the protein sequences has a known structure, the structure can be copied to the unknown protein with a high degree of confidence. Homology modeling produces an all atom model based on alignment with template protein.

Overall homology modeling procedure consists of six steps.

Template selection: Identification of homologous sequences in the protein structure database to be used as templates for modeling.

Alignment: It involves alignment of the target and template sequences.

Model build up: It involves building a framework structure for the target protein consisting of main chain atoms.

Optimization: The fourth step of model building includes the addition and optimization of side chains atoms and loops.

Refine: It involves refining and optimizing the entire model according to energy criteria.

Evaluation: The final step involves evaluating of the overall quality of the model obtained.

Repeat: If necessary alignment and model building are repeated until a satisfactory result is obtained.

A number of homology modeling programs are able to perform the complete procedures of homology modeling in an automated fashion (Xiong, 2006). Modeler (http://bioserv.cbs.cnrs.fr/HTML_BIO/frame _mod.html) is a web server for homology modeling (Douguet and Labesse, 2001). The user provides a predetermined sequence alignment of a template and a target to allow the programme to calculate a model containing all of the heavy atoms (nonhydrogen atoms). Swiss model (www.expasy.ch/swissmod/SWISS-MODEL.html) is an automated modeling server that allows a user to submit a sequence and to get back a structure automatically. The server

constructs a model by automatic alignment (first approach mode) or manual alignment (optimize mode). In the first approach mode, the user provides sequence input for modeling. The server performs alignment of the query with sequence in PDB using BLAST. After selection of suitable templates, a raw model is built. Refinement of the structures is done using GROMACS. Alternately, the user can specify or upload structures as templates. The final model is sent to the user by e-mail (Peitsch, 1995; Guex and Peitsch, 1997). In the Optimize mode, the user constructs a sequence alignment in the SwissPdbViewer and submits it to the server for model construction.

3D-JIGSAW (www.bmm.icnet.uk/servers/3djigsaw) is a modeling server that works in either the automatic mode or the interactive mode. Its loop modeling relies on the database method (Bates et al., 2001). The interactive model allows the user to edit alignments and select templates, loops, and side chains during modeling, whereas the automatic mode allows no human intervention and models a submitted protein sequence if it has an identity > 40% with known protein structure.

Modeller is a computer program that models three-dimensional structures of proteins and their assemblies by satisfaction of spatial restraints (Sali and Blundell, 1993). Modeller is most frequently used for homology or comparative protein structure modeling. The user provides an alignment of a sequence to be modeled with known related structures and Modeller will automatically calculate a model with all non-hydrogen atoms. More generally, the input to the program is restraints on the spatial structure of the amino acid sequence(s) and ligands to be modeled. The output is a 3D structure that satisfies these restraints as well as possible. Restraints can in principle be derived from a protein structures (comparative modeling), NMR experiments (NMR refinement), rules of secondary structure packing (combinatorial modeling), cross-linking experiments, fluorescence spectroscopy, image reconstruction in electron microscopy, site-directed mutagenesis, intuition, residue-residue and atom-atom potentials of mean force, etc. The restraints can operate on distances, angles, dihedral angles, pairs of dihedral angles and some other spatial features defined by atoms or pseudo atoms. Presently, Modeller automatically derives the restraints only from the known related structures and their alignment with the target sequence.

3.1 Structure of TRPV1

Rat TRPV1 is a homotetrameric integral plasma membrane protein containing 838 amino acids per subunit. It is predicted to have six (S1-S6) transmembrane helices formed by

residues 433-684 and a pore-loop region ("P loop") together with segments S5-S6 are thought to form ion selectivity filter and permeation pathways respectively. Both N- and C-terminus are located on the cytoplasmic side and comprise of 70 % of the protein.

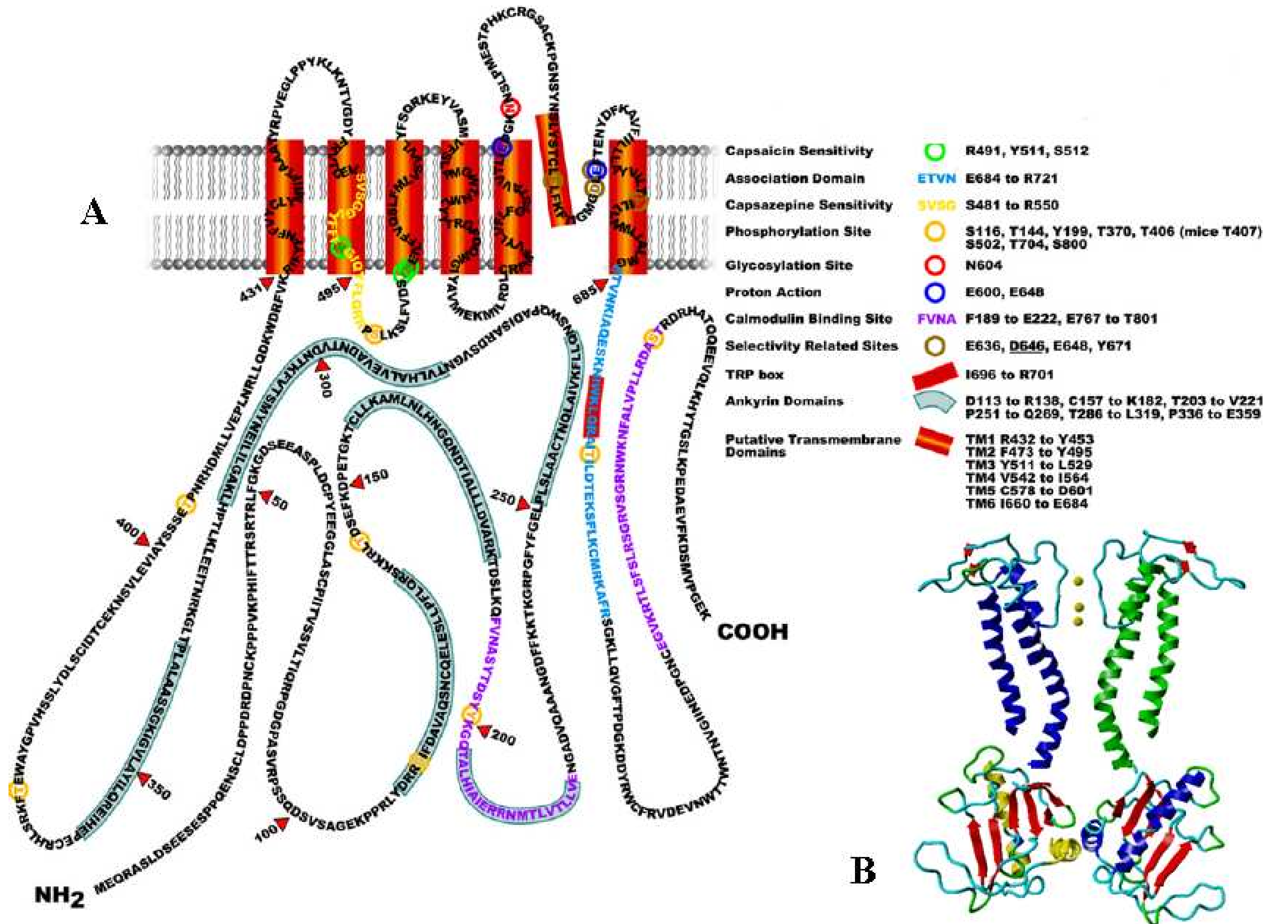

Fig. 3: Putative membrane topology of TRPV1 (A) Predicted structure and function relationship of TRPV1 **(B)** Homology model of TRPV1 S5-P-S6 region together with C-tail. Three-dimensional molecular model of the fourfold symmetric pore module $(S5\text{-}P\text{-}S6)_4$ of the TRPV1 channel was based on an identity of ~18% and a homology of ~43% between the rat TRPV1 amino acid sequence M572-E692 and the KcsA protein, the structure of which has been solved to a resolution of 1.9 Å. Homology model of TRPV1 C-terminal tail residues 690-838 were built based on 44 % sequence homology to known x-ray structure of the fragile histidine triad FHIT protein. The C-tail contains two helices, H1 and H2 and seven β strands. Only two subunits are shown for clarity. Homology model of TRPV1 S5-P-S6 region and C-tail

Full length crystal structure of TRPVs is generally lacking, however, recent report of 19 Å TRPV1 structure through electron cryomicroscopy revealed that the structure of this channel is consistent with those of other tetramers of six-transmembrane-helix channels, such as voltage-gated potassium Kv1.2 (Moiseenkova et al., 2008). In the absence of 3D structures, homology modeling and molecular dynamics study can be an indirect approach to understand structure of the channels.

3.1.1 N-terminal domain

A 47.41–kDa, 431 amino acid N-terminus protein containing ankyrin repeat, calmodulin binding domain, ATP binding pocket, a site for activation by pungent compounds and five phosphorylation sites (Fig 2, 3a) (Docherty et al., 1996; Bhave et al., 2002; Numazaki et al., 2002, 2003; Bhave et al., 2003; Mohapatra and Nau, 2003, 2005, Mandadi et al., 2004, 2006, Jin et al., 2006; Grycova et al., 2007). A shared feature of many TRP channel are long ankyrin repeats and TRP box. Ankyrin repeats are unique in the sense that they are absent in the voltage gated channels and their roles in TRPs ion channels are not very well characterized. Recent advancement in this direction is 2.7 Å crystal structure of TRPV1 ankyrin repeat. The TRPV1 ARDs have six ankyrin repeat motifs, with atypical long finger loops and a pronounced twist between the fourth and fifth repeat, such that the helices of repeats 1 – 4 and 5 – 6 are no longer nearly parallel to each other. However, in screening experiments for optimizing TRPV1-ARD crystallization conditions, it was observed that ATP altered the crystal shape, likely by changing the packing interactions between protein molecules. This new crystal form diffracted to higher resolution, allowing structure determination and refinement. The resulting electron density map indicated that an ATP molecule was indeed bound to the TRPV1-ARD on the concave surface that is typically occupied by ligand in ARD – ligand complexes.

An exclusive property of TRPV1 ARD is, a molecule of ATP is complexed to noncanonical nucleotide binding pocket with in ARD fold, shedding a light on complex regulatory mechanisms of TRPV1 tachyphylaxis “A reduced response to repeated stimuli”. Binding of an ATP molecular to ARD repeats is also confirmed by *in vitro* mutation study. An earlier understanding that ATP binding is mediated through canonical Walker box motifs is changed as no such motif is present in TRPV1 ankyrin motifs. Crystal results that ATP bound to concave surfaces formed by the repeats 1-3 (Lishko et al., 2007) is also confirmed in vitro mutation study. More interesting feature is inhabitation of ATP binding to N-terminal repeats by divalent cations in contrasting with other ATP binding pocket where this interaction is stabilized with divalents. Another clue that the TRPV1-ARD interaction with ATP is physiologically relevant is that it is conserved in the chicken homologue (Phelps et al., 2007), indicating that it is better conserved than capsaicin sensitivity because chicken TRPV1 is insensitive to capsaicin (Jordt and Julius, 2002).

3.1.2 Transmembrane region

Based on their structure, TRP's six transmembrane helices (432-684 amino acids) can be divided into two regions, S1-S4 module and S5-P-S6 module (Fig. 1). In TRPV1, transmembrane segment S2-S3 contains the binding pocket for TRPV1 agonists (capsaicin, resinferatoxin) and antagonists (e.g. capsazepine) (Jordt and Julius 2002) while proton activation involving both peripheral domain (S3-S4 linker "V538") and the pore region (pore helix "T633") region. (Ryu et al., 2007)

Recently, a homology model of the tetrameric S5-P-S6 region of TRPV1 based on ~43 % sequence homology with the KcsA channel proposed the sequence TIGMG in "P loop" analogous to the selectivity filter (TVGYG) of KcsA channels (Doyle et al. 1998). A molecular dynamics study of the tetramer complex embedded in the POPC bilayer yielded a channel with all four inner helices from N676 to A680 symmetrically bent at the intracellular ends. The pore diameter is predicted to be ~6.5 Å in the selectivity filter while ~3.8 Å in the inner pore region at I679. The denaturation protocol identifies two bending regions centered around T670 and A680 in the inner pore helix which are analogous to glycine, a "universal gating hinge" in KcsA, Shaker, Ca_v, and Na_v and to the first glycine in $G(X)_3G$ motif in gating of high voltage activated Ca^{2+} channels respectively (Susankova et al., 2007).

3.1.3 C-Terminal domain

A 17.5–kDa, 155 amino acid C-terminus protein located on cytoplasmic side comprised of coiled-coil domain, low conserved TRP domain, association domain, calmodulin binding domain, ER retention motif, two phosphorylation sites (T704, S800), an unidentified heat sensing domain (Vlachova et al., 2003; Prescott and Julius, 2003; Clapham, 2003; Garcia-Sanz et al., 2004).

In the absence of 3D structure an approximate homology model based on ~44% homology to crystal structure of 1.8 Å FHIT proteins was proposed which comprise of two helices H_1 and H_2 and seven ß strands (Fig 3b). Anti parallel strands one and two forms ß hairpin while strands three to seven form a five stranded antiparallel sheet, both helices pack on the same side of the sheet with H2 interact with strand three and a 20 residue disordered loop connecting strands two and three. According to analysis with Procheck, the model is of high quality stereochemistry (Vlachova et al., 2003).

Recent advancement in this direction is building a TRPV1 C-terminal homology model based on previous approach with walker A motif using the human cystic fibrosis

transmembrane regulator (CFTR), the transporter associated with antigen processing (TAP1), the ATP-binding subunit of histidine permease (HisP), and MJ0796, a bacterial ABC transporter cassette as template. They identified the key role of the K735 residue in the binding of the nucleotide (Grycova et al., 2007).

However different model for TRPV1 C-terminus was proposed based on sequence homology with hyperpolarized, cyclic nucleotide-gated channel HCN2 by automated Swiss-Model Protein Modeling Server at ExPASy Molecular Biology web server. Their model is composed of two distinct domains, the C-linker corresponding to the TRP-like domain (E684-R721) and the regulatory region harboring the phosphatidylinositol-4, 5-biphosphate (PIP_2) and calmodulin binding sites (E767-T801). Whereas the binding domain shows a compact structure created by a mixture of four α-helices and four anti-parallel β-strands, the TRP-like domain consists of an amphipatic α-helix connected to the modulatory domain by a flexible loop and short α -helix. In their recent study they propose a coiled-coil structure of the TRP box. However both models agree that R701 is solvent accessible and that it can interact with the membrane lipids (Vlachova et al., 2003; García-Sanz et al., 2004; Valente et al., 2008).

3.1.4 Full length structure of TRPV1

Crystal structure of full length TRPV1 is not available yet. However, in an alternative approach, full length TRPV1 structure through electron cryomicroscopy and single particle analysis reveals that TRPV1 is homologous to Kv1.2. channel (Moiseenkova-bell et al., 2008). The structure has fourfold symmetry and have two well defined domains i.e transmembrane and cytosolic. Transmembrane domain is 40 Å (length) and 60 Å diameter harboring channel pore. A large basket like cytosolic domain is 75 Å (length) and 100 Å diameter harboring both N- and C-termini together. It has a large central cavity of unknown function (Moiseenkova-Bell and Wensel, 2009).

Recently a molecular model of full length TRPV1 in closed and desensitized states has also been proposed (Fernandez-Ballester and Ferrer-Montiel, 2008). N-terminal model based on three distinct regions: 1) 111 residues forming the N-end of N-terminus, 2) crystal structure of ankyrin repeats with ATP binding pocket, 3) regulatory domain that connects the N-terminus to the membrane region. The first 111 residues (which are not crucial for capsaicin activated current) consist of proximal proline (1-35 residues) stretch and distal segment (95-111 residues) homologous to human thymidylate kinase. Amino acid residues (365-433) lack of similarity to any protein available and a small part of this domain is

modeled based on cytoplasmic beta-subunit associated to Kv1.2 channel. It serves as a connector of ankyrin repeat to the transmembrane region.

The transmembrane regions are modeled using rat Kv1.2 channel as a template. As a result of low amino acid similarities between TRPV1 and Kv1.2 channel, TMHMM algorithm was used to assign the transmembrane region. Further functional data is used to model the S2-S3 intracellular loop containing the binding sites for capsaicin and derivates near the membrane interface. The pore region of TRPV1 shows higher resemblance to the Kv1.2 channels. Overall structure shows that S5 of the one subunit interacts with the S4 of the adjacent monomer. The S6 holds at its C-end a set of hydrophobic residues that may structure the gate of the channel. The sequence TXGMG serves as a signature sequence and form the selectivity filter. The C-terminal region is modeled based on the coordinates of HCN2 channels and consists of TRP domain and a regulatory domain that are connected by a flexible linker (Fernandez-Ballester and Ferrer-Montiel, 2008)

3.2 Structure of TRPA1

Human TRPA1 is a homotetrameric integral plasma membrane protein composed of 1,119 amino acids, with a calculated molecular weight of 127.4 kDa. Each subunit is predicted to have six (S1-S6) transmembrane helices formed by residues 717-964 and a "P loop," together with segments S5-S6 is thought to form ion selectivity filter and permeation pathway respectively. Both N and C -terminus are located on the cytoplasmic side and comprise of more than 75 % of the protein. TRPA1 is a nonselective cation channel with permeability ratio ($P_{Ca}/P_{Na} \sim 0.8$) (Story et al., 2003; Nilius and Mahieu, 2006). Recent study shows TRPA1 activation by reactive or non-reactive agonists induces uptake of larger cation *i.e* Yo-Pro, which can be blocked by TRPA1 antagonists (Chen et al., 2009)**.**

3.2.1 N-terminal domain

TRPA1 has 716 residues N-terminus containing 14 predicted ankyrin repeats, EF calcium hand (Fig. 2), coiled-coil domain, a putative split pleckstrin homology domain and three cysteine residues for activation by pungent compounds (Hinman et al., 2006; Macpherson et al., 2007). A novel mode of activation by covalent modification and more numbers of ankyrin repeat together with pleckstrin homology domain in the N-terminus of hTRPA1 make channel more attractive to gain insight into TRPA1 structure. However, current knowledge in this direction is currently lacking. Crystal structure of TRPV1 ankyrin

repeats leads to identification of novel ATP binding sites and more detailed analysis of agonist induced tachyphylaxis. Similarly, 'divide and conquer' rule of getting the protein structure of difficult protein together with cell free expression system can be a good approach to gain insight into the N-terminus of TRPA1 (Chen et al., 2009).

3.2.2 Transmembrane region

In an analogy to Kv channels TRPs have six transmembrane helices (717-964). This region can be divided into two regions S1-S4 module and S5-P-S6 pore domain where TM5 is critical for menthol sensitivity in mammalian TRPA1. This study purposes a homology model of mouse TRPA1 based on homology with KcsA channel. According to sequence alignment selectivity filter of KcsA (TVGYGD) aligns with SMMLGD of mTRPA1, which may potentially form the selectivity filter of mTRPA1 (Xiao et al., 2008). Further study in this direction identifies critical residues in the putative selectivity filter of rat TRPA1 (A946, M949) and corresponding residues (S943, I946) in human TRPA1 for species specific activation and blockade.

The sequence alignment around S6 domains revealed six amino acid differences between rTRPA1 and hTRPA1. Their study is further substantiated by homology model of S5-S6 domain of rTRPA1 (Chen et al., 2008). There is substantial progress in understanding the transmembrane topology of TRPA1. A recent report showing pore dilation that occurs in TRPA1 (Chen et al., 2009) suggests that SCAM would be a good strategy to determine the pore diameter and more detailed analysis of TRPA1 pore. Two papers presented in this study deal with alanine scanning of putative inner pore region of TRPA1 to elucidate the ion permeability and channel gating.

3.2.3 C-terminal domain

TRPA1 C-terminus consists of 965-1119 residues and comprise of TRP box like motif, coiled-coil domain and a putative PH like domain. Role of the TRP box in the tetramerization of TRPV1 and its interaction with the membrane PIP_2 suggest that a low conserved TRP box in TRPA1, might have a functional role in channel regulation. Furthermore, TRPA1 modulation by PIP_2 and cluster of charge residues in close proximity to 6TM region evoke our research interests into this region. In the current study we employ an alanine scanning mutagenesis of positively charged residues together with homology model of

6TM domain and short extended C-terminal region embedded into POPC layer to gain insight into role of these regions in hTRPA1 function.

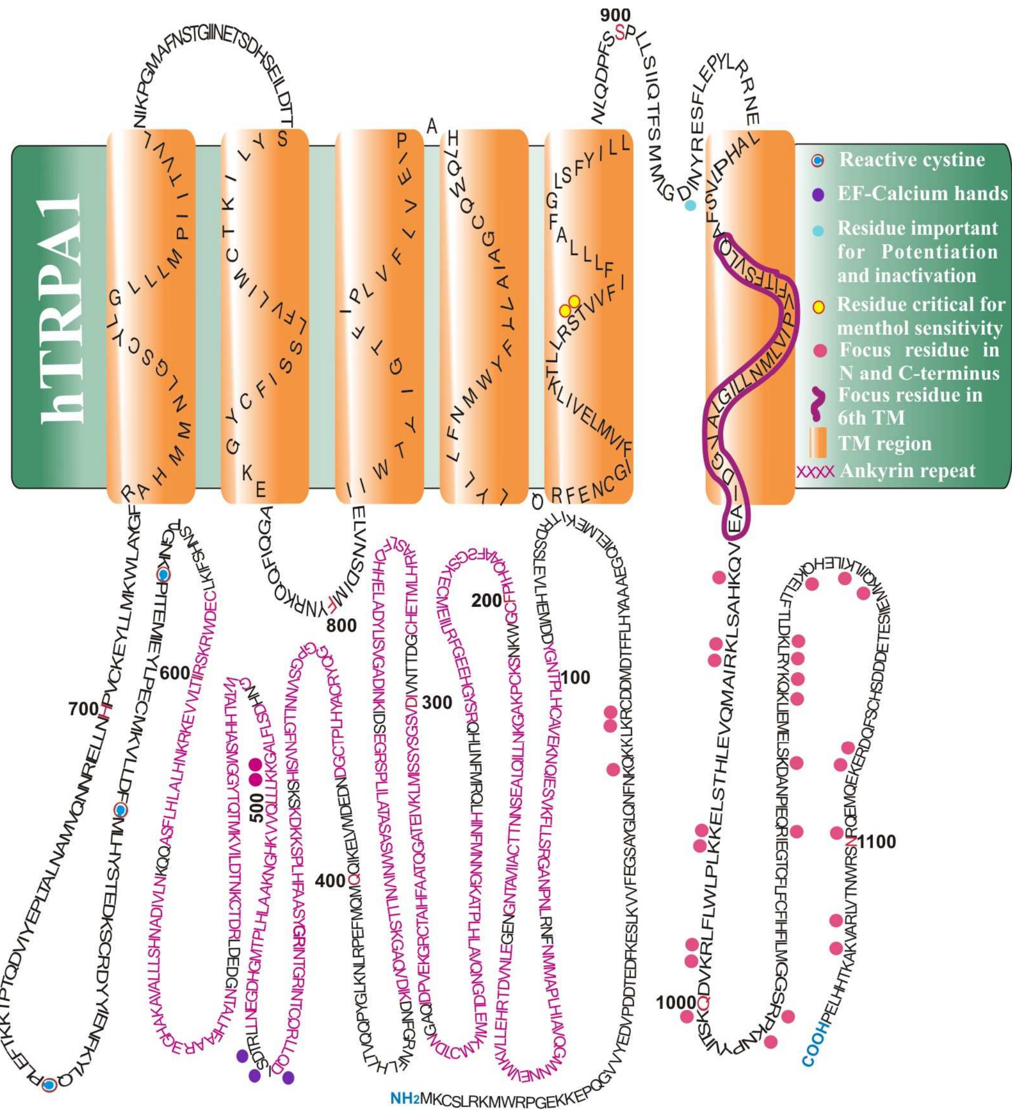

Fig.4: Putative Transmembrane topology of TRPA1. Molecular weight of TRPA1 is 125 kDa, 1119 amino acid residues, N-terminus comprise of (1-716), transmembrane region (717-964), C-terminus (965-1119) residues. Residues are numbered in sets of 100. Residues marked with filled lines in 6TM and red dots (filled) in the amino and carboxyl terminal are focus of our study.

4. General Structural and Functional Relationship of TRP Channels

Structural knowledge about TRP channels is very important for complete elucidation of their functions. Ankyrin repeats at the N-terminus play roles in protein protein interactions (TRPC1), channel tetramerization (TRPV6), membrane targeting (TRPC3) and can serve as transduction of mechanical (TRPA1,TRPN) stimuli (Engelke et al., 2002; Wedel et al., 2003; Erler et al., 2004; Corey et al., 2004; Lin and Corey, 2005). TRPM channels are devoid of ankyrin repeats and contains coiled-coil domain on N- and C-terminus required for tetramerization of TRPM2, TRPM3 and TRPM8 (Clapham, 2003). In TRPM4 C-terminus coiled-coil domain shows some similarity with the pleckstrin domain of PLC which mediates interaction with phosphatidylinositol 4, 5-bisphosphate (PIP_2) (Harlan et al., 1994, 1995).

TRP channels mediate the transmembrane flux of Ca^{2+} which can evoke downstream signaling (*e.g.* GPCR, calmodulin, various kinases and phospholipases) cascades (Ramsey et al., 2006). Calcium binds to calmodulin; Ca^{2+}-CaM complexes modulate TRP channels activity by interacting with CaMBD (Zhu et al., 2005). In TRPV6 C-terminal CaMBD region overlaps with PKC phosphorylation site. Protein kinase C dependent phosphorylation at this site alters CaM binding and delays channel inactivation. While an overexpression of CaM dominant negative mutant CaM_{1234} significantly reduced the channel activation that shows TRPM channels are modulated by calmodulin (Nilius et al., 2005b).

Like some potassium channels, TRP channels can be also regulated by membrane lipids (e.g. PIP_2). Negatively charged phosphate head groups of membrane lipids can interact with positively charged and basic amino acid sequester in the cytoplasmic domain. Calcium influx activates phospholipase C (PLC) which destabilized the interaction between the PIP_2 and cytosolic domain by removing phosphate head group. In TRPC3 the interaction with the PIP_2 occurs through split pleckstrin homology (PH) domain formed by interaction of TRPC3 N-terminus (PH_N) with PLCγ1 C-terminus (PH_C) (Lemon, 2005). While in TRPM channels interactions with PIP_2 is mediated by charged residues in the TRP box (Rohacs et al., 2005).

4.1 Known structure and function relationship of TRPV1

After cloning of capsaicin receptor it has been intensively studied to gain insight into structure and function (Caterina et al., 1997).

4.1.1 Analgesic capsaicin and the target receptor

Capsaicin, the active pungent compound in "hot" chili peppers, produces burning pain in humans by activating and sensitizing nociceptors which further leads to local release of inflammatory mediators (Sawynok, 2003; Nagy et al., 2004; Tang et al., 2008). Experimental evidence reveals that repeated application of capsaicin desensitized polymodal nociceptors to capsaicin and to other stimuli as well (Winter et al., 1995). This desensitization was suggested to occur rather due to physiological than to morphological alterations, since no evidence of degeneration was found to the site of capsaicin application (Macmahon, 1991; Kissin, 2008).

Capsaicin induced desensitization forms the basis of the therapeutic use of it to treat wide variety of pain disorders in humans (White, 2008). On the other hand, despite the burning pain, peoples find eating hot chili pepper meals highly enjoyable (Sawynok, 2003; Nagy, 2004). Scientists for more than a century had been puzzled over the diverse effects of capsaicin, but detailed understanding about this puzzle is elucidated after cloning of capsaicin activated vanilloid receptor 1 (TRPV1), a founding member of the vanilloid subfamily of TRP channels (Caterina, 1997; Montell, 2005).

4.1.2 Activation domains of TRPV1

Capsaicin is highly lipophilic compound, can pass though lipid membrane and acts on intracellular region to activate TRPV1, while a membrane impermeable capsaicin analog is only affective when applied to the intracellular side (Jung et al., 1999). According to model proposed by Jordt and Julius (2002), vanilloid binding pocket comprises of tyrosine Y511 which may interact with vanillyl functional group of capsaicin while serine S512 or arginine R491 may interact with capsaicin via hydrogen bonds.

Additional residues T550, W549, M547 in TM4 are involved in interaction with vanilloid functional group while T511 interact with the aliphatic chain of the capsaicin (Gavva et al., 2004). Other residues in N- and C-terminal domains, R114 and E761, are also involved in capsaicin binding (Jung et al., 2002).

In contrast to Kv channels which have a well defined voltage sensing domain with a conserved positively charged residues in S4 segment, TRPV1 has weak voltage dependence because of conserved single positively charge arginine residue (gating charge z =0.8, Shaker K z = 13) in the S4 segment. Since the TRPs actual voltage sensing domain is not defined yet, therefore S1-S4 is thought to be a voltage sensing domain (Nilius et al., 2005b).

Heat activation (noxious range > 43 ^{o}C) of TRPV1 is still not fully understood. No mutations have been identified yet which will selectively abolish the heat response completely. Heat activation is present in the cell free patches, indicating a membrane delimited signaling event. Cytoplasmic C-terminus is supposed to contain modulatory domains which confer temperature sensitivity phenotype to TRP channels (Caterina et al., 1997; Vlachova et al., 2003; Patapoutian et al., 2003; Hellwig et al., 2004; Pingle et al., 2007; Latorre et al., 2007).

Extracellular protons can activate and sensitize the TRPV1 channel. At pH 6 to 7, protons sensitize the heat and capsaicin response while higher concentration (pH <6) can directly activate the channel (Bevan and Yeats, 1991; Petersen and Lamotte, 1993; Martenson et al., 1994; Baumann et al., 1996; Tominaga et al., 1998). Amino acid residues E600 and E648 are crucial for potentiation and direct activation by pH (Jordt et al., 2000). Recently two discrete domains T633 in the pore helix and the other V538 in S3-S4 linker were reported to play crucial role for direct activation of the channel by low pH. Mutations in the either residue abrogated the direct activation with out affecting capsaicin and heat response and their potentiation by mild acidic pH (Ryu et al., 2007).

TRPV1 activation by exogenous ligands is very well studied but knowledge about activation through endogenous ligands was limited. Recently it has been demonstrated that TRPV1 is directly activated by diacylglycerol (endogenous ligand) by binding at Y511 (Woo et al., 2008). This further supports the view that TRPV1 is a polymodal cellular sensor which can be activated by variety of exogenous and endogenous stimuli.

4.1.3 Sensitization and desensitization of TPRV1

TRPV1 is involved in nociception and is considered vital in mediating hyperalgesia during inflammation. A variety of inflammatory mediators (see section 1.2), can sensitize the TRPV1 channels (Pingle et al., 2007). Upon activation, TRPV1 can regulate cellular Ca^{2+} levels by down regulating its own activity, a phenomenon called “desensitization”. TRPV1 exhibits two types of desensitization, both of which frequently occur in conjunction: acute desensitization, defined as diminished response during continuous vanilloid application, and tachyphylaxis, which is a reduction in the response to repeated applications. Studies in this direction have shown that cytoplasmic domains of TRPV1 have several sites that serve as switches for sensitization and desensitization of TRPV1 through phosphorylation and

dephosphorylation of channel upon activation by capsaicin (Docherty et al., 1996; Koplas et al., 1997; Bhave et al., 2003).

TRPV1 is target for several kinases like PKA (Bhave et al., 2002; Mohapatra and Nau, 2003), PKC (Numazaki et al., 2002; Bhave et al., 2003; Mandadi et al., 2004, 2006), Ca^{2+}/CaM dependent kinase II (CaM kinase II) (Jung et al., 2004), and Src kinase (Jin et al., 2004). In resting state, TRPV1 is phosphorylated and on exposure to capsaicin channel activates and Ca^{2+} permeate into the cell and start to dephosphorylate the TRPV1 by activating Ca^{2+} /calmodulin dependent Ser/Thr phosphatase B calcineurin that leads to desensitization of the channel. From these experiments, it has been concluded that the desensitization of TRPV1 is mostly a functional reflection of the dynamic balance between the Ca^{2+} dependent phosphorylation and dephosphorylation of the TRPV1 channels (Jung et al., 2004).

4.1.3.1 Protein kinase A (PKA)

Inflammatory mediators can activate the PKA pathways which influence the capsaicin or heat activated responses in sensory neurons by acting on TRPV1. Several PKA dependent phosphorylation sites have been identified among which T144, T370, and S502 (Rathee et al*.,* 2002) were found to sensitize the heat activated TRPV1 response, while S116, serves as a gate that control entry into desensitized state is prevented by phosphorylation and allowed to occur with dephosphorylation at this site (Bhave et al*.,* 2002). On contrary, residue T370 is identified as a key amino acid residue to control desensitization via Ca^{2+} dependent calcineurin phosphatase (Mohapatra and Nau, 2003).

4.1.3.2 Protein kinase C (PKC)

PKC dependent phosphorylation of TRPV1 occurs down stream activation of G protein coupled receptors (GPCRs) by inflammatory mediators. Two key serine residues (S502 and S800) were identified as target for phosphorylation of TRPV1 by PKC through direct biochemical approach. In the double mutant with alanine, sensitization or potentiation of TRPV1 activity induced by any of three different stimuli (capsaicin, proton, or heat) was abolished (Numazaki et al., 2002; Bhave et al., 2003).

4.1.3.3 Calmodulin kinase II (CaMKII)

Recently, it was reported that TRPV1 must be phosphorylated by Ca^{2+} calmodulin dependent kinase II (CaMKII) at least one of the two consensus sites, S502 and T704, before it can be activated by capsaicin (Jung et al., 2004). A mutant TRPV1 channel S502A/T704I in which two putative consensus sites for CaMKII are simultaneously (but not individually) replaced with nonphosphorylatable residues is insensitive to capsaicin. They proposed that once S502 is phosphorylated by CaMKII, TRPV1 is ready to be activated and sensitized if other key sites are phosphorylated by PKA or PKC. When only T704 is phosphorylated, TRPV1 is ready to be activated and sensitized if other key sites are phosphorylated by PKA or PKC. From these findings it was inferred that it is mostly a functional reflection of the dynamic balance between the Ca^{2+} dependent phosphorylation and dephosphorylation of the receptor protein that accounts for the desensitization of the TRPV1 channel (Jung et al*.,* 2004).

4.1.3.4 Desensitization a "Complex Phenomenon"

Later, evidence was presented that the TRPV1 desensitization might involve a much more complex Ca^{2+} dependent pathways. Acute Ca^{2+} dependent TRPV1 desensitization has been shown to be accompanied by a profound change in voltage dependence (Piper et al*.,* 1999; Gunthorpe et al*.,* 2000), loss of capsaicin binding (Jung et al*.,* 2004), and depletion of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) that indicates the involvement of Ca^{2+} dependent PLC pathways. A role for ATP was also suggested, based on the evidence that the recovery from desensitization requires a high cytoplasmic concentration of ATP to replenish PIP_2 (Liu et al*.,* 2005). A tyrosine residue Y671 within the internal pore of the TRPV1 channel was also identified to control Ca^{2+} dependent desensitization, through structural rearrangements of the channel protein complex (Mohapatra et al*.,* 2003). Although in many cases it could not be clearly distinguished which of these processes are primarily the direct cause of desensitization.

More recently a study based on crystal structure of ankyrin repeat proposes that ATP and calmodulin bind and compete for the same site on the N-terminus (Lishko et al., 2007). ATP was shown to reduce the tachyphylaxis while calmodulin increased the tachyphylaxis. The authors concluded that ATP and PIP_2 sensitize the channel and upon Ca^{2+} influx CaM activates and replaces the ATP from the N-tail and also activates the PLC pathway which

depletes the PIP_2. CaM can bind to N- and C–termini of TRPV1 and can crosslink both termini which leads to desensitization of the channels.

4.1.3.5 TRPV1 regulation by PIP_2

Charged membrane phospholipids are thought to regulate a variety of ion channels by interacting with basic, hydrophobic, and hydrophilic amino acids through phosphate head groups of PIP_2 via electrostatic, hydrogen bonding, and/or hydrophobic forces to ensure certain conformational state. For example, the interaction with PIP_2 stabilizes the inward rectifier K^+ channel, ROMK, IRK1, and G protein gated inward rectifying K^+ (GIRK) channels in the open state (Huang et al., 1989; Rohacs et al., 1999; Fan and Makielski, 1997; Huang, 2007).

Among mammalian TRP channels, at least seven have been reported to be regulated by the cellular PIP_2 levels. There are conflicting reports on the effect of PIP_2 on TRPV1 channels. TRPV1 shows a PLC/ NGF dependent inhibition, where binding of NGF to TrkA is coupled to PLC activation that leads to PIP_2 hydrolysis (Prescott and Julius, 2003). A C-terminal region of TRPV1 rich in basic residues was identified as a PIP_2 binding site. Mutations in the distal C terminal region are supposed to weaken the PIP_2 TRPV1 interaction and reduce thresholds for chemical and/or thermal stimuli., TRPV1 inhibition through PIP_2 was found critical based on the fact that PIP_2 levels are strongly reduced upon TRPV1 activation, and that the recovery of TRPV1 activity after desensitization requires and coincides with recovery of cellular PIP_2 levels (Liu et al*.,* 2005).

Others also reported that PIP_2 depletion after TRPV1 activation leads the channel to desensitize and alters the channel sensitivity to agonist. They suggest that activation of TRPV1 triggers pain sensation and Ca^{2+} influx confers adaption " A condition after attaining full desensitization " remains fully responsive to stimuli over a shifted intensity range onto TRPV1 receptor (Yao and Qin, 2009).

An alternative pathway has been proposed for the inhibition based on NGF/PI3K dependent phosphorylation of the TRPV1 C-terminal domain and a subsequent increase in membrane expression (Zhang et al*.,* 2005). These observations, together with the finding that, in excised patches, PIP_2 activates TRPV1 rather than inhibits (Nilius and Mahieu, 2006) where as sequestering PIP_2 using polylysine inhibits channel function make uncertain the existence of a specific PIP_2 inhibitory domain (Stein et al*.,* 2006). Recent related studies suggest that phosphoinositides have dual effects on TRPV1 and a balance between the

inhibitory and activating effects depends on the strength of stimulation (Lukacs et al., 2007). They further propose that regulation of TRPV1 by phosphoinositides is highly complex and additional studies are required to fully elucidate the mechanisms and the interplay between the regulating and inhibitory phosphoinositides effects. Other studied TRPV1 and PIP_2 interaction by direct application of phosphoinositides specific pleckstrin homology domains in isolated patches and PIP_2 modulators in the whole cell configuration. Results reveals that $PI(4,5)P_2$ and no other phosphoinositides is bound to TRPV1 and sequestration of $PI(4,5)P_2$ in excised patches inhibits TRPV1, whereas the addition of $PI(4,5)P_2$ stimulates TRPV1 (Klein et al., 2008).

4.2 Known structure and function relationship of TRPA1

TRPA1 is most recently joined member of TRPs family and considered an ion channel essential for sensing chemical damage. Understanding their physiology with relevance to their structure will help us to cure wide variety of pain disorder in humans.

4.2.1 Activation by pungent compounds and menthol

Plant derived products like isothiocyanates (found in mustard, horseradish, wasabi, brussels sprouts, capers), cinnamaldehyde (found in cinnamon), allium (onion), and an environmental irritant acroline activate TRPA1 through a mechanism distinct than classical ligand and receptor interaction. These compounds are membrane permeable electrophiles that can form covalent adducts with thiols, primary amines, and to some extent with hydroxyl group (Smith et al., 2001). Site directed mutagenesis and pharmacological experiments with cysteine modifying agents point out N-terminus cysteine residues (C619, C639, C663) are covalently modified by isothiocyanates via Michael addition reaction. TRPA1 cysteine modification with AITC and cinnamaldehyde is reversible while N methyl maleimide (NMM) is irreversible (Hinman et al., 2006; Macpherson et al., 2007).

A pungent thioaminal compound 4-methyl- *N*-[2,2,2-trichloro-1-(4-nitro-phenylsulfanyl)-ethyl]-benzamide (CMP1) is also a potent electrophile that can covalently modify the cysteine residues in TRPA1 channel. However its effect on the rTRPA1 and hTRPA1 is opposite. It activates rTRPA1 but not hTRPA1 rather it inhibits AITC and URB597 (non reactive agonist) evoked current in hTRPA1. A chimeric channel in which S5-P-S6 of rat was introduced into hTRPA1 was activated by CMP1 while the reverse chimeric channel was not activated rather it inhibits AITC and URB597 response. It identifies the

species specific determinants of channel gating. Further mutation experiments leads to identification of A946 and M949 in rat are critical for activation while corresponding residue S943, I946 in hTRPA1 are critical for blockage. However CMP1 can not be used as potential antagonist as it can modify cysteine residues in other protein and can have unwanted effects (Chen et al., 2008).

Variety of inflammatory mediators (15-deoxy- Δ12,14-prostaglandin J2 (15d-PGJ2), nitric oxide (NO), hydrogen peroxide (H_2O_2), and proton (H^+)) can also activate human TRPA1 by reacting with N-terminal cysteine residues (C421,C621) which shows that TRPA1 channels are targeted by an array of inflammatory mediators to elicit inflammatory pain in the nervous system (Takahashi et al., 2008).

TRPA1 is also activated by menthol, thymol and hydroxy-α-sanshool (HαSS) (Koo et al., 2007). Menthol has a dual effect on TRPA1. In a recent report, TM5 of mammalian TRPA1 is considered critical for menthol responsiveness (Xiao et al., 2008). Sequence alignment of mammalian TRPA1 with other species and mutation study reveals that in hTRPA1, Ser873/Thr874 critically determine the menthol sensitivity of the TRPA1 channel. The idea is further strengthen by homology model of mouse TM5 region and docking of menthol which makes hydrophobic contacts with Gly878 and Thr877 residues critical for menthol sensitivity in mouse and form a putative menthol binding pocket.

4.2.2 Direct activation by Ca^{2+}

Calcium directly gates recombinant TRPA1 channels in whole cell and excised patches. This activation occurs via EF hand calcium binding domain (D468-L480) in its cytoplasmic N-terminal domain and in a PLC independent fashion. EF hand is highly conserved within different species. An alanine scanning of the conserved residues in TRPA1 EF hand shows that leucine at position 474 is involved in a Ca^{2+} dependent activation.

Extracellular Ca^{2+} potentiates AITC, cinnamaldehyde, carvacrol and icilin. In the absence of Ca^{2+}, AITC- and cinnamaldehyde- induced inward currents developed slowly and were potentiated with addition of Ca^{2+} in bath solution (Doerner et al., 2007). However, icilin shows a little or no activity in the absence of Ca^{2+} which recovered when Ca^{2+} was replenished in bath. These results show that Ca^{2+} directly modulates TRPA1 and consider as coagonist with icilin. However the current study in this direction shows that effect of extracellular Ca^{2+} on TRPA1 current (*i.e.* potentiation and inactivation) is indirect and can be entirely attributed to entry through TRPA1 and subsequent elevation of intracellular Ca^{2+}.

Both processes are independent of each other and affected very much by mutation D918A which reduced Ca^{2+} influx and neither of these processes are affected by mutation of a putative EF hand motif (Wang et al., 2008).

4.2.3 TRPA1 regulation by PIP_2

TRPA1 coexpression with TRPV1 in nociceptive neurons parallels many cellular pathways between both channels *i.e.* TRPV1 is activated by NGF while TRPA1 is activated by bradykinin via PLC which depletes membrane phosphatidylinositol 4,5-bisphosphate (PIP_2). Furthermore, desensitization of TRPV1/TRPA1 entirely depends upon Ca^{2+} and is regulated by PIP_2. A recent report shows that PIP_2 has dual (inhibition and potentiation) effect on TRPV1 while reports about PIP_2 effect on TRPA1 are conflicting (Lukacs et al., 2007; Rohacs et al., 2008; Akopian et al., 2007).

Recent reports show that PIP_2 inhibits recombinant TRPA1 (Dai et al., 2007; Kim et al., 2008). In their series of experiments direct application of PIP_2 to inside out patches did not activate TRPA1 while PIP_2 inhibits AITC activated currents. PIP_2 scavenger polylysine and PIP_2 antibody have a positive effect on AITC activated whole cell currents which were significantly diminished by PIP_2 while both scavengers activate TRPA1 in inside out patches (Kim et al., 2008). On the contrary, application of PIP_2 and Mg^{2+} ATP to inside out patches induces recovery of TRPA1 activity and PIP_2 protects the channel from desensitization. In their whole cell experiments with recombinant TRPA1, desensitization was delayed when PIP_2 was supplemented via the patch pipette, whereas the PIP_2 scavenger neomycin accelerated desensitization. Wortmannin, an inhibitor of PI_4K also could not settle the conflicting issue between both studies (Karashima et al., 2008). However, both authors agree that polylysine activated TRPA1 current in inside out patches. In sum the present understanding about TRPA1 regulation through PIP_2 is interesting because one report concludes that PIP_2 has a complex and inhibitory role on TRPA1 while other suggests that PIP_2 modulates TRPA1 but to lesser extent as compared with other TRP channels (Karashima et al., 2008; Kim et al., 2008). However both studies did not mention any specific region which can interact with the membrane PIP_2.

5. Open Questions

The general objectives of the present study are to identify and characterize the key residues of TRPV1 and TRPA1 receptors that are responsible for channel regulation and to describe how the molecular structure of these receptors is related to their functional channels.

The specific aims of our experiments are:

1) Reviewing the concepts of capsaicin induced Ca^{2+} dependent TRPV1 desensitization.
2) To elucidate the role of R701 in the TRP box of rat TRPV1 in channel gating.
3) Defining the gating region in the putative inner pore domain of human TRPA1 channel.
4) Determining the functional role of basic residues in the putative cytoplasmic carboxyl-terminal tail of human TRPA1 in channel gating.

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7) List of Publications

I) Karolina Tousova, Ladislav Vyklicky, Klara Susankova, Jan Benedikt, Abdul Samad, Jan Teisinger, Viktorie Vlachova. (2007). Functional Changes In The Vanilloid Receptor Subtype 1 Channel (TRPV1) During And After Acute Desensitization. Neuroscience. 149:144-154.

Abstract: Agonist-induced desensitization of the transient receptor potential vanilloid receptor-1 (TRPV1) is one of the key strategies that offer a way to alleviate neuropathic and inflammatory pain. This process is initiated by TRPV1 receptor activation and the subsequent entry of extracellular Ca^{2+} through the channel into sensory neurones. One of the prominent mechanisms responsible for TRPV1 desensitization is dephosphorylation of the TRPV1 protein by the Ca^{2+}/calmodulin- dependent enzyme, phosphatase 2B (calcineurin). Of several consensus phosphorylation sites identified so far, the most notable are two sites for Ca^{2+}/calmodulin dependent kinase II (CaMKII) at which the dynamic equilibrium between the phosphorylated and dephosphorylated states presumably regulates agonist binding. We examined the mechanisms of acute Ca^{2+}-dependent desensitization using whole-cell patch-clamp techniques in human embryonic kidney (HEK) 293T cells expressing the wild type or CaMKII phosphorylation site mutants of rat TRPV1. The nonphosphorylatable mutant S502A/T704I was capsaicin-insensitive but the S502A/T704A construct was fully functional, indicating a requirement for a specific residue at position 704. A point mutation at the nearby conserved residue R701 strongly affected the heat, capsaicin and pH-evoked currents. As this residue constitutes a stringent CaMKII consensus site but is also predicted to be involved in the interaction with membrane phosphatidylinositol 4,5-bisphosphate (PIP2), these data suggest that in addition to dephosphorylation, or as its consequence, a short C-terminal juxtamembrane segment adjacent to the transient receptor potential box composed of R701 and T704 might be involved in the decelerated gating kinetics of the desensitized TRPV1 channel.

II) Ladislav Vyklicky, Karolina Tousova, Jan Benedikt, Abdul Samad, Filip Touska, Viktorie Vlachova. (2008). Calcium-dependent Desensitization of Vanilloid Receptor TRPV1: A Mechanism Possibly Involved in Analgesia Induced by Topical Application of Capsaicin. Physiological Research. 57: 59-68.

Abstract: The rationale for the topical application of capsaicin and other vanilloids in the treatment of pain is that such compounds selectively excite and subsequently desensitize nociceptive neurons. This desensitization is triggered by the activation of vanilloid receptors (TRPV1), which leads to an elevation in intracellular free Ca^{2+} levels. Depending on the vanilloid concentration and duration of exposure, the Ca^{2+} influx via TRPV1 desensitizes the channels themselves, which may represent not only a feedback mechanism protecting the cell from toxic Ca^{2+} overload, but also likely contributes to the analgesic effects of capsaicin. This review summarizes the current state of knowledge concerning the mechanisms that underlie the acute capsaicin-induced Ca^{2+}-dependent desensitization of TRPV1 channels and explores to what extent they may contribute to capsaicin-induced analgesia. In view of the polymodal nature of TRPV1, we illustrate how the channels behave in their desensitized state when activated by other stimuli such as noxious heat or depolarizing voltages. We also show that the desensitized channel can be strongly reactivated by capsaicin at concentrations higher than those previously used to desensitize it. We provide a possible explanation for a high incidence

of adverse effects of topical capsaicin and point to a need for more accurate clinical criteria for employing it as a reliable remedy.

III) Jan Benedikt*, Abdul Samad*, Rudiger Ettrich, Jan Teisinger*, Viktorie Vlachova. (2009). Essential role for the putative S6 inner pore region in the activation gating of the human TRPA1 channel Biochimica et Biophysica Acta (BBA)- Molecular Cell Research. 1793: 1279-1288. * Equal contribution

Abstract: The ankyrin transient receptor potential channel TRPA1 is a sensory neuron-specific channel that is gated by various proalgesic agents such as allyl isothiocyanate (AITC), deep cooling or highly depolarizing voltages. How these disparate stimuli converge on the channel protein to open/close its ion-conducting pore is unknown. We identify several residues within the S6 inner pore-forming region of human TRPA1 that contribute to AITC and voltage-dependent gating. Alanine substitution in the conserved mid-S6 proline (P949A) strongly affected the activation/deactivation and ion permeation. The P949A was functionally restored by substitution with a glycine but not by the introduction of a proline at positions −1, −2 or +1, which indicates that P949 is structurally required for the normal functioning of the TRPA1 channel. Mutation N954A generated a constitutively open phenotype, suggesting a role in stabilizing the closed conformation. Alanine substitutions in the distal GXXXG motif decreased the relative permeability of the channel for Ca^{2+} and strongly affected its activation/deactivation properties, indicating that the distal G962 stabilizes the open conformation. G958, on the other hand, provides additional tuning leading to decreased channel activity. Together these findings provide functional support for the critical role of the putative inner pore region in controlling the conformational changes that determine the transitions between the open and close states of the TRPA1 channel.

IV) Abdul Samad*, Lucie Sura*, Jan Benedikt, Rudiger Ettrich, Babak Minofar, Jan Teisinger, Viktorie Vlachova. The C-terminal basic residues regulate the chemical and Voltage-dependent activation of TRPA1. Accepted in Biochemical Journal.

Abstract: The ankyrin transient receptor potential channel TRPA1 is a nonselective cationic channel that is expressed by sensory neurons, where it can be activated by pungent chemicals, such as allyl isothiocyanate (AITC), cinnamon or allicin, by deep cooling (< 18°C) or highly depolarizing voltages (> +100 mV). From the cytoplasmic side, this channel can be regulated by negatively charged ligands such as phosphoinositides or inorganic polyphosphates, most likely through an interaction with an as yet unidentified positively charged domain(s). In the present study, we have mutated 27 basic residues all along the C-terminal tail of TRPA1, trying to explore their role in AITC- and voltage-dependent gating. In the proximal part of the C-terminus, the function-affecting mutations were at K969, R975, K988 and K989. A homology model of the transmembrane region with the proximal portion of the first intracellular helix was refined by molecular dynamics in a lipid bilayer and used to interpret the data. A second significant region was found in the predicted helix, centered around K1048 and K1052, in which single alanine mutations completely abolished AITC- and voltage-dependent activation. In the distal portion of the C-terminus, the charge neutralizations K1092A and R1099A reduced the AITC sensitivity, and, in the latter mutant, increased the voltage-induced steady-state responses at +200 mV. Together, our findings identify basic residues in the C terminus that are strongly involved in TRPA1 voltage and chemical

sensitivity, some of them may represent possible interaction sites for negatively charged molecules that are generally considered to modulate TRPA1.

V) Karolina Tousova, Jan Benedikt, Abdul Samad, Ladislav Vyklicky, Viktorie Vlachova. (2007). Analgesic effects of capsaicin: thousands years undetermined mechanism. Bolest. 2:19-24.

Abstract: Since time immemorial, people have used the effects of pungent compounds such as resiniferatoxin and capsaicin for the treatment of various painful disorders. Although the first written records on vanilloids and their therapeutic use in pain management go back thousands of years, until now, the precise mechanisms of their analgesic action remain unclear. The aim of this article is to provide an overview of the current status of research on antinociceptive effects of capsaicin at the cellular and molecular level. The most recent combination of electrophysiological data with known structural information has now indicated possible mechanisms by which acute topical application of capsaicin could result in the Ca^{2+}-dependent desensitization of the transient receptor potential vanilloid receptor-1 (TRPV1); this provides for some exciting and testable hypotheses, which will allow yet more clarification in this area: 1. Among the Ca^{2+}-activated enzymes that are believed to play pivotal roles in TRPV1 desensitization is the Ca^{2+}/calmodulin-dependent phosphatase 2B, calcineurin, which dephosphorylates TRPV1 receptors. 2. The second hypothesis proposes that TRPV1 Ca^{2+}-dependent desensitization is mediated by calmodulin which interacts with TRPV1 at amino- and carboxyl-terminal regions. 3. The third hypothesis is based on the finding the activation of TRPV1 results in potent depletion of phosphatidylinositol 4,5-bisphosphate (PIP2), presumably due to the Ca^{2+} influx, and that PIP2 is required to recover the activity of the channel after desensitization.

8. Summary of Papers

The aim of the studies incorporated in the presented doctoral thesis was to gain insight into structural and functional aspects of two ion channels, rat transient receptor potential cation channel, subfamily V, member 1 (TRPV1), also known as the capsaicin receptor, and human transient receptor potential cation channel, subfamily A, member 1 (TRPA1), and to answer the following three specific research questions:

1. How to reconcile the concepts of capsaicin induced TRPV1 desensitization with our evidence that mutating the two putative Ca^{2+}-CaM dependent phosphorylation sites does not affect TRPV1 functioning?

Compared with their initial state, desensitized channels have reduced apparent affinity for agonist which is evident from the fact that the full response of the desensitized channels can be restored by using supersaturating concentration of the agonist. Dephosphorylation of TRPV1 at S502/T704 by calcineurin was suggested to control the binding capacity of capsaicin. In a series of experiments with double mutant TRPV1, mutant S502A/T704I was capsaicin insensitive while S502A/T704A was fully functional. This finding did not support the original hypothesis and instead suggested a new structural notion for the requirement of a specific residue at the T704 position (Paper I).

The new hypothesis was further supported by a mutation of the nearby residue R701. Being positively charged, its location in the conserved TRP box and the part of consensus sequence for phosphorylation via CaMKII and PKC make it a significantly important residue to control channel gating. Charge neutralization of R701A slowed down the capsaicin-dependent activation and deactivation of the TRPV1 channel. Further, R701A did not respond to pH 5.0 but still can potentiate the capsaicin response and has a minor response to heat while voltage signature completely resembles wild type. Based on its strategic location very close to the 6th TM domain and the phenotype of the residue in combination with structural analysis of homology model of TRPV1 C-terminus based on the FHIT crystal structure, that was published by our group earlier, we hypothesize that R701 interacts with negatively charged membrane lipids, phosphatidylinositol 4,5-bisphosphate or PIP_2, and control the downstream gating mechanism after channel activation through its specific stimuli (Paper II).

2. Is the gating mechanism of TRPV1 universal for transient receptor potential channels and therefore the same for human TRPA1, or has TRPA1 a unique mechanism?

Capsaicin does not activate TRPA1 rather it is activated by isothiocyanates (horseradish, mustard), cinnamaldehyde (cinnamon) and allicin (garlic). Agonist induced TRPA1 response is modulated by Ca^{+2} and PIP_2. A series of experiments are conducted to explore TRPA1 gating and its modulation with PIP_2. The experimental design and interpretation is guided by a three-dimensional homolgy model refined by moleclar dynamics simulation. The study reveals that TRPA1 has several residues in the putative inner pore region and C-terminal domain, responsible for proper channel function.

A sequence comparsion of the sixth transmembrane spanning region of TRPA1 with other P-loop containing channels identifies a conserved P949 and a distal bi-glycine (GXXXG) motif to be potentially involved in channel gating. P949 in TRPA1 is structurally in the same position as the highly conserved glycine hinge located near the middle of transmembrane spanning helix S6 in many Kv channels. A mutation in this position of proline to alanine shows the mutant channel P949A to have a strong defect to agonist AITC and voltage while mutant channel P949G has a signature close to wild type. Proline, an amino acid not found in clasical α-helical structure, results in a slight bending and deviation from optimum when present in helical structure. These results suggest that the bending is not by chance and that TRPA1 needs helical bending at position 949 for proper functioning.

In TRPA1 the distal biglycine G958XXXG962 is analogous to a bi-glycine motif in several K_v and Ca_v channels. This motif has a substantial oligomerization potential and high affinity for association of transmembrane helices. Mutations in this motif directly influence channel functioning. TRPA1 channel with the mutation G958A remains open for a longer time as compared to wild type while G962A has short flickery openings. This can be structurally interpreted in a way that G958A does not form a flexible hinge for a bending motion in the lower region of the pore while G962A may narrow the region that severly affects ion permeation through the channel. G962 therefore is required in the open conformation of the channel (Paper III).

TRPA1 is a polymodal non-selective Ca^{+2} permeable channel. Extracellular Ca^{2+} potentiates and inactivates AITC-induced currents and permeating Ca^{+2} ions interact at some of the key places and control gating. In order to follow that region we introduced charges at the critical sites (P949E, P949H), that reduce AITC response and slow down the channel kinetics. Interestingly, a mutant P949K hinders the inward flow of cations but shows a strong

outward rectification. The charges at P949 probably interfere with permeating cations and influence channel gating as well. A mutation G958K converts the channel to prominent inward rectification by attraction to the nearby residues D963 and E966. In inward rectifiers channel a double ring of negative charges blocks the outward current by $[Mg^{+2}]_i$. A mutation D963K blocks the inward flow of cations while exhibiting a prominent outward rectification, demonstrating the region's importance for channel gating in an interplay with other regions. Charge reversion by mutation at E966 to positively charged lysine slows down channel kinetics without affecting the channel signature. E966 therefore is not involved in forming the ion conduction pathway which is also confirmed by our TRPA1 homology model.

With the structural approach of homology modeling and *in vitro* site directed mutagenesis in the sixth transmembrane spanning helix we are able to map the regions involved in TRPA1 gating. Gating significantly differs from the mechanism described for TRPV1. Cations permeability is controlled by centrally located P949 which makes the channel pore flexible to open and close. This happens in an interplay with distal G962 serving as stabilizer for the open conformation while G958 is required for normal packing or oligomerization of the inner helices. The key difference from TRPV1, however, is the extension of the gate to cytoplasmic part of the channel, up to E966 which resides near the putative inner mouth of the channel (paper III).

3. What is the functional role of basic residues in the putative cytoplasmic carboxyl-terminal tail of human TRPA1?

The cytoplasmic C-terminus of TRPA1 is hypothesized to play a vital role in gating and regulation of the channel by phosphoinositides or inorganic polyphosphates by interacting with unidentified positively charged domains. To unravel role of basic residues in AITC and voltage dependent gating, we mutated all 27 positively charged residues in the C-terminus of TRPA1 to alanine. An initial screening with the agonist AITC showed significantly reduced AITC response for 9 mutants. Among them, K969 is the most critical residue which does not tolerate alanine, isoleucine or arginine. Reversion of the charge in mutant K969E speeds up channel kinetics as compared to K969A and K969I. AITC induced steep secondary phase in K989E and K989N was only observed upon prolonged (> 40s) application. In the mutants K969R, R975A, K989A, K1071A and K1092A, the secondary phase was not observed and prolonged AITC exposure led to inactive channels (Paper IV).

Voltage dependent gating was studied in a voltage range from -80 mV up to + 200mV by +20 mV step, applied first in control solution and then in the presence of 200 μM AITC. In the initial screen, we identified residues located in the most proximal region to the transmembrane segments that have a critical role in voltage dependent gating. Under control conditions, R975A, K989A, K989E exhibited higher outward currents and stronger outward rectification than wild type. Depolarization led to instantaneous outward activation while repolarization induced very small inward currents in the R975A mutant indicating that the inward flow of cations was blocked at negative membrane potentials. Furthermore, in mutants K988A and K989A, voltage induced activation was faster than in wild type and not significantly different from K989E or K989N, implying that charge alone cannot explain the voltage dependent functional changes in these cases.

Further characterization of voltage dependent component of gating was performed by measuring the conductance to voltage (G-V) relations in control solution and in the presence of AITC. In control solution, G-V relations were shifted toward less depolarizing potentials in K969A, R975A, and K989A which indicates enhanced voltage dependent activity at more physiological potentials. In the presence of AITC, G-V relations were virtually voltage independent in wild type TRPA1 and in K988A. The voltage dependent component of AITC induced gating was strongly increased in R975A, K989A, K989E and K989N. Mutant K969E exhibited saturation followed by a decrease in the maximum outward conductance at higher voltages +120-140 mV. The negative charge at the C-terminus proximal region either interferes with the permeation of cations in the outward direction or changes in the voltage dependent gating.

Altogether, wild type TRPA1 channel showed voltage independent gating in the presences of saturating concentration of AITC, charge reversion from positive to negative at position R975 leads to a non functional channel while K969E has strong defects in channel opening and a decrease in conductance at higher voltage (>+100 mV). Interestingly, K969E response to AITC at -70 mV resembles the wild type channel. All these results converge to the interpretation that voltage dependent gating in TRPA1 can be described by a model having anionic molecules blocking the inner pore at a negative potential and release at strong depolarization.

The activation of gating of TRPA1 channel is fine tuned by several positively charged residues located in the putative C terminal tail. Impaired gating of TRPA1 is also observed in K1009, K1046, K1071, K1092 and K1099. It has been shown that TRPA1 is modulated by PIP_2 and this interaction is maintained by electrostatic interactions between a positively

charged lipid binding pocket and PIP_2. TRPA1 C-terminus sequence analysis identifies a pleckstrin homology domain [K/R]-X3-11-[K/R]-X-[K/R]-[K-R]. Within this region the mutation of K1092 leads to reduced AITC sensitivity. Furthermore, the putative pleckstrin homology domain motif K-Xn-[K/R]-X-R can be found in two places, K1001-R1011 and K1046-K1052. In these regions a mutation of K1009 exhibits reduced AITC and voltage dependent gating, while mutations of K1048, K1052 lost the channel function completely.

Conclusions:

The studies in this thesis investigated the molecular mechanisms that regulate two members of the TRP ion channel superfamily: TRPV1 and TRPA1. Understanding which mechanisms control the responses of these channels may lead to new ideas about future drug targets and therapeutics to modulate their function.

a) The mechanisms of acute Ca^{2+} -dependent desensitization of the rat vanilloid receptor TRPV1 is investigated and a short C-terminal juxtamembrane segment adjacent to the TRP box comprised of R701 and T704 is demonstrated to be possibly involved in the decelerated gating kinetics of the desensitized TRPV1 channel in addition to dephosphorylation, or as its consequence.

b) Evidence that the putative inner-pore S6 region of the ankyrin receptor channel A1 plays an active role in AITC and voltage-dependent channel gating is provided and several residues that are critical for controlling the processes by which this channel opens and closes its pore in response to chemical and voltage stimuli are identified.

c) Several significant regions within the C-terminus in which positively charged amino acids confer both chemical and voltage sensitivity to TRPA1 are identified.

These key findings and the underpinning experimental expertise are a good starting points for further research with the aim to determine the mechanisms of activation and modulation of these two physiologically important ion channels.