1. Introduction

1.1. General overview about the TRP Channels.

The Transient Receptor Potential (TRP) family of ion channels is named after the *Drosophila melanogaster* ion channel that is mutated in the *trp* mutant. This mutant was first reported by Minke (Minke B. 1977). It was further shown that the photoreceptors carrying a mutation in the *trp* gene exhibited a transient-voltage response to continuous light (Montell et al. 1985). Later on, the presence of TRP channels has been reported in lower eukaryotes too. In yeast, TRP channels are used to perceive and respond to hyper-tonicity (Denis and Cyert. 2002; Zhou et al. 2003). In nematodes, TRP channels are located at the tips of the neuronal dendrites (as their "noses") and are responsible for detecting noxious chemicals (deBono et al. 2002). Male mice use a pheromone-sensing TRP channel to communicate with other males and also for sex-discrimination (Stowers et al. 2002). Humans use TRP channels not only to sense different tastes (Zhang et al. 2003), but also to discriminate warmth, cold and hot. In all these cases, TRP channels mediate the sensory transduction not only for multi-cellular organism, but also at the level of single cells. TRP channels in eukaryotes are mainly involved in sensory processes. TRP-channels can be identified by overall homology in their amino-acid sequences, similar structural features, and by the presence of a TRP-box and other related-motifs. Mammalian TRP-channels have been classified in different subfamilies based on their sequence similarity. In humans at least 28 genes have been considered as conventional TRP channels (Clapham D.E. 2003).

The mammalian TRP family consists of six related-subfamilies, with sequence identity as low as 20% (Figure 1.1) (Clapham D.E. 2003). The six subfamilies are TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPP (Polycystin), TRPML (Mucolipin) and TRPA (ANKTM1) ion channels. With the exception of some polycystins, all TRP channels are predicted to have four subunits of 6-transmembrane (6TM) polypeptides that assemble into a tetramer forming the cation-permeable-pore. The most conserved region among all these TRP channels is the S6 transmembrane sequence. Most of the TRP channels are non-selective cation channels.

Both the N- and C-terminus of TRP-channel polypeptides form cytoplasmic domains. The N-terminal cytoplasmic domain of the TRPV and the TRPC channels contain single to multiple ankyrin repeats. The C-terminal cytoplasmic domain contains a TRP-box (in many TRP channels), which is well conserved in members of the TRPC subfamily, but less conserved within TRPM and TRPV subfamilies. Other sequence features to occur commonly

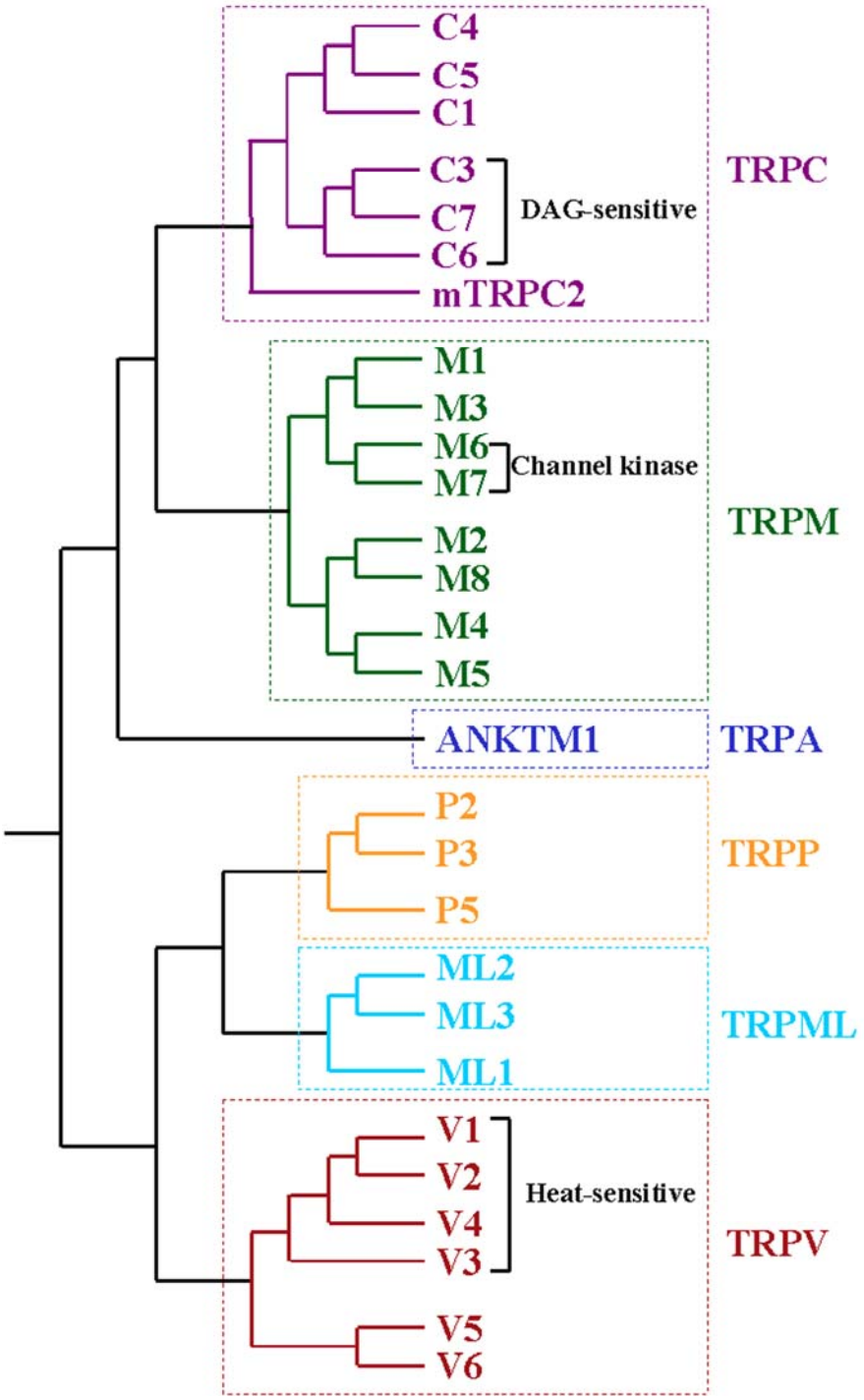

Figure 1.1. The TRP family tree (from mammals). The TRP-family is divided in 6 subfamilies: TRPC (Canonical), TRPM (Melastatin), TRPA (ANKTM1), TRPP (Polycystin), TRPML (Mucolipin) and TRPV (Vanilloid). Image adapted from Clapham D.E. (2003).

in some TRP channels are: a Ca^{2+}-sensing EF-hand motif, different phosphorylation sites and the Calmodulin binding sites.

1.2. TRPV1.

Capsaicin, the main pungent ingredient of chilli, is known to excite pain by exciting a particular subset of Dorsal Root Ganglion (DRG) neurons. Later on capsaicin-sensitive DRG neurons were characterized by their small size, their ability to take up cobalt in response to capsaicin, and also by the lack of immunoreactivity against the monoclonal antibody RT97 that stains neurofilament 200 kDa (Winter J. 1987; Winter et al. 1988). Subsequently, the "capsaicin-sensitivity" of certain nociceptors, mainly the small-diameter neurons present within the sensory ganglia, was used to distinguish them from other (capsaicin-insensitive) neurons. Subsequently it became clear from a number of studies that the capsaicin-sensitive nociceptors express a membrane-bound receptor, which can recognize capsaicin and acts as a calcium channel upon capsaicin binding (Wood et al. 1988). It was also demonstrated that Resinifera-Toxin (RTX) can act as an ultra-potent capsaicin-analogue, and specific binding of radio-labelled RTX to the DRG membrane was demonstrated (Szallasi and Blumberg. 1990a-b; Szolcsanyi et al. 1990).

Table 1.1: TRPV1 orthologues

Species	NCBI accession number	♣References
Homo sapiens (Human)	AAM89472	Hayes et al. (2000)
Rattus norvegicus (Rat)	AAC53398	Caterina et a1. (1997)
Mus musculus (Mouse)	AY445519	Correll et al. (2004)
Cavia porcellus (Domestic Guinea pig)	AAU43730	Savidge et al. (2002)
Oryctolagus cuniculus (Rabbit)	AY487342	Gavva et al. (2004)
Canis familiaris (Dog)	Q697L1	Phelps et al. (2005)
Sus scrofa (Porcine)	Not submitted!	Ohta et al. (2005)
Gallus gallus (Chicken)	AAL78069 *	Jordt and Julius. (2002)

∗ Due to capsaicin insensitivity, the chicken orthologue is often considered as TRPV2 orthologue.
♣ TRPV1 has been cloned by many groups, but selected references are either first to report or due to significant characterization.

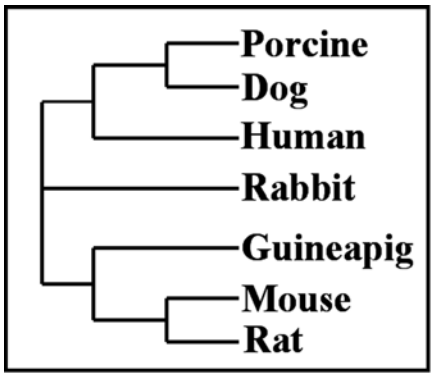

Figure 1.2. A family tree of the mammalian TRPV1. An alignment of all mammalian TRPV1 orthologues reveals more than 80% homology. Adapted from Ohta et al (2005).

Based on these information's, Caterina et al. (Caterina et al. 1997) screened a DRG-derived c-DNA expression-library (from rat) to find a receptor that can account for a robust Ca^{2+} influx upon capsaicin treatment. This receptor was initially named capsaicin receptor or vanilloid receptor 1 (VR1) as it was activated by other components containing vanilloid-like moieties as well. This ion channel also displayed other characteristics of a predicted capsaicin receptor, such as sensitivity to noxious heat and low pH.

The analysis of the VR1 amino acid sequence revealed that it contained a six transmembrane sequence ion channel with both N- and C-terminal sequences forming cytoplasmic domains. Due to the overall similarity, it was grouped into the TRP super family. The capsaicin receptor forms a new TRP ion channel subfamily named TRPV (V stands for vanilloid). Since this is the founder member of the TRPV subfamily, this receptor was named TRPV1 by the HUGO gene nomenclature committee (Montell et al. 2002). TRPV1 has been cloned from various species which include human, rat, mouse, chicken, guinea pig, rabbit, dog and porcine (table 1.1, figure 1.2). Later on five more closely related homologues (TRPV2-6) are identified from a number of species forming the TRPV-subfamily (figure 1.1).

Later studies from many groups not only revealed the presence of full-length, but also many splice variants of TRPV1 (Schumacher et al. 2000; Xue et al. 2001; Tian et al. 2006;Wang et al. 2004). An N-terminal splice variant of TRPV1 (VR.5'sv) has also been identified and cloned. This variant differs from the full-length TRPV1 by a lack of the major part of the intracellular N-terminal domain and the ankyrin repeat elements (Schumacher et al. 2000; Xue et al. 2001). However, the N-terminal splice variant was reported to be insensitive to capsaicin, RTX, and to heat (Schumacher et al. 2000) indicating that the splice variants can act as dominant negative subunits when co-expressed with the full-length receptor (Wang et al. 2004). These splice variants regulate surface expression of the full-length receptor, possibly by preventing the self-assembly (Wang et al. 2004).

1.2.1. Molecular structure of TRPV1.

The full-length TRPV1 polypeptide is composed of 838 amino acids (based on the sequence from rat). Sequence analysis of TRPV1 reveals the presence of six transmembrane sequences with a pore-loop (P-loop) between the 5th and 6th transmembrane sequences (figure 1.3). Both the N- and the C-terminal sequences of TRPV1 form cytoplasmic domains. The N-terminal cytoplasmic sequence consists of 432 amino acids with three ankyrin repeats. The ankyrin repeats are known to be involved in the protein-protein interaction (Mosavi et al. 2004). The importance of these ankyrin repeats for the surface-expression and for the channel assembly (for TRPV4 and TRPV5 receptors) has been reported (Chang et al. 2004; Erler et al. 2004; Arniges et al. 2006). The specific function of the ankyrin repeats in TRPV1 is not well established. However, TRPV1 has been shown to interact with the synaptic vesicular proteins (Snapin and Syt IX) through these ankyrin repeats (Morenilla-Palao et al. 2004).

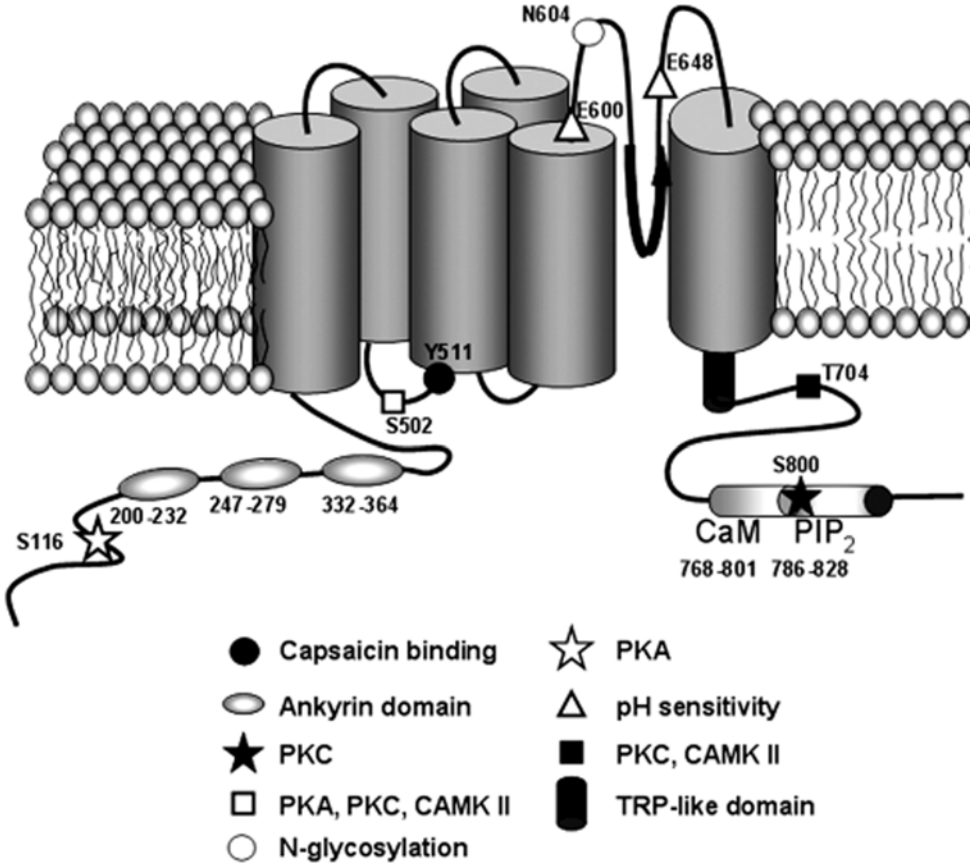

Figure 1.3. Topological organization of a TRPV1 channel subunit with different important domains and residues. The model consist of an N-terminal domain (amino acids 1–414) containing three ankyrin repeats and a phosphorylation site for protein kinase A, six transmembrane-spanning segments (cylinders) and a large stretch connecting the S5 and S6 membrane segments that hold a short amphipathic fragment (curved arrow), and a cytosolic C-terminus domain carrying calmodulin (CaM) and phosphatydylinositol-4,5-bisphosphate (PIP_2) binding sites. Highlighted are molecular determinants of TRPV1 properties. Numbers denote the amino acid number in the deduced rat primary sequence. Image adapted from Ferrer-Montiel et al; 2004.

The C-terminal sequence is relatively short and consists of 155 amino acids. The C-terminus has apparently no known structural motif or domain. However, a putative EF-hand motif exists within the C-terminal sequences. The amino acid sequence 684 to 721 forms the TRP-domain (figure 1.3), which is highly conserved in many TRP channels (Garcia-Sanz et al. 2004).

The TRP-like domain contains the conserved TRP-box with the sequence IWKLER. Though the crystal structure of TRPV1 is not available so far, the structure of the C-terminal

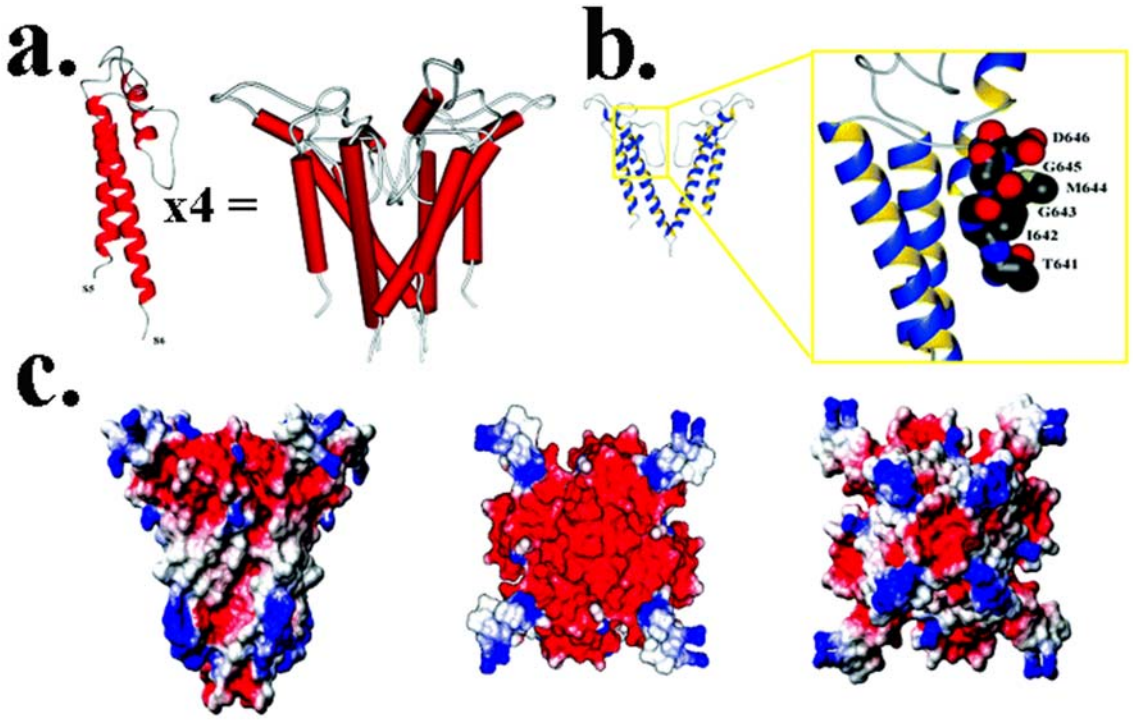

Figure 1.4. A plausible structural model of the TRPV1 pore module. a. Secondary and tertiary structures of the S5-P-S6 motif of a TRPV1 subunit (left). The functional channel would be formed by the assembly of four identical subunits around a central aqueous pore (right). The P-loop configures the narrowest part of the pore conduit at the extra cellular side, whereas the S6 segment is the inner α-helix that structures the walls of the pore at the cytosolic side. **b.** Amino acids that cast the selectivity-filter of the channel. **c.** Electrostatic surface potential of TRPV1 pore domain. Side view parallel to the four-fold axis of symmetry and perpendicular to the plane of the membrane (left), views of the pore conduit from the extra cellular and intracellular sides (middle and right), respectively. Red colour denotes negative potential, whereas blue indicates positive potential and white neutral potential. The atomic structure of the *Streptomyces lividans* K^+ channel was used as a scaffold. The homology modelling was performed in the SWISS-MODEL Protein Modelling Server at ExPASy Molecular Biology web server. The resulting models were energy minimized using GROMOS 43B1 implemented in SWISS-PDB VIEWER V3.7, and evaluated in terms of energy by FOLD-X web page (http://foldx.embl.de). Image taken from Ferrer-Montiel et al; 2004.

cytoplasmic domain has been modelled by two independent groups (Vlachova et al. 2003; Garcia-Sanz et al. 2004). These predicted structures are described later (see chapter 3.1.4, figure 3.5 and 3.6). The pore-module consisting the transmembrane sequences and the P-loop has also been modelled (figure 1.3 and figure 1.4, Ferrer-Montiel et al. 2004).

Like other TRP channels, TRPV1 has the ability to form a tetrameric structure. From biochemical experiments it has been shown that TRPV1 forms a homo-tetramer (Kedai et al 2001; Jahnel et al. 2001). Hetero-tetramer formation with the TRPV2 and the TRPV3 has also been reported (Hellwig et al. 2005; Rutter et al. 2005; Smith et al. 2002). Interestingly, a smaller region of the C-terminal cytoplasmic domain of TRPV1 (discussed later, see Figure 3.8) was reported to form an oligomeric structure (*in vitro*) including a tetramer (Garcia-Sanz et al. 2004).

1.2.2. TRPV1 as a non-selective cation channel.

Several studies demonstrated that the tetrameric structure of TRPV1 forms a non-selective cation-permeable channel (figure 1.3 and 1.4) and the activation of TRPV1 receptor results in the influx of various cations through this channel. However, the permeability of different cations differs considerably (Caterina et al. 1997). The channel formed by TRPV1 does not discriminate among mono-valent cations, but exhibits a notable preference for divalent cations (order of permeability sequence: $Ca^{2+} > Mg^{2+} > Na^{+} \approx K^{+} \approx Cs^{+}$, Caterina et al. 1997). The TRPV1 channel has a much higher permeability for Ca^{2+} ($pCa^{2+}/pNa^{+} = 9.6$) than for all other cations ($pMg^{2+}/pNa^{+} = 4.99$). Recently, direct proton (H^{+}) influx through TRPV1 in acidic solution has also been shown (Hellwig et al. 2004). The TRPV1 is also known to be permeable for cobalt ions too. Previously “cobalt-uptake” was routinely used for the characterization of the capsaicin-sensitive DRG neurons.

1.3. Expression and distribution of TRPV1.

The TRPV1 was first cloned by screening of a c-DNA-library isolated from rat dorsal root ganglia (DRG) (Caterina et al. 1997). At that time a number of studies speculated that the expression and distribution of TRPV1 was restricted to a subset of DRG neurons (Caterina et al. 1997), predominantly to small- and medium-sized (c and A-δ nociceptors) DRG neurons. However, many recent studies demonstrate that the expression of TRPV1 is not restricted to DRG neurons. The expression and distribution of TRPV1 was successfully detected in many parts of the spinal cord and in the brain, albeit at much lower levels (table 1.2). Synaptosomes prepared from brain and spinal-cord were reported to be capsaicin-sensitive (Li and Eisenach. 2001; Schmid et al 1998), suggesting the presence of functional TRPV1 in the brain and in the spinal-cord. Additionally, functional expression of TRPV1 is reported in many sensory and peripheral neurons projecting to different parts of the body.

Recently, using several sensitive methods like reverse-transcriptase PCR (RT-PCR), Ca^{2+}-influx due to capsaicin-responsiveness and immuno-detection using TRPV1-specific antibodies, a large number of studies have also demonstrated the presence of TRPV1 in many non-neuronal tissues and cells, but at a much lower level than in the DRG (table 1.3). The functional significance of TRPV1 expression in most of these non-neuronal tissues and cells has also been established (table 1.3). These widespread and non-neuronal expressions not only indicate the importance of TRPV1 function in different tissues, but also suggest that the same receptor may be subject to tissue-specific regulation.

Table 1.2. Neuronal expression of TRPV1 (other than DRG, peripheral and sensory neurons).

Area	References
1. Cerebral cortex	Liapi and Wood 2005.
2. Hippocampus, cortex, cerebellum, olfactory bulb, mesencephalon and hindbrain	Toth et al. 2005
3. Air way nerves	Groneberg et al. 2004
4. Brain endothelium	Golech et al 2004
5. Amygdala, cerebellum, hippocampus and other	Szallasi and Marzo 2000

Table 1.3. Non-neuronal expression of TRPV1.

Tissue/Cell	Proposed function	References
1. Kidney	Modulation of TRPV1 responsiveness	Tien et al. 2006
2. Hair folicle	Hair growth control	Bodo et al. 2005
3. Skeletal muscle	Ca^{2+} release from store	Xin et al. 2005

4. Organ of croti	Hearing	Zheng et al. 2003,
5. Prostate	?	Stein et al 2004
6. Testis	?	Stein et al 2004
7. Penis	?	Stein et al 2004
8. Urinary bladder	Urinary bladder function	Stein et al 2004
9. Male genital track	?	Stein et al 2004
10. Dental pulp cell	NF(k)-B signalling	Miyamoto et al. 2005
11. Pancreatic islet β-cells	Insulin secretion	Akiba at al. 2004
12. Keratinocytes	Pro-inflammatory signalling	Southall et al. 2003
13. Dendritic cells	Immunological	Basu and Srivastava. 2005
14. Prostate cell	?	Sanchez et al. 2005
15. Sperm tail	Signalling? Sperm motility ?	Maccarrone et al. 2005
16. Astrocyte	Signalling?	Doly et al 2004
17. Mast cell	?	Stander et al. 2004
18. Epidermal keratinocytes	Pro-inflammatory signalling	Stander et al. 2004
19. Dermal blood vessels	Blood pressure?	Stander et al. 2004
20. Differentiated sebocytes	?	Stander et al. 2004
21. Sweat gland	?	Stander et al. 2004

1.4. Stimuli and ligands of TRPV1.

TRPV1 is unique due to its ability to get activated by many physical and chemical stimuli. Remarkably, TRPV1 shows strong synergistic effects with responding to various physical and chemical stimuli (figure 1.5). The ability of TRPV1 to integrate different physical and chemical stimuli makes it an ideal pain receptor (discussed below, see chapter 1.6). Each of these physical and chemical stimuli is discussed here.

1.4.1. Physical stimuli (high temperature and low pH).

Several studies have shown that the TRPV1 is activated by high temperature (Caterina et al 1997), in the noxious heat region above 43°C. The ability of TRPV1 to act as a channel in response to high temperature makes it an ideal molecular thermosensor that might be involved in thermal nociception. TRPV1 is characterized by having gating mechanisms, which shows sensitivity to heat. TRPV1 has a very high temperature coefficient ($Q10$ value), of around 27 (Liu et al. 2003). This value is much greater than 2, the standard $Q10$ value for the majority of biochemical reactions including most of the ion-channel-activities (Benham et al. 2003). Experiments with rapid temperature jump show that TRPV1 is activated relatively rapidly with currents reaching a plateau after less than 500ms (Hayes et al. 2000). Conversely, it has been shown that lower temperature reduces the probability of channel opening (Babesa et al. 2002). Though a number of studies have demonstrated that TRPV1 can act as a molecular thermosensor, the exact mechanisms underlying the temperature-sensitivity of TRPV1 is not yet established.

Recently it has been demonstrated that the temperature-sensitivity of TRPV1 is strongly dependent on the transmembrane voltage. This phenomenon arises from a tenfold difference in the activation energies associated with the voltage-dependent opening and closing (Voets et al. 2004). Voets et al. demonstrated that chemical ligands could alter the voltage-dependent properties of the channel. For example, ligands like capsaicin actually reduce the voltage-dependency of the channel, and thus could allow opening of the channel at 24°C due to much lower activation energy required.

In addition to high temperature, TRPV1 is also known to be activated by extra cellular protons, especially at a pH less than 5.9. Two amino-acid residues, mainly E600 and E648 located on the extra-cellular side between the 5th and 6th transmembrane sequences are believed to act as the proton-sensors (Jordt et al. 2000). The exact mechanism of proton-sensing is not known yet. Remarkably, low pH makes the TRPV1 channel more sensitive to the other physical and chemical agonists (figure 1.5). Though there is no report which can

suggest that wild type TRPV1 is involved in the mechanosensation, a recent report suggested that a splice variant of TRPV1 is important for osmosensory transduction (Naeini et al. 2006).

The properties of TRPV1 to sense high temperature, low pH and many other endogenous ligands (discussed later, see chapter 1.6 and table 1.4) makes TRPV1 an ideal receptor which can sense, integrate and amplify signals during inflammation.

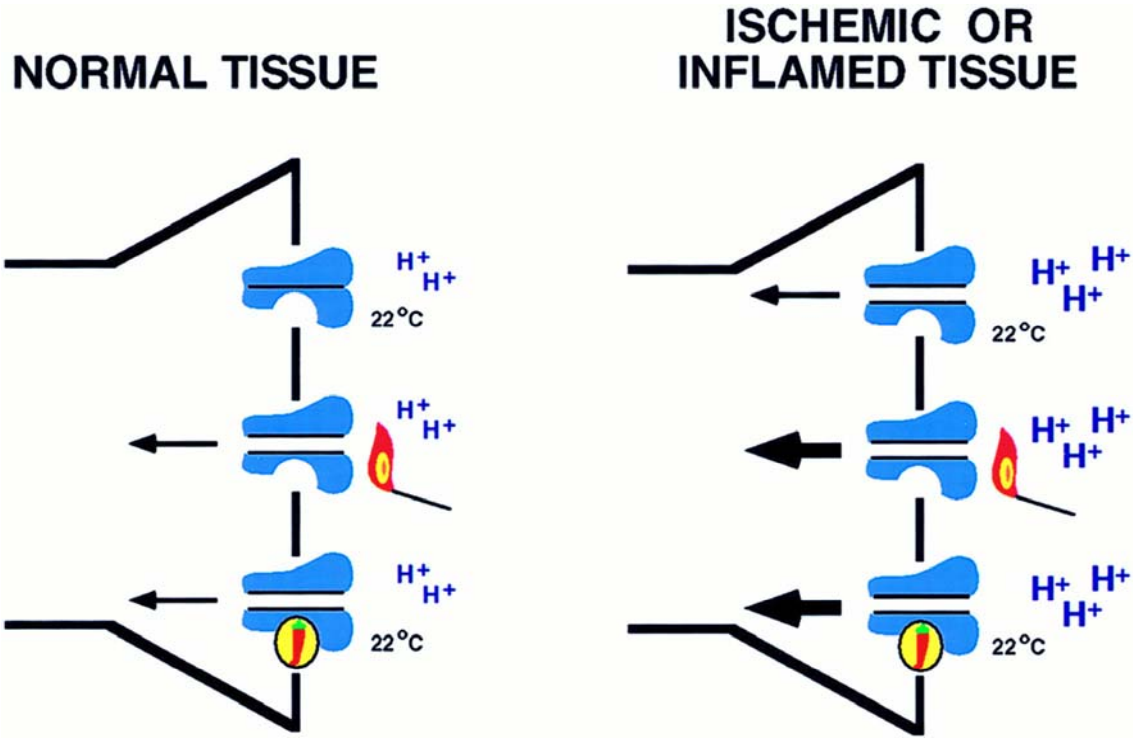

Figure 1.5. Proposed synergistic model for TRPV1 integration of painful stimuli in normal and ischemic or inflamed tissues. Heat (flame) or capsaicin (pepper) can evoke pain in normal animals by activating TRPV1 in nociceptors terminals at physiological pH (left). In ischemic and inflamed tissue (right), TRPV1 is activated at room temperature by the elevated proton (H^+) concentrations. In addition, protons potentiate responses evoked by heat or capsaicin, resulting in increased activity of nociceptors. Thickness of arrows corresponds to relative magnitude of nociceptors activity, which in turn contributes to the magnitude of the resultant pain response. Image taken from Tominaga et al. 1998.

1.4.2. Chemical stimuli

TRPV1 is one of the few receptors that are known to be modulated by multiple chemical ligands. Many of these ligands are natural components (table 1.5). Some of these natural components are often used in different culinary preparations to enhance the food taste. For example, capsaicin (present in hot chilli pepper), piperine (present in black pepper),

allicin (present in garlic) and gingerol (present in ginger), have been reported to activate TRPV1 (see table 1-4 for references). Resinifera toxin (RTX), the most potent agonist of TRPV1 is a natural product too, isolated from a plant of the euphorbia family (*Euphorbia resinifera*). Ethanol, another natural compound widely used by many, is also reported to act on TRPV1 (see table 1.4 for references).

The number of reported ligands of TRPV1 is increasing rapidly, mainly due to the commercial interest in pain management. Apart from the natural and synthetic exogenous components, a large number of endogenous compounds are reported to act on TRPV1. The majority of these endogenous compounds are products and intermediates of fatty acid metabolism. Some of these ligands are listed in table (table 1.4).

∗**Table 1.4. Different ligands of TRPV1.**

Component	Effect	Source	References
Capsaicin	↑	Exogenous (Red hot chilli)	Caterina et al. 1997
Allicin	↑	Exogenous (Garlic)	Macpherson et al. 2005,
Gingerols	↑	Exogenous (Ginger)	Dedov et al. 2002
Piperine	↑	Exogenous (Black Pepper)	Szallasi A. 2005
RTX	↑	Exogenous (*E. Resinifera*)	Caterina et al. 1997
Ethanol	↑	Exogenous (Natural)	Trevisani et al. 2002
2-APB	↑	Exogenous (Synthetic)	Hu et al. 2004
Capsazepine	↓	Exogenous (Synthetic)	Dickenson and Dray. 2001
5' I-RTX	↓	Exogenous (Synthetic)	Wahl et al. 2001
Camphor	↑	Exogenous (*C. camphora*)	Xu et al 2005
12-HPETE	↑	Endogenous	Hwang et al. 2000
NAEs	↑	Endogenous	Movahed et al. 2005
NADA	↑	Endogenous	Huang et al. 2002
OLDA	↑	Endogenous	Chu et al. 2003
OEA	↑	Endogenous	Ahern G.P. 2003
Anandamide	↑	Endogenous	Zygmunt et al. 1999

∗ There are many more ligands known which act on TRPV1. Not all of these ligands are mentioned in the table. Ligands acting as agonists are indicated by an upside arrow (↑) and antagonists are indicated by down side arrow (↓).

1.5. Modulation of TRPV1 by post-translational modifications:

Apart from the modulation of TRPV1 channel by various ligands, TRPV1 is also modulated by post-translational modifications, such as phosphorylation and glycosylation. All these post-translational modifications modulate the channel properties to a large extent (discussed below) and add another degree of complexity to the regulation of this channel. The TRPV1 receptor is reported to be phosphorylated by a number of kinases, such as PKA, PKC and CamKII at various residues, which in turn determine it's biological functions, predominantly the sensitization of the ion-channel (discussed in chapter 1.8, see also figure 1.3 and 3.9). In contrast, dephosphorylation by Calcineurin is reported to be involved in the desensitization of the channel. Additionally, involvement of tyrosine kinases, c-Src kinases and protein phosphatase 2 in the regulation of TRPV1 have been reported, but evidence demonstrating direct phosphorylation-dephosphorylation by these kinases *in vivo* is missing (Jin et al. 2004). Recently, NGF-induced phosphorylation of tyrosine residue (Y200) of TRPV1, by Src kinase has been reported (Zhang et al. 2005). This phosphorylation is reported to be important for surface expression of the receptor (Zhang et al. 2005).

Apart from phosphorylation, TRPV1 is subject to glycosylation. Multiple studies reported that in Western Blot analysis, anti-TRPV1 antibodies detect a specific immunoreactivity at around 115 kDa. This immunoreactivity at higher molecular weight suggested the presence of a post-translationally modified form of TRPV1. Later it was demonstrated that TRPV1 is subject to N-type glycosylation exclusively at the amino-acid residue 604 (Asn), which lies between 5th and 6th transmembrane region and oriented towards extracellular site (Jahnel et al. 2001). This N-type glycosylation on the TRPV1 has been reported to influence the channel properties, especially the sensitivity of TRPV1 to chemical ligands and to the pH (Wirkner et al. 2005).

1.6. Role of TRPV1 in nociception: TRPV1 as a pain receptor *in vivo*.

It was known that capsaicin, the main pungent ingredient of hot chilly pepper, could excite a subset of DRG neurons that act as nociceptors. Apart from the capsaicin-sensitivity, cloning of the TRPV1 c-DNA by Caterina et al. and their studies proved that TRPV1 could also be activated directly by many noxious chemical and physical stimuli (Caterina et al. 1997). Subsequent studies demonstrated that the TRPV1 has unique ability to integrate multiple pain producing stimuli (Tominaga et al. 1998; Tominaga and Julius. 2000). The TRPV1-knockout mice (*trpv1* -/-) reveal an impaired nociception and pain sensation (Caterina et al. 2000).

Much information about the TRPV1 function and importance originated from the study of the TRPV1-knockout mice. The TRPV1-knockout mice were first prepared by Caterina et al. (Caterina et al. 2000). Knocking-out TRPV1 is not lethal and the knockout mice are fully fertile, develop normally without major complications. The knockout mice are studied and characterized by several groups. Many of their biological functions, mainly pain-related functions were shown to be dependent on TRPV1 (table 1.5). Interestingly, apart from the pain-related functions, some other functions have also been shown to be dependent on TRPV1, like certain immunological functions of dendritic cells, function of urinary bladder (see table 1.5) etc. However, many studies confirmed that the knockout mice exert normal heat response (Woodbury et al. 2004). The TRPV1-knockout mice appeared normal in a wide range of behavioural tests, including responses to acute noxious thermal stimuli; but their ability to develop carrageenan-induced thermal hyperalgesia was completely abolished (Davis et al. 2000). This is probably due to the presence of many other thermo-sensitive receptors and probably indicates that heat response is not exclusively dependent on TRPV1.

The ability of TRPV1 to be activated by temperature within the range of 43°C not only makes TRPV1 a thermal sensor for noxious temperature but also links it to thermal hyperalgesia (Pogatzki-Zahn et al. 2005). Other studies also demonstrated the importance of TRPV1 functions in the pain pathways. The role of the TRPV1 in inflammatory thermal hyperalgesia has also been reported (Davis et al. 2000). In diabetic mice, both the thermal allodynia and the hyperalgesia have been reported to be TRPV1-dependent phenomena (Kamei et al. 2001).

Several studies reported that both the expression and the function of TRPV1 are subjected to tight regulation in experimental paradigms of pain. For example, expression of TRPV1 increases in undamaged DRG neurons after partial nerve injury (Hudson et al. 2001). Inflammatory mediators, such as ATP, Bradykinin or NGF, have all been shown to sensitize

Table1.5. Summary of functions dependent on (and independent of) TRPV1

Functions	References
Dependent on TRPV1	
Impaired nociception and pain sensation	Caterina et al. 2000
Altered urinary bladder function.	Birder et al. 2002
Activation of bronchopulmonary vagal afferent nerves	Kollarik et al. 2004
NGF-signalling.	Bonnington and McNaughton. 2003.
Immunological role on dendritic cells.	Basu and Srivastava. 2005
Adjuvant-induced chronic arthritis.	Szabo et al. 2005
Thermal hyperalgesia after incision	Pogatzki-Zahn et al. 2005
Chronic nociceptive processes	Bolcskei et al. 2005
Protective role in a cutaneous contact allergic dermatitis	Banvolgyi et al. 2005
Vascular and hyperalgesic components of joint inflammation.	Keeble et al. 2005
Releasing calcitonin gene-related peptide from isolated hearts.	Strecker et al. 2005
Neurogenesis	Jin et al. 2004
Independent of TRPV1	
Heat responses	Woodbury et al. 2004
Daily body temperature rhythm and heat tolerance	Szelenyi et al. 2004
Circadian body temperature fluctuation, tolerance, fever response	Lida et al. 2005
Response to irritant air polluants	Symanowicz et al. 2004
Cardiovascular profile	Pacher et al. 2004

TRPV1 (Cortright and Szallasi. 2004). As mentioned before, the presence of many different endogenous components activating TRPV1 and the existing cross talk between different pain signalling pathways suggests that TRPV1 is involved in nociception. For example, a bradykinin-/12-lipoxygenase-TRPV1-signalling pathway has been implicated in inflammatory hyperalgesia (Shin et al. 2002). In another study, the ATP-evoked pain and hyperalgesia has been linked to the ability of metabotropic ATP receptors to potentiate downstream TRPV1 activity (Tominaga et al. 2001). Moreover TRPV1 is activated by low pH (below 5.9) and is sensitized by many other endogenous components including bradykinin and prostaglandin. This makes TRPV1 an ideal receptor for sensing tissue damage and inflammation (figure 1.5 and figure 1.6). A number of studies not only proved the importance of TRPV1 in different neuropathic and inflammatory pain, but also from multiple tissues and organs (figure 1.6).

Recently, the involvement of TRPV1 in different cancer conditions and cancer-induced pain has been reported (discussed in detail in chapter 3.2.5). Human breast pain has been linked to TRPV1 (Gopinath et al. 2005). In addition, pain resulting from a variety of

diseases like osteoarthritis, or from chronic cough, have been linked to TRPV1 (Fernihough et al. 2005; Szallasi A. 2002; Mitchell et al. 2005). Though there is currently no evidence, which can suggest a direct role of TRPV1 in mechanosensation, capsazepine, the TRPV1 antagonist, has been reported to reverse mechanical hyperalgesia in rat models of inflammatory and neuropathic pain (Walker et al. 2003).

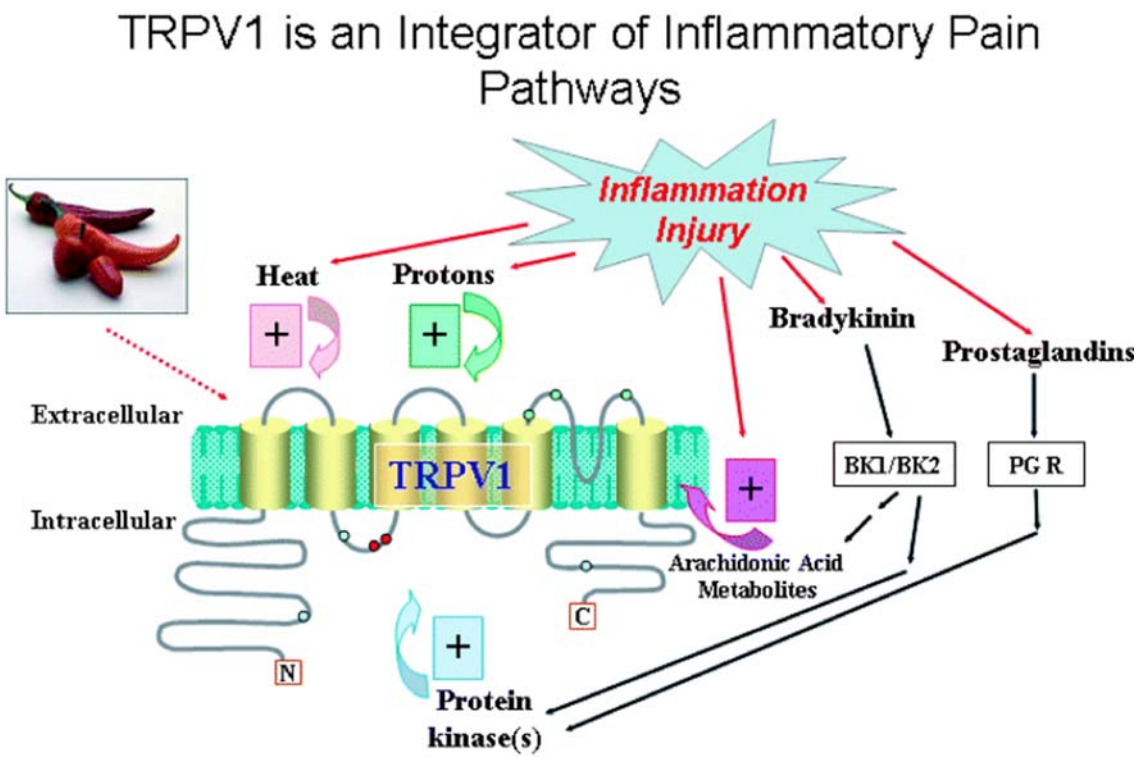

figure 1.6. TRPV1 is an integrator of inflammatory pain pathways. The figure shows a schematic of TRPV1 and its activators in inflammatory conditions. TRPV1 is directly activated by a multiplicity of stimuli including vanilloids, such as capsaicin, low pH, elevated temperature, and arachidonic acid metabolites such as anandamide. TRPV1 is indirectly activated by prostaglandin and bradykinin receptors via protein kinase activity. As noted in the text, several amino acids are important for activation of TRPV1 by different mediators. These are shown as filled circles. Red circles correspond to residues important for capsaicin activation, green circles for low pH and blue circles for protein kinase phosphorylation sites. Image taken from Cortright and Szallasi. (2004).

1.7. TRPV1 as a potential drug target.

The expression of TRPV1 in nociceptors strongly indicates that TRPV1 is involved in the pain pathway. In addition to its role in nociceptors, the endogenous expression of TRPV1 in other tissues, for example, in epidermal keratinocytes, suggests that TRPV1 acts as a pro-inflammatory mediator in pain sensation (Southall et al. 2003; Denda et al. 2001; Inoue et al. 2002). TRPV1 activity was also reported to be involved in the expression of cyclooxygenase-2 (COX-2), and in the release of prostaglandin E_2 (PGE_2) and interleukin-8 (IL-8) (Southall et al. 2003). TRPV1 activity is also linked to release of substance-P (Nathan et al. 2001). In addition, the immunological roles of dendritic cells are shown to be dependent on TRPV1 (Basu and Srivastava. 2005). Taken together, all these information suggest that TRPV1 is involved in pain pathways, both directly and indirectly.

In recent times, the TRPV1 receptor has been recognized as a potential drug target for the treatment of pain, cough, airway disease and also for urinary incontinence (Jia et al. 2005). More importantly, the involvement of TRPV1 in cancer and cancer-related pain has been reported in several studies (see in chapter 3.2.5). All these observations make TRPV1 an important therapeutic drug target (Krause et al. 2005; Maggi C.A. 1992; Jia et al. 2005; Wood J.N. 2000; Robbins W. 2000; Szallasi A. 2001; Sawynok J. 2005; Yoshimura and Yonehara. 2001; Walker et al. 2003; Vogel G. 2000; Rashid et al. 2003a; Lopez-Rodriguez et al. 2003).

Many pharmaceutical companies and individual laboratories aimed at identifying and/or synthesizing potent TRPV1 antagonists to block TRPV1-mediated pain and signalling (Pomonis et al. 2003; Lopez-Rodriguez et al. 2003; Krause et al. 2005; Jia et al. 2005; Wood J.N. 2000; Robbins W. 2000; Szallasi A. 2001; Walker et al. 2003; Vogel G. 2000). For example, thermal nociception and hyperalgesia are reported to be attenuated by the TRPV1 blockers (Garcia-Martinez et al. 2002). In another approach, over-stimulation of the TRPV1 was reported to be effective in the relief of pain, mainly due to the deletion of TRPV1-expressing neurons (Karai et al. 2004). This approach of deleting TRPV1-expressing cells by over-stimulation was recently suggested to be also successful in the case of treating pancreatic-cancer where cancerous cells become TRPV1-positive and start over-expressing the receptor (Hartel et al. 2005). Additionally, TRPV1 is reported to be expressed in the pancreas and also involved in different pancreas-related diseases, like diabetes (Akiba at al. 2004; Davidson et al. 2005).

Capsaicin sensitive-sensory neurons have also been shown to be involved in the regulation of blood pressure (Vaishnava and Wang. 2003). Therefore it has been speculated

that a disorder, like hyper-tension (in this condition blood pressure is abnormal) can be treated pharmacologically by targeting TRPV1 (Wang D.H. 2005).

The use of capsaicin and capsaicin-derived products, like ectopically applied capsaicin cream to treat pain has gained popularity in recent times. A low intraperitoneal dose of capsaicin is effective in the treatment of sickness syndrome (Romanovsky A.A. 2004). Other disease conditions in which capsaicin has been tried as a treatment comprise arthritis, diabetes, bleeding cuts, herpes zoster, lumbago, shingles, trigeminal neuralgia, rheumatism and ease the pain following mastectomy surgery. Capsaicin cream also turned out to be effective against pain in many physio-pathological conditions.

The realization that TRPV1 and cannabinoid CB1 receptors have overlapping ligand recognition properties has far-reaching implications for vanilloid therapy. In fact, arvanil, a combined agonist of TRPV1 and CB1 receptors, has already proved to be a powerful analgesic drug in mice (Szallasi A. 2001). Therefore, capsaicin and structurally related components have been used as lead structures in the development of small molecule antagonists or agonists (Maggi C.A. 1992; Robbins W. 2000; Szallasi A. 2001; Vogel G. 2000).

Recent studies demonstrated that the capsaicin cream exerts an analgesic effect, mainly due to the deletion of existing TRPV1-expressing neurons and also by novel expression of TRPV1 in afferent fibres (Karai et al. 2004; Rashid et al. 2003a-b). In fact, the use of chilli-paste as herbal medicine and as analgesic is known for a long time to the human kind. It is not surprising that humans adopted the use of chilli since 7500 B.C. (MacNeish R.S. 1964). A lot of progress has been made in the last few years towards pain management via TRPV1. However, the wide distribution of TRPV1 in many tissues, the lack of detailed information on the identity of the signalling complex formed with TRPV1, and the poor knowledge on down-stream targets of TRPV1 activation, all makes a specific pain management through TRPV1 difficult.

1.8. The TRPV1 signalling complex: Known Protein Interactors of TRPV1.

Molecules, which interact with TRPV1 and form the downstream signalling network of TRPV1, are likely to play important roles in pain processing. Though a number of kinases and phosphatases are known to affect TRPV1, so far direct and physical interaction has been reported for only a few proteins. Among all the known interactors, some are known Ca^{2+}-signalling effectors, and others are structural proteins. These interactors (figure 1.3, see figure 3.8 also) are described below.

Calmodulin: Calmodulin, a well-known Ca^{2+} binding protein, was reported to interact with a number of receptors and channels. Its association with various ion-channels is so widely extended that often it has been referred to as a "sub-unit" of ion channels (Saimi and Kung. 2002). With some contradictions, two different studies reported the interaction of TRPV1 with calmodulin (Numazaki et al. 2003, Rosenbaum et al. 2004).

Numazaki et al. reported that the calmodulin binding site in TRPV1 is located in a 35 amino-acid segment located at the C-terminus of TRPV1. Interestingly, this is the region, which has been previously implicated in the desensitization of the channel. While desensitization of TRPV1 is a Ca^{2+}-dependent phenomenon, the calmodulin binding to this region was shown to be Ca^{2+}-independent. In addition, Numazaki et al. demonstrated no effect of mutant-calmodulin (which can not bind Ca^{2+}) as well as other calmodulin inhibitors on TRPV1 desensitization.

Rosenbaum et al. also showed that TRPV1 interact with calmodulin, but with the binding site located within the N-terminal domain of TRPV1. They assigned the amino acid sequence range required for calmodulin binding to amino acids 189 to 222. In addition, the interaction of calmodulin with this segment of TRPV1 was found to be Ca^{2+}-dependent. They could also demonstrate that the channel opening-probability of TRPV1 decreases as a result of Ca^{2+} - calmodulin interaction. Additionally they demonstrated that mutant calmodulin lacking the Ca^{2+} binding-site results in altered channel properties. This explains the role of Calmodulin in a Ca^{2+}-dependent desensitization of TRPV1.

Results from both these studies suggest a direct binding of calmodulin to the TRPV1. They also indicate that the TRPV1 may contain multiple calmodulin binding sites (Ca^{2+}-dependent and -independent).

Protein kinase A (PKA): The prominent and similar roles of TRPV1 and PKA in inflammatory hyperalgesia suggest that potentiation of TRPV1 by PKA may be an important molecular event involved in the development of thermal hypersensitivity. Consistent with this

hypothesis, PKA activation amplifies the capsaicin currents and enhances the capsaicin-induced peptide release in cultured dorsal root ganglion neurons (Hingtgen et al. 1995; Hu et al. 2002; Lopshire and Nicol, 1998).

However, recent studies have reported some conflicting results concerning PKA modulation of TRPV1. Lee et al. claimed that the cAMP-dependent kinase pathway does not sensitize the cloned TRPV1 expressed in *Xenopus* oocytes or *Aplysia* neurons (Lee et al., 2000). However, Petrocellis et al. found that the TRPV1-mediated effects of anandamide applications are enhanced by protein kinase A (De Petrocellis et al., 2001). Finally, Bhave et al. demonstrate that the TRPV1 is directly phosphorylated by PKA (Bhave et al. 2002). Most importantly, they demonstrate that the phosphorylation of Serine 116 of TRPV1 by PKA is involved in the desensitization of TRPV1. In the same study they also demonstrated that TRPV1 contains many putative PKA phosphorylation sites present in both the N- and C-terminal cytoplasmic domains.

Protein kinase C (PKC): The importance of the PKC in the pain pathway is well studied and characterized. In agreement with that, a number of studies indicated the importance of PKC in TRPV1 regulation. It has been shown that PKC is important for TRPV1 sensitization of TRPV1. Activation of PKC was reported to induce the TRPV1 activity at room temperature even in the absence of any other agonist (Premkumar et al. 2000, Crandall et al. 2002). A similar observation was also reported where it has been shown that application of PMA alone can activate an inward current in the HEK cell transiently-expressing TRPV1 (Vellani et al. 2001). These investigators demonstrated that the current could be suppressed by the TRPV1 antagonist capsazepine. Vellani et al. proposed that the PKC activation potentiates the gating of the TRPV1 by capsaicin, protons, heat and anandamides. Finally Numazaki et al. showed that TRPV1 is a substrate for PKCε and they also identified two Serine residues at the position of 502 and 800 as the target for PKCε phosphorylation (Numazaki et al. 2002). However, according to the report by Olah et al. (Olah et al. 2002), it is PKCα and not PKCε, which is required for the TRPV1 activation. They showed that loss of PKCα correlates well with the loss of TRPV1 responses. In another study it has been shown that it is the PKCmu, which interacts with TRPV1 (Wang et al. 2004). Very recently, it has been shown by Morenilla-Palao et al that PKC-signalling promotes the cell surface expression and SNARE complex-dependent surface expression of TRPV1 (Morenilla-Palao et al. 2004). This suggests that different PKC isotypes regulate the TRPV1 functions, at least in part by regulating the membrane translocation and sensitization of TRPV1.

CamKII and Calcineurin: Previously, it was observed that the pharmacological blockade of Ca^{2+}-calmodulin-dependent protein kinase II (CaMK II) activation also prevented NGF-induced sensitisation of TRPV1, indicating that CamKII is involved in the regulation of TRPV1 (Bonnington and McNaughton. 2003). Subsequently, Jung et al demonstrated that TRPV1 is subject to phosphorylation by CamKII (Jung et al. 2004). Two important residues, namely Ser-502 and Thr-704, were demonstrated as the potential sites of phosphorylation by CamKII. They could also demonstrate that phosphorylation by CamKII is important for sensitization of the receptor while de-phosphorylation by calcineurin is important for desensitization, and thus contributes to channel regulation.

Snapin and Synaptogamin IX: Recently, the two synaptic vesicle proteins snapin and syanptogamin IX have been detected in a yeast two-hybrid assay as TRPV1 interactors (Morenilla-Palao et al 2004). Both snapin and synaptogamin interact with the N-terminus of TRPV1, at the ankyrin repeat-rich regions of TRPV1. These interactions were further confirmed by co-immunoprecipitation and also by *in vitro* interactions. Interestingly, surface expression and protein kinase C-mediated potentiation of the TRPV1 has been reported to be influenced by the interaction with snapin and synaptogamin IX.

Eferin: Recently, Eferin, a little characterized molecule has been reported to interact with the TRPV1 (Lee S.Y. 2005). Eferin (EF-hands-containing Rab11/25-interacting protein) is a 756-aa protein that contains a proline-rich region in its N-terminus, and two EF hands and an ERM domain in its C-terminal region. This interaction between eferin and TRPV1 has been confirmed by a yeast-two hybrid assay, GST-pull-down as well as by co-immunoprecipitation assay. Though it has been reported that eferin interacts with the TRPV1 at its C-terminal sequence, the exact location within the C-terminus and functional significance of this interaction have not been demonstrated yet.

1.9. Aim of the study.

TRPV1, the polymodal pain receptor detects multiple noxious physical and chemical stimuli. TRPV1 has unique properties of integrating different stimuli and transmitting pain signals. It is believed that this receptor interacts with various proteins and forms a multi-protein signalling complex (signalplex) through which it recruits and regulates pain signals. Though several interactions and their functional significance are shown, the mechanism of pain signalling as a whole remains elusive.

Therefore, in order to understand pain as a molecular mechanism, it is very important to identify individual proteins which interact with TRPV1, and to know how these interactions can modulate TRPV1 or vice versa. It is also equally important to characterize these interactions in terms of pain-transmission mechanisms. In this thesis work, an initial attempt was taken to identify TRPV1-interacting proteins, which may acts as downstream effectors of TRPV1-mediated pain signalling. As the TRPV1 receptor acts as a Ca^{2+} channel, I focused on the identification of novel Ca^{2+}-dependent/sensitive interactors and on the characterization of these interactions in terms of its role in TRPV1-mediated signalling.