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DISSERTATION

Defining a function for the ion channel TRPA1

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Sandra Zurborg
aus Rinteln

Gutachter: 1.: Prof. Dr. P. A. Heppenstall

2.: Prof. Dr. G. Lewin

3.: Prof. Dr. med. C. Nau

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0 Abbreviations

$[Ca^{2+}]_i$	intracellular calcium
℃	degree centigrade
µl	microliter
µM	micromolar
AgCl	silver chloride
AITC	allyl isothiocyanates
AMH	Aδ mechano- and heat-sensitive neuron
ANKTM1 / p120	old names for TRPA1
ANOVA	analysis of variance
ATP	adenosine triphosphate
bp	base pairs
Br^{2+}	barium
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CA	cinnamaldehyde
$CaCl_2$	calcium chloride
CaM	calmodulin
$CaM_{1,2,3,4}$	dominant negative CaM mutant
cDNA	copy desoxyribonucleic acid
CFA	Complete Freund's Adjuvant
CGRP	calcitonin gene-related peptide
contra	contralateral side of DRG
CTR	control
DAG	diacylglycerol
ddH_2O	double distilled water
DEPC	diethylpyrocarbonat
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleosidtriphosphate
DRG	dorsal root ganglion
E. coli	*Escherichia* coli
EC_{50}	half-maximal effective concentration
EDTA	ethylene-diamine-tetra-acedic acid
EGTA	ethylene glycol-bis (2-amino-ethylether)N,N,N',N'-tetra acetic acid
ESC(s)	embryonic stem cell(s)
F340/F380	ratio of fluorescence at 340nm to that at 380nm
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gram(s)
G	conductance
GDNF	glial cell line-derived neurotrophic factor
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
HEK 293	human embryonic kidney cells 293
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid

I	current
IB4	isolectin B4
IP_3	ionositol triphosphate
ipsi	ipsilateral side of DRG
KCL	potassium chloride
kDA	kilo Dalton
LB	Luria-Bertani
LPS	lipopolysaccharide
m/sec	meter per second
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter(s)
mM	millimolar
Mn^{2+}	manganese
MO	mustard oil
mosmol/l	milli osmolarity per liter
mRNA	messenger RNA
MS	menthol-sensitive
ms / msec	milliseconds
mV	milli volt
n	N number
N52	marker for neurofilament/myelinated neurons
nA	nanoampere(s)
NaCl	sodium chloride
NaOH	sodium hydroxide
NGF	nerve growth factor
nM	nanomolar(s)
nm	nanometer(s)
pA	picoampere(s)
PCI	phenol:chlorophorm:isoamylalcohol 25:24:1
PCR	polymerase chain reaction
PFA	paraformaldehyde
PIP_2	phosphatidylinositol bisphosphate
PKA	protein kinase A
PLC	phospholipase C
PUFA	polyunsaturated fatty acid
Q_{10} value	change in the rate of reaction when temperature is increased by 10℃
RR	ruthenium red
RT	room temperature
s / sec	second(s)
s.e.m	standard error of the mean
SERCA	sarco- and endoplasmic reticulum Ca^{2+} transport ATPase
SOCE	store-operated calcium entry
SP	substance P
Sr^{2+}	strontium
TG	trigeminal ganglion
TM(D)	transmembrane (domain)
TNFα	tumor necrosis factor alpha

TRIS	tris (hydroxymethyl) amino-methane
TRP	transient receptor potential
TRPA1	transient receptor potential ankyrin 1
TRPL	TRP-like
Tween®	polyoxyethylene-sorbitan monolaurate
V	voltage
$V_{1/2}$	voltage for half-maximal activation
W-7	CaM antagonist
YFP	yellow fluorescent protein
ΔEF	deletion of the EF-hand domain

1 Introduction

1.1 Somatosensation and Nociception

In each sensory system the initial contact with an external stimulus occurs with a sensory receptor cell. The receptor cell transforms stimuli energy into electrical signals, a process called sensory transduction. Somatic sensibility has four major modalities: discriminative touch, proprioception (the sense of body position), nociception (signaling of tissue damage, physiological pain), and temperature sensation. These senses allow us to detect chemical, mechanical, and thermal stimuli and we are able to discriminate between innocuous and noxious stimuli.

Sensory transduction is mediated by neurons with distinct nerve terminals of dorsal root ganglia (DRG) and trigeminal ganglia (TG) (Lumpkin and Caterina, 2007). These pseudounipolar neurons transmit information of the four modalities in separate ascending pathways to the dorsal horn of the spinal cord (DRG) or to the brainstem (TG) and then via the thalamus to the primary sensory cortex or other subcortical structures. Sensory neurons with encapsulated terminals are responsible for mediating the information of touch and proprioception, whereas those with bare nerve endings mediate nociception and temperature sensation (see figure 1) (Scholz and Woolf, 2002; Lumpkin and Caterina, 2007). Somatosensory transduction is generally thought to occur in terminals of sensory neurons, but recently it has been suggested that also non-neuronal cells might play a role as primary transducers, such as keratinocytes (Peier et al., 2002b; Chung et al., 2004b; Lumpkin and Caterina, 2007).

Cutaneous primary sensory neurons are broadly classified as Aα/β-, Aδ, or C-fibers based on the degree of myelination and the speed of action potential transport (see figure 1). Proprioception and detection of innocuous stimuli applied to skin, muscle, and joints is mediated by fast conducting (>10m/sec) and thickly myelinated Aα- and Aβ-fibers, respectively. Both types of neurons have large diameters and do not contribute to pain. On the contrary, stimulation of large diameter fibers, for instance by rubbing the aching area, can also inhibit pain. In contrast, neurons with small- and medium-diameter cell bodies give rise to most of the nociceptors. There are basically two groups of nociceptors, the unmyelinated and slowly conducting C-fibers (<1m/sec) and the thinly myelinated and more rapidly conducting Aδ-fibers (1-10m/sec) (Koltzenburg et al., 1997; Julius and Basbaum, 2001; Lumpkin and Bautista, 2005). Nevertheless, the

conduction velocities of these fibers can be influenced by species, age, size, nerve type and the temperature of the preparation (Djouhri and Lawson, 2004).

Nociceptors can be further classified according to their various response profiles. The fiber subcategories are relatively diverse because it is the differential expression of ion channels and peptides that are responsible for unique functional attributes of these neurons (Julius and Basbaum, 2001). Two main subclasses of Aδ nociceptors exist. Both respond to noxious mechanical stimuli, but can be distinguished by their differential responsiveness to intense heat and how they are affected by tissue injury. Most C-fiber nociceptors are polymodal and are therefore responsive to noxious thermal and mechanical stimuli and often also to harmful chemical stimuli. Nevertheless, some C-fibers are solely responsive to noxious heat. Some nociceptors are difficult to activate by natural stimuli and are thus called 'silent' nociceptors because they are responsive only when sensitized by tissue injury (Julius and Basbaum, 2001).

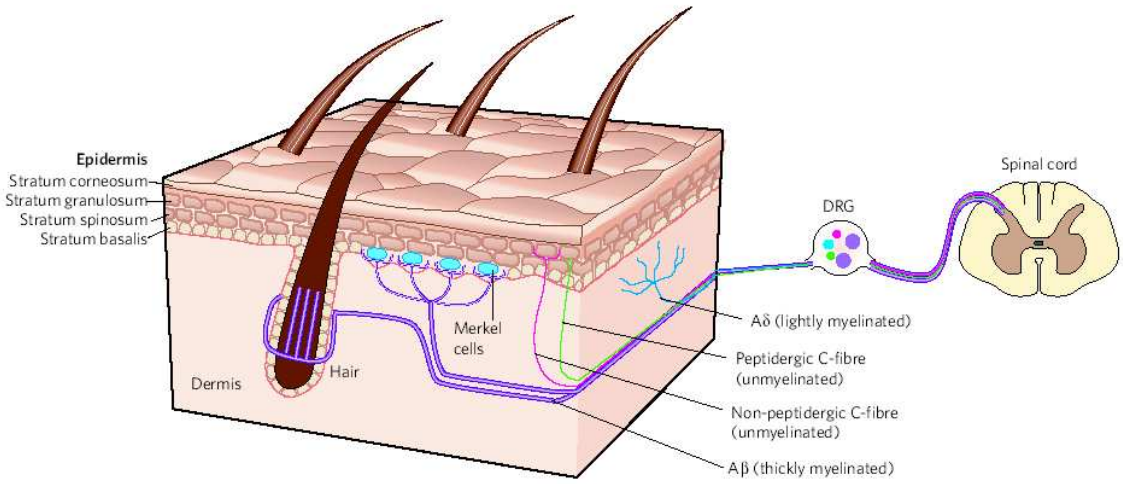

Figure 1 Somatosensory DRG neurons innervate the skin and project to the dorsal horn of the spinal cord. Thickly myelinated Aβ-fibers are mostly mechanosensitive receptors. Aδ- and C-fibers include thermoreceptors and nociceptors (obtained from Lumpkin and Caterina, 2007).

1.2 Physiological pain and clinical pain states

We as humans use the term pain to describe any unpleasant feeling, but in fact pain is a very complex entity and the term 'pain' is defined by the International association for the Study of Pain (IASP) as an 'unpleasant and emotional experience associated with actual or potential tissue damage'. It is unquestionably a physiological sensation, but it is also always unpleasant. Therefore, it has also a subjective emotional component. By contrast, the term 'nociception' refers more to the sensory component of transduction

and conduction of noxious stimuli. In addition, pain can be subdivided into several categories such as physiological pain, and clinical pain states including inflammatory pain, or neuropathic pain.

Physiological or acute nociception serves as a warning device that alerts us to the presence of damaging stimuli such as chemicals, intense pressure, and temperatures (Scholz and Woolf, 2002). Physiological pain is initiated by the firing of specialized Aδ- or C-nociceptors and activated only by noxious stimuli (Woolf and Salter, 2000). It is essential for survival and the noxious threshold has to be high enough that it does not interfere with normal activity and low enough to avoid severe tissue damage (Woolf and Salter, 2000).

Inflammatory pain is initiated by tissue damage and inflammation, whereas neuropathic pain is caused by lesions or disease in the peripheral or central nervous system. Both pain states are characterized by hypersensitivity at the site of damage and in adjacent normal tissue. Pain may appear spontaneously, stimuli that never normally produce pain begin to do so (allodynia) and noxious stimuli evoke a greater and more prolonged pain (hyperalgesia) (Woolf and Mannion, 1999). The inflammatory hypersensitivity usually disappears if the disease process is controlled, whereas neuropathic hypersensitivity still persists and becomes pathological after the initiating event has healed (Scholz and Woolf, 2002).

During inflammation (due to mechanical damage, an infection, ischemia, tumor growth, or an autoimmune process) nociceptors can become sensitized. The sensitization is triggered by release of multiple chemical mediators from damaged or inflammatory cells. This so-called ‘inflammatory soup’ contains protons, adenosinetriphosphate (ATP), prostaglandins, cytokines (tumor necrosis factor alpha (TNFα), interleukin-1 and -6, chemokines), nerve growth factor (NGF) and other proinflammatory mediators released from adjacent cells, which decrease the threshold of nociceptors (see figure 2) (Schaible and Richter, 2004). Release of peptides such as calcitonin gene-related peptide (CGRP) and substance P (SP) from nerve endings induces vasodilation, plasma extravasation, attraction of macrophages, or degranulation of mast cells (Schaible and Richter, 2004). Therefore, the inflammation produced by nociceptors is called neurogenic inflammation (Foreman, 1987).

In order to investigate peripheral inflammation several animal models can be used. Nonspecific peripheral inflammation can be induced by subcutaneous application of Complete Freund’s Adjuvant (CFA), lipopolysaccharide (LPS), zymosan, glycogen and

carrageenan (Rittner et al., 2005). In my thesis the CFA model was used to induce inflammation in mice because it is a well established model in our lab. CFA solution contains dried heat-inactivated mycobacteria (*Mycobacterium butyricum*) (Stein et al., 1988b) and is normally injected subcutaneously in the right hindpaw of the animal. Subsequently, unilateral inflammation occurs within hours after application and can last up to 2-3 weeks (Stein et al., 1988a).

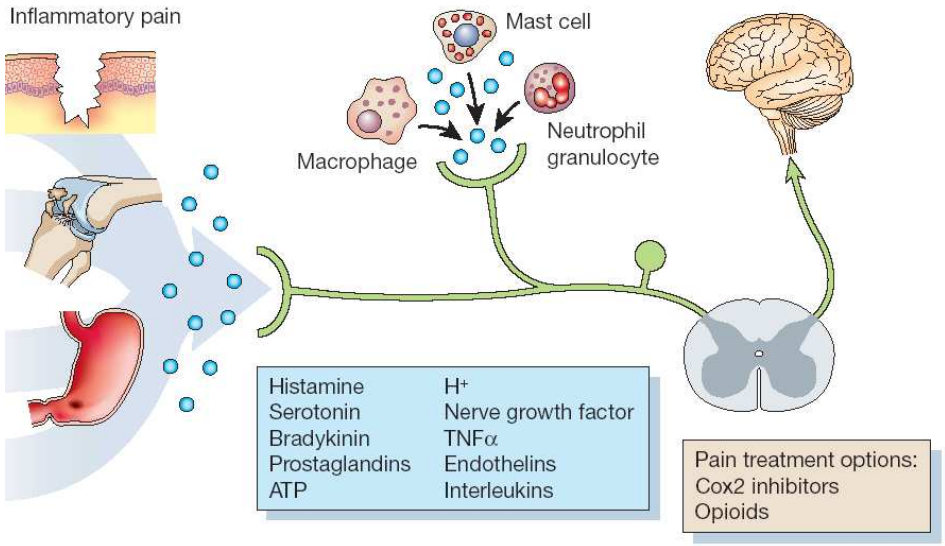

Figure 2 Inflammation by damaged tissue leads to a release of inflammatory mediators by adjacent cells called the 'inflammatory soup'. These mediators activate or modify the stimulus response of nociceptive sensory neurons (obtained from Scholz and Woolf, 2002).

1.3 Temperature sensation

The perception of external and internal temperature is an important sensory mechanism that has implications in cellular and metabolic homeostasis, avoidance, and survival (McKemy, 2007). Organisms sense temperature for many reasons. The ability to sense accurate thermosensation is required to control the body temperature of a mammal. Simpler animals sense the external temperature to search for appropriate environments to carry out activities such as hunting, feeding, mating or sleeping. Moreover, warm-blooded animals must maintain body core temperature constant within a narrow range, while cold-blooded animals must look for a thermal environment, which will enable them to reach an appropriate body temperature (Huang et al., 2006). In general, damaging extremes of temperature must be avoided. Therefore, nociceptors detect very noxious temperatures and induce an avoidance response (Cesare et al., 1999).

Humans and animals are able to detect temperatures ranging from painful cold, cool, and warm to painful heat. Humans can detect very small temperature changes of a few degrees and perceive heat as painful when it is above 43℃ and as pleasantly warm between 32℃ and 42℃. Furthermore, we perceive skin surface temperatures in the range of 15-30℃ as cool and below 15℃ as painful cold (Hensel, 1981; Spray, 1986; Davis and Pope, 2002; Dhaka et al., 2006). The perception of cooling initiates when the skin is cooled as little as 1℃ from normal body temperature (Campero et al., 2001).

With the help of single-fiber recordings, action potentials from thermoreceptors have been recorded and analyzed for the first time in the 1950s (Hensel and Zotterman, 1951b; Hensel and Zotterman, 1951c). These classical studies were performed in cats and showed that cold and warm thermoreceptors fire action potentials with a static firing frequency at normal skin temperature. Since the publications of Hensel and Zottermann in the 1950s, several studies have been carried out in primates (Hensel and Iggo, 1971; Darian-Smith et al., 1973; Kenshalo and Duclaux, 1977). In the 1990s, it became possible to use non-invasive microneurography in order to investigate human thermoreceptors (Campero et al., 1996; Serra et al., 1999; Campero et al., 2001).

A direct method of studying the detection of temperature stimuli is to ask subjects at what temperature a warm or cool stimuli begins to feel noxious, or to record the action potentials of nociceptors while a thermal stimulus is applied to the receptive field. The responses of warm and cold thermoreceptors to temperature changes are mirror images of one another. While warm-activated fibers increase their firing rate upon heating and decrease the firing rate upon cooling, the opposite is true for cold thermoreceptors (Hensel, 1981; Spray, 1986; Campero et al., 2001). Moreover, cold and warm receptors adapt quickly to constant skin temperatures, i.e. the firing frequency decays rapidly within a few seconds (Darian-Smith et al., 1973; Campero et al., 2001).

Psychophysical experiments have revealed that a painful sensation occurs around 43–45℃ and below 15℃ and that the intensity of the pain increases thereafter (Treede et al., 1992; Davis and Pope, 2002). By means of electrophysiological recordings, basically two types of heat sensitive afferents have been identified. One that responds to warm temperatures and another that responds to noxious heat. Recordings of action potentials from some nociceptors revealed a threshold for initiation of action potentials at 43–45℃ and a steep increase in firing rate as the temperature rises further (Treede et al., 1992). In primates, these heat sensitive fibers fire action potentials continuously

at a low rate at normal skin temperature (34℃). The rate of action potentials increases when the temperature rises. 'Warm' sensory afferents have their maximum response at ~41℃, whereas the action potential firing rate of 'heat' sensory fibers still increases at greater temperatures (Hensel and Iggo, 1971). Interestingly, also some cold receptors fire action potentials upon heating (Dodt and Zotterman, 1952).

Neuronal fibers that respond to non-painful cool temperatures are thinly myelinated Aδ- and unmyelinated C-fibers (Hensel and Zotterman, 1951a; Iggo, 1969; Hensel and Iggo, 1971; Darian-Smith et al., 1973; Hensel et al., 1974). Painful cold temperatures below 15℃ induces sensations of burning, cold aching, and pricking (Chery-Croze, 1983; Yarnitsky and Ochoa, 1990; Morin and Bushnell, 1998; Davis and Pope, 2002) and stimulate Aδ- and C-fiber nociceptors as well (LaMotte and Thalhammer, 1982; Leem et al., 1993; Simone and Kajander, 1996; Simone and Kajander, 1997). Furthermore, all Aδ nociceptors are excited by extremely cold stimuli of ≤0℃ with a threshold ranging from 0 to -12℃, while most C-fibers are excited by temperatures at or above 0℃ with a threshold of 12 to -6℃ (Simone and Kajander, 1996). The exact proportion of nociceptors responding to noxious cold varies in many reports and ranges from 10 to 100% of Aδ- and C-fibers (LaMotte and Thalhammer, 1982; Leem et al., 1993; Simone and Kajander, 1996; Simone and Kajander, 1997).

In order to study ionic currents, a preparation of isolated nociceptors would be useful. Other sensory receptors can be isolated more or less intact, but unfortunately nociceptors have sensory terminals that are fine and embedded in a cellular matrix and their disruption during dissection releases the signaling molecules that the nociceptor nerve terminal is supposed to detect (Cesare et al., 1999). Thus, studies on isolated nociceptors have all been on isolated cultured neuronal cell bodies of DRGs and TGs (Baccaglini and Hogan, 1983). The cell bodies of these ganglia were cultured and proteins normally destined for the receptor terminal begin to appear in the soma and can be studied with patch clamping or calcium imaging techniques (Baccaglini and Hogan, 1983).

Two different types of noxious heat-sensory neurons have been identified on the basis of the temperature threshold. In cultured sensory neurons, ~45% of small and medium diameter neurons have a mean activation temperature of 43℃, whereas a smaller group of medium and large diameter neurons have their threshold at 52℃ (Nagy and Rang, 1999).

Approximately 10-20% of DRG/TG cell bodies respond to cold temperatures between 15-30℃ (Suto and Gotoh, 1999; Reid and Flonta, 200 1b; McKemy et al., 2002; Thut et al., 2003). Two populations of cold sensitive neurons with different response properties exist. The first population activates near 30℃ and the second population responds below 20℃ (Nealen et al., 2003; Thut et al., 2003; Babes et al., 2004). The low-threshold cells were predominantly sensitive to menthol, a cooling compound, and the high-threshold population is largely insensitive to menthol (Babes et al., 2004).

These studies performed at the physiological level gave no information about the molecular mechanisms of the thermotransduction process. Until recently, nothing was known about transduction channels for temperatures. A major breakthrough in understanding thermal transduction came with the identification of the heat-activated ion channel TRPV1 in 1997 that belongs to the transient receptor potential superfamily (Caterina et al., 1997). Since then, several temperature-sensitive ion channels have been identified and will be described in the following chapters.

1.4 Transient receptor potential (TRP) ion channel superfamily

1.4.1 The discovery of the transient receptor potential superfamily

Transient receptor potential (TRP) channels constitute a superfamily of cation permeable channels. The term ‘transient receptor potential’ was used for the first times in the late 1960s, when they were first discovered in *Drosophila melanogaster* (Hardie, 2007). The name is derived from a spontaneously occurring mutation in the fly *Drosophila* that lacks TRP and shows a defect in visual transduction. The photoreceptor of these mutant flies responds with only a transient current response to continuous light. Current recordings from wild-type *Drosophila* photoreceptors show a peak response and a plateau phase (Cosens and Manning, 1969; Minke et al., 1975). The lack of the plateau phase is due to a defect in light-induced calcium influx. The mutation that caused this phenotype was identified as an ion channel and named ‘TRP’ (Montell and Rubin, 1989; Wong et al., 1989; Hardie and Minke, 1992). Montell and Rubin (1989) cloned and sequenced the gene 20 years later and it encodes a transmembrane protein that is involved in *Drosophila* phototransduction.

However, the light response is not completely abolished in TRP mutants. Philips and colleagues (1992) found a TRP-like (TRPL) homologue of TRP that is also responsible

for phototransduction in *Drosophila*. Double mutants of TRP and TRPL are unresponsive to light (Niemeyer et al., 1996; Reuss et al., 1997). Thus, TRP is necessary for the major calcium selective component of the light response (Hardie and Minke, 1992) and TRPL for the residual response (Niemeyer et al., 1996; Reuss et al., 1997).

1.4.2 Structure and function of the TRP superfamily

The sequencing of different genomes accelerated the access to homologous proteins in worm and mammals, and soon the TRP channels grew to become a superfamily (Harteneck et al., 2000). Petersen et al. (1995) reported for the first time the occurrence of TRP-related proteins (TRPC1) in vertebrates such as in mouse brain and *Xenopus* oocytes. Later in 1995, two groups described the full sequence of the human homolog TRPC1 (Wes et al., 1995; Zhu et al., 1995). Meanwhile, many vertebrate TRP homologs have been identified. The trp gene was preserved during evolution and the superfamily comprises at least 28 related channels in mammals and 13 in *Drosophila* (Harteneck et al., 2000; Moran et al., 2004; Montell, 2005). TRP proteins are present in yeast, *Drosophila*, *Caenorhabditis elegans* (*C. elegans*), fish, and mammals (Flockerzi, 2007).

TRP channels can be divided in six subclasses in mammals and seven classes in invertebrates. Almost all members are non-selective cation channels and many are weakly voltage sensitive. Nevertheless, the functions of these channels are extremely diverse. Therefore, TRP channel classification was based on amino acid sequence similarity and not on functionality or selectivity (Moran et al., 2004). The similarities among members of one subfamily can be more than 90%, but a corresponding similarity is in most cases not given among members of different subfamilies (Flockerzi, 2007). The six to seven subfamilies in mammals and invertebrates are (Moran et al., 2004) (see also figure 3):

- classical TRPs (TRPCs)
- vanilloid TRPs (TRPVs)
- melastatin TRPs (TRPMs)
- polycystin TRPs (TRPPs)
- ankyrin repeat TRPs (TRPAs)

- mucolipin TRPs (TRPMLs)
- TRPNs (NOMPC) (not present in mammals)

A crystal structure for most TRP proteins is largely unavailable (Flockerzi, 2007), except for rat TRPV1 and TRPC3 (Mio et al., 2008; Moiseenkova-Bell et al., 2008). Most TRP channels have six transmembrane domains (TMD), and thus resemble voltage-gated potassium channels in their transmembrane architecture (Minke and Cook, 2002; Clapham, 2003; Flockerzi, 2007). Moreover, TRP channels form a pore within the extracelluar linker that seperates the fifth and sixth transmembrane domains. It is thought that four TRP subunits must be complexed into a functional pore-forming channel (Flockerzi, 2007). Many channels preferentially homo-tetramerize (e.g. TRPV1, TRPV2, TRPV3, and TRPV4), whereas others often hetero-tetramerize (e.g. TRPC1 with TRPC4 and TRPC5) (Lepage and Boulay, 2007). Most channels are non-selectively permeable for cations, but exceptions are TRPM4 and TRPM5 that are impermeable for divalent cations and TRPV5 and TRPV6 that are only calcium selective (Flockerzi, 2007).

Carboxy (C)- and amino (N)-terminals of the channels are both on the intracellular side of the membrane and TRP channels have diverse cytoplasmic domains. Those often include, amongst others, 3-4 ankyrin repeats, coiled-coil domains, and calmodulin or inositol triposphate (IP_3) binding sites (Clapham et al., 2003; Flockerzi, 2007). Some TRP channels contain a so-called ‘TRP domain’ of 25 amino acids following the sixth putative transmembrane domain that has a TRP box with six invariant amino acids EWKFAR.

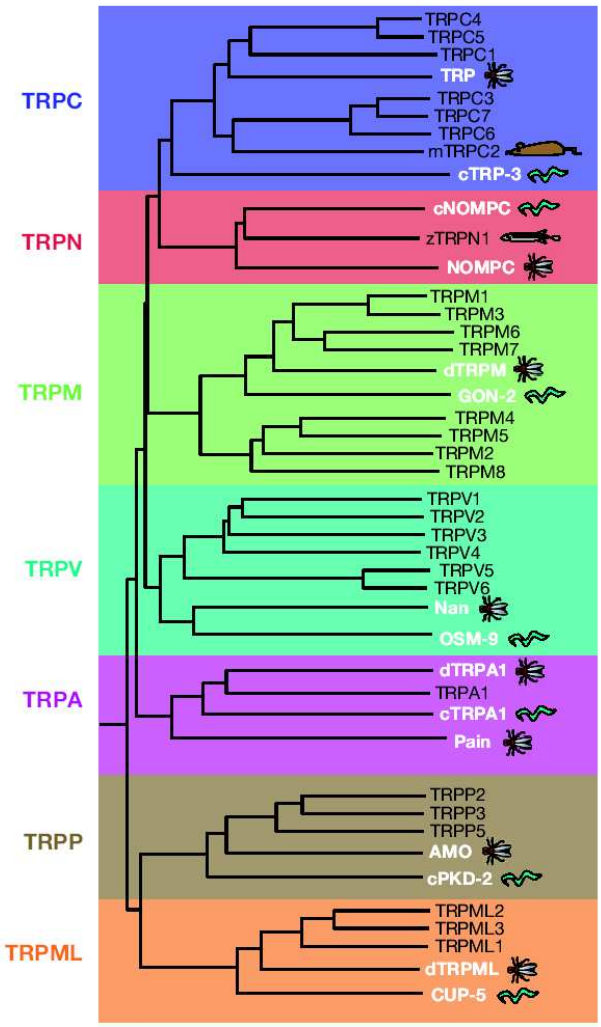

Figure 3 The phylogenetic tree showing the relation of TRP ion channel subfamilies of different species (obtained from Venkatachalam and Montell, 2007).

The functions of these channels are very diverse. They are almost ubiquitously expressed and many have splice variants. Several TRP channels are expressed in the peripheral nervous system but they also occur in the central nervous system and are expressed in many organs. Almost every TRP subtype is represented in the brain. Most of these TRP channels have not been studied in detail yet (Woodard et al., 2007), but many are key players in sensory transduction processes (Montell, 2005). They mediate for instance responses to light, chemicals, osmolarity, nerve growth factors, pheromones, temperature, pH, noxious stimuli, taste, and olfaction (Minke and Parnas, 2006). TRP channels are also widely expressed in non-neuronal cells such as endothelial, epithelial, gland and smooth muscle cells (Minke and Cook, 2002). For instance, they play a role in vasorelaxation, blood pressure, fluid secretion and smooth muscle contractility (Flockerzi, 2007; Venkatachalam and Montell, 2007).

Several TRP channel mutations have been linked to human diseases and uncontrolled activity of some channels may have pathological consequences. Those 'channelopathies' are for instance mucolipidosis type IV and autosomal dominant polycystic kidney disease, which are caused by mutations in TRPML1 and TRPP2, respectively (Kiselyov et al., 2007). Further examples for the involvement of TRP channels in diseases are the upregulation of TRPM8 and TRPV6 in prostate cancer and mutation of TRPM6 causes hypomagnesemia with secondary hypocalcemia (Kiselyov et al., 2007). However, we are still at the very beginning of understanding TRP channel functions in cellular processes and organs and their roles in disease (Flockerzi, 2007).

A mystery of TRP channels is their gating mechanism. There are some theories how TRP channels are gated, but the exact mechanism of the endogenously expressed channels is only poorly understood (Clapham et al., 2003). TRP channels are activated by a number of stimuli, such as receptor activation, ligand activation, store depletion-mediated activation, and direct activation (Woodard et al., 2007). Several TRP channels (e.g. TRPCs and *Drosophila* TRPs) can be activated by G-protein-coupled receptors (GPCR). These receptors activate phospholipase C (PLC) and this results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), generation of lipid messengers such as diacylglycerol (DAG) and polyunsaturated fatty acids (PUFAs), synthesis of IP_3 and release of calcium from intracellular stores. This pathway can in turn modulate the activation of TRP channels (Woodard et al., 2007). TRP channel activation can be regulated by many exogenous and endogenous ligands, for example by capsaicin, which activates TRPV1, or by lipids and ions (Woodard et al., 2007). Moreover, it has been proposed that TRPs are part of store-operated channels and conduct store-operated calcium entry (SOCE). Calcium release from intracellular stores is regulated by a signaling cascade that involves activation of PLC, synthesis of IP_3, which in turn binds to a receptor and discharges the stores (Berridge, 2005; Woodard et al., 2007). During that cascade TRP channels are gated, which is not necessarily associated with calcium store depletion. TRPC1, for instance, has been proposed to be part of a store-operated channel. Nevertheless, the involvement of TRP channels in SOCE remains controversial (Woodard et al., 2007). Last but not least, several physical stimuli are able to directly activate TRP channels. We will see in the following chapter that channels, belonging to the TRPV subfamily for example, are sensitive to heat changes. Further examples are *Drosophila* TRPN, which can be activated by mechanical stimuli, and TRPV4 that can be gated by osmotic changes (Woodard et al., 2007).

Natural products have played an important role in the identification of TRP channels (Calixto et al., 2005). It has been also shown that TRP channels might be new potential targets for the development of therapeutic drugs. Up to now, only TRPV1 has been targeted in the treatment of pain, gastrointestinal, and bladder disease (Nilius et al., 2007).

1.5 'Thermo-TRP' ion channels

As discussed above, temperature sensation is transmitted via peripheral nerves that are located in the DRG and TG (Brauchi et al., 2006). Moreover, initiation of thermosensation seems to be mediated by direct activation of thermally-gated ion channels expressed in the terminal endings of sensory neurons, rather than by more indirect mechanisms (Patapoutian et al., 2003). Thus far, the majority of identified temperature-sensitive ion channels belong to the TRP superfamily (Huang et al., 2006). Almost the entire range of temperatures that mammals are exposed to is covered by the temperature thresholds of these so-called 'thermo-TRP' channels ranging from below 10℃ to above 52℃ (see figure 4). The putative 'th ermo-TRP' channels are TRPV1-V4, TRPM8, TRPA1, TRPM2, TRPM4, and TRPM5. In addition to temperature, they also respond to many chemical or other physical stimuli and function as polymodal sensors. TRPV1-V4, TRPM8, and TRPA1 are expressed in peripheral sensory neurons. TRPV3 and TRPV4 are also expressed in skin keratinocytes (Denda et al., 2001; Xu et al., 2002; Patapoutian et al., 2003). It has been suggested that these keratinocytes may act as thermal receptors. Keratinocytes often surround DRG nerve endings and a signal might be transduced by these skin cells through direct chemical signaling. ATP has been proposed to be involved in that transduction mechanism from skin to neuronal cells because sensory nerve endings express the ATP-gated channel ($P2X_3$) and $P2X_3$ knockout mice show a deficit in coding of warm temperature (Peier et al., 2002a; Xu et al., 2002; Patapoutian et al., 2003; Koizumi et al., 2004). TRPM2, TRPM4, and TRPM5 are not expressed in peripheral somatosensory neurons or keratinocytes (Saito and Shingai, 2006; Caterina, 2007). Moreover, the repertoire of 'thermo-TRP' homologs has changed through vertebrate evolution and seems to have adapted to respective environments (Saito and Shingai, 2006).

The sensitivity of an ion channel to temperature is characterized by its Q_{10} value, which is defined as the change in the rate of a reaction when the temperature is increased by

10℃ (Hille, 2001). TRPV1 and TRPV2 have Q_{10} values between 10-20 and TRPV3 above 20, whereas temperature insensitive proteins have a Q_{10} value around 2-3 (Clapham, 2003).

The gating mechanism of these 'thermo-TRP' channels is unknown. TRPV1, TRPV3, and TRPM8 seem to be directly activated, whereas TRPV4 seems to be not (Caterina et al., 1997; Reid and Flonta, 2002; Watanabe et al., 2002; Xu et al., 2002). Temperature activation of TRPV1 and TRPM8 is linked to voltage-dependent gating, in such a way that elevations in temperature result in graded shifts of the voltage-dependent activation curves towards physiological potentials (Tominaga et al., 1998; Voets et al., 2004). Additionally, the C-terminus of TRP channels might be important for temperature sensitivity because swapping the C-terminal domains of TRPV1 and TRPM8 exchanges their temperature sensitivity and channel gating kinetics (Brauchi et al., 2006).

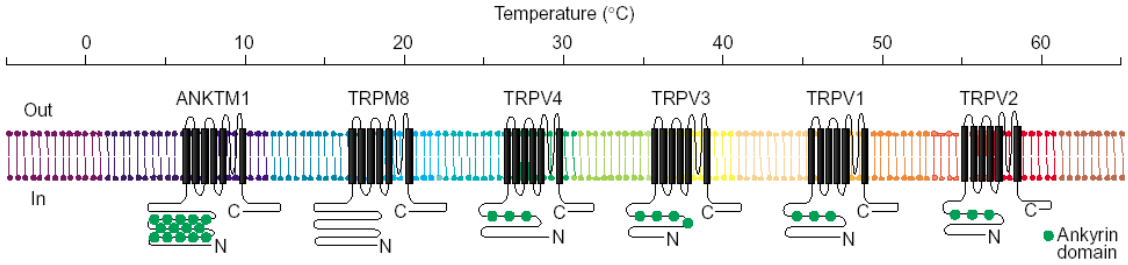

Figure 4 Line up of the known mammalian sensory 'thermo-TRP' channels, which respond to a wide range of temperatures. ANKTM1 is the old name for the ion channel TRPA1 (adapted from Jordt et al., 2003).

1.5.1 TRPV1, TRPV2, TRPV3, and TRPV4

TRPV1 was the first temperature activated channel identified and marked a milestone in thermosensory research (Caterina et al., 1997). It is the best understood temperature-activated channel at the moment. TRPV1 is a vanilloid receptor that is activated by heat above 43℃, by protons and by capsaicin the pungent ingredient of "hot" chili peppers (Caterina et al., 1997; Caterina et al., 1999; Clapham, 2003; Vlachova et al., 2003). It is mainly expressed in the endings of small diameter sensory neurons of the DRG and TG (Caterina et al., 1997). Additionally, it occurs to a lesser extent also on terminals of afferent fibers projecting to laminae I and II of the spinal cord, in the brain (Sasamura et al., 1998; Mezey et al., 2000), and in epithelial cells of the skin and bladder (Birder et al., 2001; Denda et al., 2001). Two independent groups developed TRPV1 knockout

mice (Caterina et al., 2000; Davis et al., 2000). These mice lack nocifensive responses to vanilloids and capsaicin-mediated hypothermia, which indicates a role in pain sensation and injury-induced thermal hyperalgesia. Surprisingly, TRPV1 knockout mice show nocifensive behavior to temperatures around 43-48℃ and show only mildly reduced responsiveness to temperatures above 50℃ (Tominaga et al., 1998; Caterina et al., 2000; Davis et al., 2000).

TRPV2 is not activated by vanilloids, protons or moderate heat stimuli, but it can be activated by very high temperatures above 52℃. It has 49% identity to TRPV1 (Caterina et al., 2000) and is expressed in medium- to large-diameter rat DRG neurons (Caterina et al., 1999; Ahluwalia et al., 2002; Lewinter et al., 2004). TRPV2-positive neurons can be co-stained with the neurofilament antibody N52, a marker for myelinated A-fibers. Aδ-mechanoheat-sensitive neurons (AMH) have a medium- to large diameter and the type I category can be activated around 53℃ (Treede et al., 1995). TRPV2 is a candidate transducer of extreme hot temperatures (>52℃) and mainly expressed in those lightly myelinated type I Aδ-fibers (Caterina et al., 1999; Ahluwalia et al., 2002). Moreover, it is associated with inflammation-induced hyperalgesia (Kanzaki et al., 1999). So far, there is no direct evidence that TRPV2 functions as a thermosensor (Shimosato et al., 2005), but it might form heteromeric complexes with TRPV1 (Huang et al., 2006). Behavioral responses of TRPV2 knockout mice have not been reported.

TRPV3 is activated by warm temperatures around 31-39℃, camphor, oregano, thyme, and cloves (Moqrich et al., 2005; Xu et al., 2006). TRPV3 is predominantely expressed in skin keratinocytes. Some studies also could detect TRPV3 expression in primate and human DRGs (Smith et al., 2002; Xu et al., 2002), but not in rodents (Peier et al., 2002b). Both, TRPV3 and TRPV4, have been suggested to play a role in thermosensation by keratinocytes as mentioned before (Guler et al., 2002; Peier et al., 2002a; Smith et al., 2002). *In vivo*, TRPV3 is partially necessary for thermotaxis towards warmer environments because TRPV3 knockout mice stay at room temperature (RT) instead of preferring warmer regions like wild-type mice (Lee et al., 2005a). Unexpectedly, TRPV3 knockout mice also show a deficient response to noxious acute thermal stimulation above 50℃ (Moqrich et al., 2005).

TRPV4 is activated around 27-35℃ in heterologous expression systems. The channel is expressed in many tissues, in sensory and hypothalamic neurons, and in keratinocytes (Liedtke et al., 2000; Guler et al., 2002; Peier et al., 2002b; Watanabe et

al., 2002; Xu et al., 2002; Alessandri-Haber et al., 2003; Shimosato et al., 2005). TRPV4 has originally been identified as an osmolarity-activated channel and it is also activated by protons, arachidonic acid, anandamide, and epoxyeicosatienoic acid (Chung et al., 2003; Liedtke and Friedman, 2003; Chung et al., 2004b; Moqrich et al., 2005). Gene-disruption or antisense oligonucleotide experiments of TRPV4 revealed an involvement in mechanical stimulus- and hypotonicity-induced nociception in rodents (Alessandri-Haber et al., 2003; Suzuki et al., 2003b; Alessandri-Haber et al., 2004). TRPV4 knockout mice show deficits in thermal hyperalgesia but do not display a change in withdrawal latencies when placed on a hot plate (35-50℃) (Todaka et al., 2004). The absence of TRPV4 leads to a tolerance of higher temperatures (Lee et al., 2005a) and TRPV4 knockout mice prefer warmer temperatures than wild-type mice (32℃ vs. 28.1℃). Moreover, they show longer withdrawal latencies to noxious temperatures near 47℃. The channel also mediates some acute avoidance of noxious heat and thermotactic behavior (Guler et al., 2002).

1.5.2 TRPM2, TRPM4, and TRPM5

TRPM2 is activated by warm temperatures greater than 34℃ by direct gating of the channel. B-NAD+ and ADP-ribose activate the channel but the activity of the channel is enhanced by co-application of heat (Togashi et al., 2006). TRPM4 and TRPM5 are non-selective cation channels that are impermeable to calcium and are activated by increased levels of intracellular calcium. Both can be activated between 15-35℃. TRPM5 is expressed in epithelial taste cells of the tongue and is required for the ability of warm temperatures to enhance sweet tastant-evoked firing of gustatory neurons in mice (Togashi et al., 2006). Nevertheless, all three channels are not expressed in peripheral sensory neurons and thus are not involved in cutaneous thermosensation (Xu et al., 2001; Zhang et al., 2003; Talavera et al., 2005; Saito and Shingai, 2006). Therefore, their function in thermosensation or pain remains unknown.

1.5.3 TRPM8

Another exciting milestone in the understanding of thermotransduction was the identification of the ion channel TRPM8, which was first discovered in the prostate (Tsavaler et al., 2001).

Two independent laboratories cloned TRPM8 in 2002 and discovered that it is activated by cold temperatures below ~26℃ down to ~8℃ and t hus span innocuous as well as noxious cold temperatures (<15℃). The channel is a lso activated by several cooling compounds such as menthol, eucalyptol, and icilin (McKemy et al., 2002; Peier et al., 2002a). Furthermore, the channel shows adaptation, ion selectivity, outward rectification, and menthol shifts its activation temperature (Dhaka et al., 2008). TRPM8 is expressed in small- to medium diameter size neurons in the DRG and TG. Some groups found some overlap of TRPV1 and TRPM8 (Peier et al., 2002a; Okazawa et al., 2004) but this remains controversial. TRPM8 is not co-expressed with the nociceptive markers CGRP or isolectin B4 (IB4) (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007).

Recently, three independently created TRPM8 knockout mice were generated. TRPM8 deficient mice show severe deficits in behavioral cool thermosensation and cultured DRGs from these mice are less sensitive to cold stimulation (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Moreover, TRPM8-deficient mice show reduced nociceptive-like behavior upon acetone application. The acetone test is used to assess cold allodynia by local application of acetone to the hind paw, which cools the skin when it evaporates. The knockout studies showed that TRPM8 is the predominant detector of *innocuous* cold temperature (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007) but one study suggests that TRPM8 also meditates a component of *noxious* cold (Colburn et al., 2007). Colburn and colleagues (2007) demonstrated that TRPM8 is necessary for cold hyperalgesia and cold allodynia as well. In summary, TRPM8 is now widely accepted to be essential for cold sensation.

In 2003, TRPA1 was suggested to play a role in noxious cold sensation. Since then this putative function of the channel has been much debated and even two independently created knockout studies were not able to clarify the role of TRPA1 in noxious cold sensation. I will write about TRPA1 in more detail in the next chapter and discuss its potential role as a painful cold sensor throughout my thesis.

1.6 TRPA1

1.6.1 Identification, structure and expression

TRPA1 was previously called ANTKM1 and p120 and was first cloned in 1999 (Jaquemar et al., 1999). The channel was identified as an ankyrin-like protein that is lost after oncogenic transformation of human fibroblasts (Jaquemar et al., 1999). The human channel has 1119 amino acids (AA) (1125 AA in rat and mouse) and the molecular weight is 127.4kDa. The TRPA1 gene is on human chromosome 8q13. Besides the occurrence of TRPA1 in humans, it also occurs in rodents, flies, *C. elegans*, and zebrafish. Genes encoding mammalian TRPA1 are around 50kb big and have 27 exons (Garcia-Anoveros and Nagata, 2007).

The architecture of the channel is typical for most TRP channels, as mentioned before. It has six transmembrane domains, a presumed pore loop between TMD 5 and 6, and the N- and C-termini are predicted to be on intracellular side of the cell. One distinct characteristic of the channel is the occurrence of up to 18 ankyrin repeats in the N-terminus in humans (and up to 14 repeats in rodents). The function of these 33 AA long repeats is still unclear, but a potential role in protein-protein interaction and molecular spring formation has been suggested (Garcia-Anoveros and Nagata, 2007). Like most other TRP channels, TRPA1 is permeable to monovalent and divalent cations, although the ionic selectivity has not been calculated in detail (Garcia-Anoveros and Nagata, 2007) and it has many putative phosphorylation sites as judged from the amino acid sequence (Story et al., 2003).

During development, TRPA1 mRNA can be first detected relatively late at postnatal day 0 (P0) and the channel becomes functional in the first postnatal week in CD1 and C57/BL6 mice (Hjerling-Leffler et al., 2007).

TRPA1 is expressed in many cells and tissues but it predominantly occurs in nociceptors of DRG and TG sensory neurons, such as non-myelinated C- or lightly myelinated Aδ-fibers (Story et al., 2003; Kobayashi et al., 2005). Furthermore, TRPA1 always co-localizes with the painful heat-activated channel TRPV1 but rarely with the cool-activated channel TRPM8 (Story et al., 2003; Kobayashi et al., 2005; Linte et al., 2006; Hjerling-Leffler et al., 2007). Peptidergic nociceptors contain inflammatory peptides such as CGRP and SP, whereas non-peptidergic nociceptors can be labeled with IB4. TRPA1 can be found in both types of nociceptors (Story et al., 2003; Bautista et al., 2006; Hjerling-Leffler et al., 2007).

Several groups investigated the expression pattern of TRPA1 in TG and DRG neurons. Surprisingly, they reported different percentages of TRPA1-positive neurons in these cells ranging from 3.6% up to 56.5% (Story et al., 2003; Jordt et al., 2004; Kobayashi et al., 2005; Nagata et al., 2005), a fact, which might be explained due to species and methodological differences.

Besides the expression in DRG and TG neurons, TRPA1 is lightly expressed in several other neuronal and non-neuronal cells. Additional TRPA1 positive neuronal cells have been detected in the murine sympathetic neurons from the superior cervical ganglion (Smith et al., 2004), in the nodose ganglia (Nagata et al., 2005), and in hair cells in the inner ear and the vestibular system (Corey et al., 2004). Recently, TRPA1 was also found in human spinal cord motor neurons, ventral roots and basal keratinocytes (Anand et al., 2008).

Moreover, TRPA1 is expressed in neurons throughout the bladder together with TRPV1 (Du et al., 2008; Streng et al., 2008) and was also detected in the rat geniculate ganglia (Katsura et al., 2006), vagal nerve afferents innervating the airways (Nassenstein et al., 2008) and in the spinal cord (Andrade et al., 2008).

Non-neuronal expression of TRPA1 has been shown in human joint cells (synoviocytes) (Kochukov et al., 2006), and in tissues such as the small intestine, the colon and the duodenum (Stokes et al., 2006). Moreover, Stokes and colleagues (2006) also found TRPA1 protein in heart, brain, lung, skeletal muscles and pancreas.

1.6.2 Agonists and gating

Many chemical components that activate TRPA1 cause a pungent burning sensation when applied to the skin. These include ingredients of garlic (allicin), mustard oil (MO), gingerol, and eugenol (Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2005; Macpherson et al., 2005) and environmental irritants such as acrolein and 2-pentenal (Bautista et al., 2006). TRPA1 is activated by many exogenous and endogenous substances and the list is still growing (see table 1). Unfortunately, many compounds that were initially thought to be specific to some TRP channels have a promiscuous relationship to other TRP channels as well (Macpherson et al., 2006). For instance menthol was thought to be specific for TRPM8, but it can also inhibit and activate TRPA1 at different concentrations (McKemy et al., 2002; Macpherson et al., 2006; Karashima et al., 2007). In order to investigate TRPA1 by pharmacological experiments,

cinnamaldehyde (CA) and MO are frequently applied (Bandell et al., 2004; Jordt et al., 2004). Most of the other agonists (see table 1) are unspecific.

Until recently, there was no specific antagonist for TRPA1 available. In most studies, TRPA1 has been blocked by ruthenium red (RR), an antagonist that also blocks many other TRP channels. Several months ago, a few selective blockers of TRPA1 have been reported such as AP18 (Petrus et al., 2007) and HC-030031 (McNamara et al., 2007). All other known antagonists of TRPA1 also block many other TRP channels.

Besides chemical compounds, TRPA1 has been demonstrated to be activated by physical stimuli such as noxious cold temperature below 17℃ (Story et al., 2003; Kwan et al., 2006; Sawada et al., 2007). Nevertheless, many other groups could not confirm this result (see also chapter 1.6.3 and discussion) (Story et al., 2003; Jordt et al., 2004; Bautista et al., 2006; Kwan et al., 2006; Hill and Schaefer, 2007; Sawada et al., 2007). Moreover, another physiological activator of TRPA1 is bradykinin. Bradykinin is an inflammatory mediator that activates the channel through a GPCR signaling pathway (see also chapter 1.6.3) (Bandell et al., 2004; Bautista et al., 2006).

I have already mentioned that the gating mechanisms for most TRP channels are largely unresolved (see chapter 1.4.2). This is also the case for the ion channel TRPA1. TRPA1 agonists are usually structurally unrelated. For some of the substances, activation might occur by interaction of molecules with the receptor via the classical ‘lock-and-key’ principle. However, this model has been challenged by two recent publications (Cebi and Koert, 2007). They show that activation of the channel by some agonists results from covalent modification of TRPA1 (Hinman et al., 2006; Macpherson et al., 2007a). The group of David Julius demonstrated that structurally distinct electrophiles (e.g. isothiocyanates, N-methyl-maleidmide) are strong activators of human TRPA1. The nucleophilic counterparts of these electrophiles are cysteine residues and site-directed mutagenesis studies showed that three cysteines and a lysine residue within the N-terminus (C619, C639, C663, and K708) are critical for the activation of the channel (Hinman et al., 2006). Thus, these amino acids of TRPA1 are directly modified by the electrophilic agonists and the activation does not depend on structure but on reactivity. The second group also identified three cysteines in the N-terminus of mouse TRPA1 to be the target for elecrophilic agonists, such as isothiocyanates, cinnamaldehyde types, and iodoacetamides (Macpherson et al., 2007a). The position of the modified cysteines (C415, C422, and C622) was identified by means of mass spectrometry, pull-down click labeling and site-directed mutagenesis

experiments. Many electrophilic reactions are irreversible and how the channel is afterwards inactivated remains open (Macpherson et al., 2007a). Recent experiments revealed that endogenously produced hydrogen peroxide, reactive oxygen species and reactive nitrogen species directly activate TRPA1 by oxidation of intracellular cysteine residues (Andersson et al., 2008; Sawada et al., 2008). However, mutational analysis revealed that structurally unrelated TRPA1 agonists, such as 2-aminophenyl borane (2-APB) and δ-9-tetrahydrocannabinol (THC), are able to activate the channel by an independent biochemical pathway (Hinman et al., 2006).

Kim and Cavanaugh (2007) claimed that intracellular free polyphosphates may serve a crucial role by keeping TRPA1 in the needed conformation for channel gating by pungent chemicals such as AITC and allicin.

Agonist of TRPA1	**Antagonist of TRPA1**	**Reference**
	AP18 (specific for TRPA1)	(Petrus et al., 2007)
	Camphor	(Xu et al., 2005) (Macpherson et al., 2006)
	Gadolinium	(Nagata et al., 2005) (Bang et al., 2007)
	HC-030031 (specific for TRPA1)	(McNamara et al., 2007)
	Ruthenium red	(Story et al., 2003)
15-deoxy-$\Delta^{12,14}$-Prostaglandin J2 5,8,111,14 eicosatetraynoic acid Clordantoin Disulfiram Farnesyl thioacetic acid Farnesyl thiosalicylic acid		(Maher et al., 2008)
15-deoxy-$\Delta^{12,14}$-Prostaglandin J2, isoprostane 8-iso prostaglandin A_2, PGD_2 metabolites (A and J)		(Taylor-Clark et al., 2008b)
2-aminophenyl borane (2-APB)		(Hinman et al., 2006)
4-HHE (4-hydroxyhexenal), 4-ONE (4-oxononenal)		(Taylor-Clark et al., 2008a)
4-HNE (4-hydroxynonenal)		(Macpherson et al., 2007a)
Acetaldehyde		(Bang et al., 2007)
Acrolein (2-propenal), 2-pentanal		(Bautista et al., 2006)
Allyl isothiocyanates (Mustard oil, AITC) Eugenol		(Jordt et al., 2004) (Bandell et al., 2004)

Gingerol Methyl salicylate (wintergreen oil)		
Benzyl isothiocyanate (BITC)		(Hinman et al., 2006)
Bradykinin		(Bandell et al., 2004) (Bautista et al., 2006)
Cannabinoid WIN 55,212-2		(Jeske et al., 2006)
Chlorine		(Bessac et al., 2008)
Cinnamaldehyde		(Bandell et al., 2004)
Citral	inhibition at higher concentrations	(Stotz et al., 2008)
Clotrimazole		(Meseguer et al., 2008)
Crotonaldehyde, cigarette smoke aqueous extract (CSE)		(Andre et al., 2008)
Delta-9-tetrahydrocannabinol (THC) and cannabinol		(Jordt et al., 2004)
Formalin		(McNamara et al., 2007) (Macpherson et al., 2007b)
Garlic (allicin)		(Macpherson et al., 2005) (Bautista et al., 2005)
Hydrogen peroxide (H_2O_2),		(Andersson et al., 2008) (Sawada et al., 2008) (Bessac et al., 2008)
Hydrogen sulfide H_2S NaHS (donor of H_2S)		(Streng et al., 2008)
Icilin		(Story et al., 2003)
Iodoacetamide IA		(Macpherson et al., 2007b)
Isovelleral, polygodial (sesquiterpene)		(Escalera et al., 2008)
Lidocaine		(Leffler et al., 2008)
Menthol (low concentration activates TRPA1)	Menthol (high concentration blocks TRPA1)	(Karashima et al., 2007) (Macpherson et al., 2006)
Morphantridine tear gases (CN, CR, CS, EBA, CA-BBC)		(Brone et al., 2008)
MTSEA methane thiosuphonate		(Hinman et al., 2006) (Macpherson et al., 2007a)
Nifedipine		(Fajardo et al., 2008)
N-methyl maleimide (NMM)		(Hinman et al., 2006)
Parabens, Methyl-p-hydroxybenzoate		(Fujita et al., 2007)
Phytocannabinoids		(De Petrocellis et al., 2008)
Reactive oxygen species, nitric oxide, reactive nitrogen species		(Sawada et al., 2008)
TCEB (AMG9090; AMG5445)	TCEB (AMG9090; AMG5445;	(Klionsky et al., 2007)

partially activate rat TRPA1.	AMG2504, AMG7160). All inhibit human TRPA1 and last two inhibit rat TRPA1	
Thymol 2-tert-butyl-5-methylphenol carvacrol 2,6-dimethylphenol 2,5-dimethylphenol		(Lee et al., 2008)
Trinitrophenol Toxin GsMTx-4		(Hill and Schaefer, 2007)

Table 1 Alphabetical list of all published TRPA1 agonists and antagonists. References are shown in brackets.

1.6.3 Putative functions for TRPA1

Since the identification of TRPA1, researchers have suggested several functions for the channel. Most importantly, TRPA1 has a role in pain sensation but it has also been proposed to be a mechanically gated channel in hair cells of the cochlea (Corey et al., 2004), to be a sensor for noxious cold temperatures (Story et al., 2003), or a receptor operated channel (Jordt et al., 2004).

Evidence that TRPA1 has a function in pain sensation comes from its expression pattern in small to medium diameter nociceptive sensory neurons together with the capsaicin receptor TRPV1, the bradykinin receptor and nociceptive markers such as CGRP, SP, and IB4 (see 1.6.1) (Story et al., 2003; Bandell et al., 2004; Bautista et al., 2006; Hjerling-Leffler et al., 2007). Moreover, it is activated by many exogenous and endogenous ligands that can elicit a painful sensation, for example cinnamaldehyde, AITC, bradykinin, acetaldehyde, 4-HNE, acrolein, hydrogen peroxide and methyl p-hydroxybenzoate (see table 1 for more details) (Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2006; Bang et al., 2007; Fujita et al., 2007; Trevisani et al., 2007; Sawada et al., 2008). Injection of these compounds leads to acute pain behavior in animals. Mice with a deletion in the pore of TRPA1 display a decreased nocifensive response to pain-producing agonists like MO, allicin, and acrolein, whereas the nocifensive behavior to the TRPV1 agonist capsaicin remains normal. Thus, TRPA1 certainly mediates the response of nociceptors to these pungent chemicals (Bautista et al., 2006; Kwan et al., 2006). In addition, TRPA1 deficient mice show reduced response to punctuate mechanical stimulation and have lower withdrawal responses in

comparison to wild-type mice (Kwan et al., 2006), although Bautista and colleagues (2006) could not observe a deficit in mechanical pain.

TRPA1 functions also as a receptor-operated channel that depolarizes nociceptors in response to proalgesic and proinflammatory agents. Bradykinin is such an endogenously produced agent that activates and sensitizes TRPA1 (Garcia-Anoveros and Nagata, 2007; Wang et al., 2008). This occurs via the bradykinin receptor, a GPCR, which in turn activates PLC and protein kinase A (PKA) second messenger signaling pathways (Bandell et al., 2004; Jordt et al., 2004; Wang et al., 2008). TRPA1 knockout mice did not show hyperalgesia after treatment with bradykinin (Bautista et al., 2006; Kwan et al., 2006). Many other proinflammatory agents such as ATP, monoamines, and neurotrophins also stimulate the PLC pathway, suggesting that they may exert their effects via TRPA1 as well (Jordt et al., 2004).

It has been shown in two studies that TRPA1 has an altered expression profile in rat inflammatory and neuropathic pain models. TRPA1, and also TRPV2 and TRPM8, mRNA levels in DRGs are increased in mice 7 and 14 days after chronic constriction injury, a commonly used neuropathic pain model (Frederick et al., 2007). Moreover, TRPA1 was found to be down-regulated in injured neurons, but upregulated in uninjured neurons in the L5 spinal nerve ligation model (Obata et al., 2005). The same group also reported that CFA-induced inflammation results in an increased percentage of TRPA1 mRNA-positive neurons in ipsilateral DRGs. A recently performed human investigation demonstrated TRPA1 upregulation in DRGs after avulsion injuries (Anand et al., 2008), which also supports a role of TRPA1 in nociception and chronic pain.

Corey and colleagues (2004) suggested that TRPA1 plays a role in auditory transduction in mammals. The TRPN1 homologue in zebrafish, like TRPA1, has many ankyrin repeats and participates in hair-cell function. Due to the fact that mammals do not have TRPN1, TRPA1 seemed to be a likely candidate for the mechanically gated transduction channel in hair cells. The ankyrin repeats could serve as a gating spring that pulls on the transduction channel (Sotomayor et al., 2005). It has been shown that TRPA1 is located in mechanosensory sensory hair cells of the inner ear and in supporting cells and acute inhibition of TRPA1 by means of small interfering RNA in mouse hair cells reduced the mechanotransduction current (Corey et al., 2004). Pharmacological studies have shown that TRPA1 has similar properties compared to other mechanotransduction channels, such as conduction properties and similar antagonists (Nagata et al., 2005). Nevertheless, two independently generated TRPA1

knockout studies failed to report a possible function in auditory sensation (Bautista et al., 2006; Kwan et al., 2006). Mice with a deletion of the TRPA1 pore region show no vestibular or auditory deficits, as determined by behavioral experiments. In addition, auditory brainstem recordings were comparable to wild-type animals and the hair cells in cochlea and utricle had functional transduction channels (Kwan et al., 2007). Current amplitude, range, time course and adaptation properties of hair cells were normal as well (Kwan et al., 2007). Furthermore, a direct activation of TRPA1 by mechanical forces has not been described so far (Garcia-Anoveros and Nagata, 2007). Thus, the contribution of the channel to mechanotransduction is still unclear (Bautista et al., 2006; Kwan et al., 2006).

Originally, Story and colleagues reported that TRPA1 functions as a noxious cold sensor and responds to the super-cooling synthetic agent icilin (Story et al., 2003). They claimed that TRPA1 is activated by cold temperatures with a threshold of ~17℃ (Story et al., 2003), a temperature that elicits a painful sensation when applied to the skin (Hensel, 1981; Spray, 1986; Davis and Pope, 2002). This result could not be confirmed by calcium imaging and electrophysiological studies of some other research groups (Babes et al., 2004; Jordt et al., 2004; Nagata et al., 2005; Hill and Schaefer, 2007) and since then, many groups tried to resolve the issue whether TRPA1 really functions as a noxious cold sensor.

Babes and colleagues (2004) classified cold-sensitive neurons in a menthol-sensitive, and thus TRPM8 containing group, and a menthol-insensitive group of DRG cells. If TRPA1 is cold-sensitive, it should belong to the menthol-insensitive group because TRPM8 and TRPA1 are rarely co-expressed (Kobayashi et al., 2005), but these neurons did not respond to the TRPA1 agonists icilin and MO. Another group (Nagata, 2007) observed no current in TRPA1-expressing HEK293 cells activated by a decrease in temperature in whole-cell and single-channel experiments. Moreover, robust cold responses were present in neurons from E12.5, before the onset of TRPM8 and TRPA1 expression, suggesting there are additional cold transduction pathways (Hjerling-Leffler et al., 2007).

Some studies however support the putative function of TRPA1 as a cold sensor (Bandell et al., 2004; Smith et al., 2004; Elitt et al., 2006; Sawada et al., 2007). One group (Elitt et al., 2006) reported that mice over-expressing artemin express more TRPV1 and TRPA1 and also show an enhanced behavioral response to heat and cold. Thus, they claim that TRPA1 has a role in cold perception.

Interestingly, a *Drosophila* orthologue of TRPA1 is not activated by cold but by warm temperature around 27℃ (Viswanath et al., 2003) and it is also responsible for thermotactic behavior in *Drosophila* (Rosenzweig et al., 2005). Therefore, an evolutionary conservation in temperature sensation seemed likely. Most groups used cultured neurons and heterologous expression studies, and therefore knockout studies for TRPA1 have long been awaited to clarify this issue. Two independently created knockout mice from the laboratories of David Julius and David Corey further complicated the story and showed again controversial results (Bautista et al., 2006; Kwan et al., 2006). In one study, TRPA1 knockout mice showed normal behavior to acetone, cold plate, avoidance tests, and cold activation of cultured neurons (Bautista et al., 2006). In the other study, female TRPA1 knockout mice showed a reduction in the cold response (Kwan et al., 2006). Thus, in mammals there is no definite evidence that TRPA1 mediates thermal sensation.

In summary, besides the fact that TRPA1 mediates the response to many chemicals and has a role in pain sensation, its function is still relatively unknown and even two independently knockout studies show controversial results and failed to identify the precise endogenous function of TRPA1.

1.7 Objectives

The overall aim of my Ph.D. thesis was to determine a function for the ion channel TRPA1. Thus, my project was to systematically characterize the function of TRPA1 in vitro with the help of calcium microfluorimetry and patch clamping in whole-cell and excised patch configurations.

It has been demonstrated that TRPA1 has a role in the pain pathway but nevertheless its endogenous function remains uncharacterized. Additionally, it has been shown that TRPA1 can be gated by both, thermal stimuli and GPCR signaling. Whether TRPA1 is activated by temperature is controversially discussed in the literature (Story et al., 2003; Babes et al., 2004; Jordt et al., 2004; Nagata et al., 2005; Bautista et al., 2006; Kwan et al., 2006; Hill and Schaefer, 2007; Sawada et al., 2007).

Our aim was to characterize how stimuli activate the channel and whether we could identify endogenous compounds that gate the channel. Therefore, we wanted to clarify whether the channel is activated by temperature at all and whether this involves a direct or an indirect mechanism. We tested whether the model of temperature-dependent gating proposed by Voets and colleagues (2004) could be extended to TRPA1. It has also been suggested that TRPA1 functions as mechano-sensor in inner hair cells (Corey et al., 2004), although a direct activation of the channel by mechanical forces is still lacking. Subsequently, we asked whether TRPA1 is mechanically activated by osmolarity. Moreover, the activation of TRPA1 by endogenous compounds such as divalent cations should be investigated as it has been shown for other TRP channels.

Finally, we wanted to investigated whether the expression pattern of the ion channel TRPA1 changes in an animal model of inflammatory pain. We injected CFA to induce inflammation and afterwards DRGs were cultured and investigated with *in situ* hybridization and pharmacological means in calcium imaging experiments.

After analyzing TRPA1 systematically *in vitro* at the cellular level, I started to establish an *in vivo* approach to define a role of TRPA1 in the mammalian somatosensory system (see Appendix B) in collaboration with the laboratory of Prof. Dr. Carmen Birchmeier-Kohler at the Max-Delbrück-Center (MDC), Berlin.

2 Animals, materials and methods

2.1 Animals

Wild-type C57BL/6j mice were bred in FEM (Forschungseinrichtung für experimentelle Medizin, Berlin, Germany) and were housed in cages lined with ground corncob bedding. Standard laboratory rodent chow and tap water were available *ad libitum*. Rooms were maintained at 22°C and at a relative humidity between 40% and 60%. A 12/12 hr (8 a.m./8 p.m.) light/dark cycle was used. Experiments were approved by the animal care committee of the Senat of Berlin and strictly followed the guidelines of the *International Association for the Study of Pain* (IASP) (Zimmermann, 1983).

2.2 Materials

In the following chapter the materials are listed in alphabetical order and the distributors' names are given. For more details on distributors (city, country) please see appendix A.

2.2.1 Bacteria and cell lines

Escherichia coli (*E. coli*) MAX Efficiency® DH5α™ Competent cells F⁻Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k-, m_k+) *pho*A *sup*E44 λ⁻ *thi*⁻1 *gyr*A96 *rel*A1	Invitrogen
Escherichia coli (*E. coli*) XL10-Gold® Ultracompetent Cells Tet^{r}Δ(*mcr*A)183; Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* Hte [F´ *pro*AB *lac*I^{q}ZΔ*M*15 Tn*10* (Tet^r) Amy Cam^r]	Stratagene
HEK293 cell line (human embryonic kidney cells 293)	Deutsche Sammlung von Mikrooraganismen und Zellkulturen (DSMZ)

2.2.2 Oligonucleotides

The primers used for polymerase chain reaction (PCR) were purchased from TIB MOLBIOL. The name, sequence and application are listed in table 2.1.

Table 2.1

Name	5' - 3' sequence	Application
TRPA1 full length upper	ggc gtc cag gtg gag tca	cloning & *in situ* hybridization
TRPA1 full length lower	ggg tga ggt tgc agg aac ta	
TRPA1 probe upper	aaa gga gct aag ctg tat aaa tc	cloning & *in situ* hybridization
TRPA1 probe lower	gtg gag agg ggt cat ccc	
TRPA1 263 upper	cgt gtg aag tgc tga ata taa tgg	cloning & *in situ* hybridization
TRPA1 263 lower	cca tca tgt cga tgt gtg c	
TRPA1 673 upper	aac ggc tac agc aag gag ac	cloning & *in situ* hybridization
TRPA1 673 lower	gga gca aat tca cca tgt tcc	
ef-Hand 5' fw	atc aat acc tgt cag agg ctc cta caa cat gga atg act cct ctc cat ctg gca	deletion of the EF-hand domain (hTRPA1)
ef-Hand 3' rev	tgc cag atg gag agg agt cat tcc atg ttg tag gag cct ctg aca ggt att gat	
ef-D466A-5' fw	gag gct cct aca agc cat aag tga tac gag	substitution mutation of hTRPA1 at D466A
ef-D466A-3' rev	ctc gta tca ctt atg gct tgt agg agc ctc	
ef-D477A-5' fw	ctt ctg aat gaa ggt gcc ctt cat gga atg ac	substitution mutation of hTRPA1 at D477A
ef-D477A-3' rev	gtc att cca tga agg gca cct tca ttc aga ag	
ef-S468A-5' fw	gct cct aca aga cat agc tga tac gag gct tct g	substitution mutation of hTRPA1 at S468A
ef-S468A-3' rev	cag aag cct cgt atc agc tat gtc ttg tag gag c	
ef-T470A-5' fw	caa gac ata agt gat gcg agg ctt ctg aat g	substitution mutation of hTRPA1 at T470A
ef-T470A-3' rev	cat tca gaa gcc tcg cat cac tta tgt ctt g	
T470A-S468A-5' fw	ctc cta caa gac ata gct gat gcg agg ctt c	double mutation of hTRPA1 at T470A and S468A
T470A-S468A-3' rev	gaa gcc tcg cat cag cta tgt ctt gta gga g	
mEF-D466A-5' fw	gag act tct gca agc cat aag tga tac gag	substitution mutation of mTRPA1 at D466A
mEF-D466A-3' rev	ctc gta tca ctt atg gct tgc aga agt ctc	
mEF-D477A-5' fw	ctt ttg aat gaa ggg gct ctc cat ggg	substitution mutation of

	atg ac	hTRPA1 at D477A
mEF-D477A-3' rev	gtc atc cca tgg aga gcc cct tca ttc aaa ag	
TRPM4_NotI_5'	ata gcg gcc gca aat aca cgg agc ctc ag	cloning of TRPM4 into pTRE2
TRPM4_EcoRV_3'	gcg ata tcc tca gtc ttt gga acc agt gg	
Cam5'	atg ata tca cca tgg ctg acc aac	cloning of Calmodulin receptor into pTRE2
Cam3'	atg cgg ccg ctc act tcg ctg tca tc	
Humark 5'	tat gcg gcc gca cca tga agc gca gcc tg	cloning hTRPA1 in pTRE2
Humark 3'	cgt cta gac taa ggc tca aga tgg tgt g	
cYFP5'	atc cgc gga cca tgg tga gca ag	cloning YFP into pTRE2 with mutant TRPA1
Humc2YFP3'	atg cgg ccg ctg gac ttg tac ag	
GFP5'	ctg gat cca cca tgg cta gca a	cloning GFP into pTRE2
GFP3'	atg cgg ccg cct aat cca tgc cat	

2.2.3 Enzymes

Collagenase IV	Sigma-Aldrich
DNase I	Qiagen, Roche
DpnI	Stratagene
PfuUltra polymerase	Stratagene
Proteinase K	Sigma-Aldrich
Restriction enzymes	Invitrogen, Promega, NEB
SP6 RNA polymerase	Roche
T4 DNA ligase	Promega
T7 RNA polymerase	Roche
Taq polymerase	Roche
Trypsin	Sigma-Aldrich

2.2.4 Antibodies

Anti-CGRP (polyclonal)	Sigma-Aldrich
Anti-NF200 (clone 52)	Sigma-Aldrich
Fluorescin anti-mouse IgG (H+L) made in horse	Vector Laboratories
Fluorescin anti-rabbit IgG (H+L) made in goat	Vector Laboratories

2.2.5 Plasmids

pEYFP-C1	Clontech
pGEM-T-Easy	Promega
pGEM-T-Easy with mTRPA1	O. Caspani, Charité, Berlin
pTetOn	Clontech
pTRACER-SV40	Invitrogen
pTRE2	Clontech
pTRE2 with mouse and human TRPA1	O. Caspani, Charité, Berlin

2.2.6 Antibiotics

Ampicillin	Sigma-Aldrich
Doxycycline	Sigma-Aldrich
Geneticin G418	Sigma-Aldrich
Penicillin (10.000U)/Streptomycin (10.000 µg/ml) (P/S)	Biochrom AG

2.2.7 Pharmacological agents

Bradykinin	Sigma-Aldrich
Carbachol	Sigma-Aldrich
Cinnamaldehyde	Sigma-Aldrich
Human calmodulin (CaM)	Calbiochem
Mustard oil	Sigma-Aldrich
Ruthenium red	Sigma-Aldrich
Thapsigargin	Sigma-Aldrich
W-7 hydrochloride	Calbiochem

2.2.8 Chemicals, reagents and media

10x ligase buffer	Promega
2-methylbutane	Sigma-Aldrich
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich
Acetic acid	Sigma-Aldrich
Acetic anhydride	Sigma-Aldrich
Agarose LE	Roche
Albumin from bovine serum (BSA)	Sigma-Aldrich
Anti-DIG antibody conjugated with horseradish peroxidise	Roche
Anti-digoxigenin conjugated to alkaline phosphatase	Roche
Blocking reagent for nucleic acid hybridization	Roche
Boric acid	Sigma-Aldrich

Bromophenol blue	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
CHAPS	Sigma-Aldrich
Complete Freund's Adjuvant (CFA)	Calbiochem
D-(+)-glucose	Sigma-Aldrich
D-(+)-saccharose	Carl Roth GmbH
Denhardt's solution	Sigma-Aldrich
Deoxy-nucleotide triphosphate mix (dNTP)	Roche
Dextrane sulphate	Sigma-Aldrich
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich
DIG RNA labeling mix	Roche
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich
D-mannitol	Sigma-Aldrich
DMEM/Hams-F12 medium	GIBCO Invitrogen Corporation
DNA molecular weight marker (XVII, 500 bp)	Roche
Dulbecco's modified eagle's medium	Sigma-Aldrich
Ethanol 99.9%	Carl Roth
Ethidium bromide	Sigma-Aldrich
Ethylendiaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethyleneglycol tetraacetic acid (EGTA)	Sigma-Aldrich
Fetal bovine serum (FBS) tet system approved	Clontech
Formamide	Sigma-Aldrich
FURA-2/AM dye	Invitrogen
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
Heat-inactivated horse serum	Biochrom AG
Heparin	Braun
Hydrochloride (HCL) 25%	Merck AG
Incomplete Freund's Adjuvant	Difco Laboratories
Isofluran curamed	Rhodia Organic Fine Ltd
Isopropanol	Sigma-Aldrich
L-glutamine	Sigma-Aldrich
Luria Bertani (LB) AGAR Lennox L AGAR	Invitrogen
Luria Bertani (LB) Broth Base (Lennox L Broth Base)	Invitrogen
Magnesium chloride hexahydrate	Sigma-Aldrich
Maleic acid	Sigma-Aldrich
MEMα medium with ribonuclease and deoxyribonuclease	GIBCO Invitrogen
Methanol	Carl Roth GmbH
Monopotassium phosphate (KH_2PO_4)	Sigma-Aldrich

Mowiol 4-88 ®	Calbiochem
NBT/BCIP solution	Roche
Normal goat serum	Vector Laboratories Inc.
Normal horse serum	Vector Laboratories Inc.
Paraformaldehyde	Invitrogen
Pluronic F-127 (20% solution in DMSO) ®	Invitrogen
Poly-L-lysine hydrobromide	Sigma-Aldrich
Potassium chloride (KCL)	Sigma-Aldrich
S.O.C. medium	Invitrogen
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich
Sodium chloride	C. Roth GmbH
Sodium citrate	Sigma-Aldrich
Sodium hydroxide (NaOH)	Sigma-Aldrich
Sodium-ATP	Sigma-Aldrich
Triethanolamine (TEA)	Sigma-Aldrich
Tris-Base	Sigma-Aldrich
Tris-hydroxymethyl- aminomethane (TRIS)	Roche
TRIZMA® hydrochloride	Invitrogen
Trypsin (0.05%)/EDTA(0.02%) Solution	Biochrom AG
Tween®-20	Sigma-Aldrich
Yeast tRNA	Sigma-Aldrich
β-mercaptoethanol	Carl Roth GmbH

2.2.9 Kits and reaction systems

FuGene® 6 Transfection Reagent	Roche
High Pure PCR Product Purification Kit	Roche
QIAfilter Plasmid Maxi Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QuikChange® II XL Site Directed Mutagenesis Kit	Stratagene
QuikChange® Multi Site Directed Mutagenesis Kit	Stratagene
Tyramide Signal Amplification System	PerkinElmer

2.2.10 Buffers and solutions

1.32% Triethanolamine TEA (50ml)	49ml DEPC H_2O, 660µl triethanolamine, 370µl of 25% HCl
10x PBS-RNA (Phosphate buffered	137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM

saline)	KH_2PO_4, in 1l DEPC-H_2O, pH 7.4, mix well, sterilize by autoclaving
10x TN buffer (Tris sodiumchloride solution)	0.1M Tris-HCl, 0.15 M NaCl, pH 7.5
1M Triethanolamine (pH 8.0)	Add 66.5ml triethanolamine and 20ml conc. HCL to 413.5ml DEPC-water in an RNAse free bottle
1x TBE	100mM tris base, 100mM boric acid, 2.5 mM EDTA
20x SSC	3M NaCl, 0.3M sodium citrate
4% Fixation buffer	4% PFA in 1x PBS (60℃), pH 7.4
70% ethanol	70% ethanol in DEPC-H_2O
Acetic anhydride solution	200ml DEPC-H_2O, 1.48ml HCL (25%), 2.64ml triethanolamine, add 480µl acetic anhydride dropwise under constant stiring
active DEPC solution	1ml of DEPC in 1 l ddH_2O, mix well
Agarose gel	1-2% agarose, 1xTBE, 0.0001% ethidium bromide
Blocking solution (1%)	1% (w/v) of blocking reagent for nucleic acid hybridization in maleic acid buffer
Calcium imaging buffer (CIB)	140mM NaCl, 4mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM Glucose, 10mM HEPES, pH 7.4
Carbonate buffer	60mM Na_2CO_3, 40mM $NaHCO_3$, pH 10.2
CGRP blocking buffer	7% normal goat serum in maleic acid buffer
Complete Freund's Adjuvant (CFA) solution	50µg of desiccated M. Butyricum was diluted into 20µl Incomplete Freund Adjuvant (Difco Laboratories Detroit, USA)
DEPC H_2O	0.1% DEPC in ddH_2O, mix well, sterilize by autoclaving
DEPC-PBS	1ml DEPC in 1l 1xPBS-RNA, mix well and autoclave
Detection buffer	100mM Tris-HCL, 100mM NaCl, pH 9.5 (store at 20℃)
DRG medium	10% horse serum, 1mM glutamine, 100u penicillin, 100µg/ml streptomycin, 0.8% glucose, in DMEM/HAM's F12 without glutamine
ECS (extracellular solution) for whole-cell patch clamping and ICS (intracellular solution) in inside-out patch recordings	156mM NaCl, 6mM CsCl, 2.5mM $MgCl_2$, 10mM glucose, 10mM HEPES, 1mM EGTA, pH 7.4. In some experiments EGTA and 2.5mM $MgCl_2$ were replaced with 1.5mM $CaCl_2$ and 1mM $MgCl_2$
EDTA/NaOH washing solution	0.744g EDTA, 8g NaOH in 2l ddH_2O

Fura-2 solution	50µg Fura-2/AM diluted in 10µl pluronic F-127 (20% solution in DMSO) and 50µl DMSO
Gel loading buffer	0.25% bromophenol blue, 50mM Tris pH 7.6, 60% glycerol, stored at 4℃
Hybridization buffer (10ml)	50% deionized formamide, 5x SSC, 1mg/ml yeast tRNA (Sigma), 100µg/ml heparin, 1x Denhardt's solution, 0.1% Tween® 20, 0.1% chaps, 5 mM EDTA, add DEPC-H_2O to final volume, 10% (w/v) dextrane sulphate
Hydrolysis-neutralization buffer	3M sodium acetate, 1% (v/v) acetic acid, pH 6.0
ICS (Intracellular pipette solution) for whole cell recordings and ECS (extracellular solution) for inside-out patch recordings	20mM CsCl, 100mM CsAsp, 1mM $MgCl_2$, 4mM Na_2ATP, 10mM HEPES, 10mM EGTA, pH 7.2. Ca^{2+} concentration was then adjusted by adding appropriate amounts of $CaCl_2$ as calculated using the CaBuf program (ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip)
LB medium	20g of LB Broth Base (Lennox L Broth Base) in 1l of ddH_20, then divided into 10x 100ml solutions and autoclaved. The appropriate antibiotic was added before inoculation with the bacterial clone
Maleic acid buffer (1l)	100mM maleic acid, 150mM NaCl, in 1l ddH_2O, pH 7.5
Maleic acid buffer with Tween 0.03 % (1l)	100mM maleic acid, 150mM NaCl, in 1l ddH_2O, pH 7.5, add 0.03% Tween20
Mowiol embedding solution	12g glycerine and 4.8g Mowiol 4-88 in 24ml 0.2M Tris/HCL solution (pH 8.5). Stir at 50℃ for 30-40 minutes. Clarify by centrifugation at 4000-5000 rpm for 20 min and store the supernatant at 4℃
NF200 blocking buffer	7% normal horse serum in maleic acid buffer
Osmolarity buffer	88mM NaCl, 5mM KCl, 1mM MgCl2, 5.5mM glucose, 0.1% BSA, 10mM HEPES, 1mM $CaCl_2$, pH 7.4 with NaOH. Add the appropriate concentration of mannitol to adjust the solutions to the desired osmolarity. 40mM of mannitol for 240mosm buffer, 70mM for 270mosm buffer and so on
Phosphate buffered saline (1x PBS)	Dissolve the following in 800ml ddH_2O: 80g of NaCl, 2.0g of KCl, 14.4g of Na_2HPO_4, 2.4g of KH_2PO_4. Adjust pH to 7.4. Adjust volume to 1l with

	additional ddH_2O. Sterilize by autoclaving.
Proteinase K buffer (PK buffer)	50mM Tris-HCl, pH 7.5, 5mM EDTA
RNA-dilution buffer	DEPC-H_2O, 20x SSC and formaldehyde mixed in a volume ratio of 5+3+2
Saturation buffer	5x SSC, 50% formamide
Tet-On medium	MEMα medium with ribonuclease and deoxyribonuclease containing 10% FBS tet system approved, 1% P/S and 1% glutamine. The antibiotic G418 (2µg/ml) was added to the cultured cells.

2.2.11 Consumable materials

50ml plastic tubes	Sarstedt AG & Co.
Borosilicat glass capillaries with filament	Hilgenberg
Cell culture bottles	Sarstedt AG & Co.
Cell culture dishes	TPP AG
Cell culture plates	Sigma-Aldrich
Cell scraper	Sigma-Aldrich
Cell strainer (40µm) Nylon	BD Biosciences
Cellular Incubator 6000	Heraeus
Coverslips	Carl Roth GmbH
Cryo tubes (1.5ml)	Nunc GmbH
Frozen section medium	Richard Allan Scientific
Needles	Becton-Dickinson GmbH
PCR tubes and caps	Applied Biosystems
Pipettes (1-25ml, one-way)	Sarstedt AG & Co.
Polysin microscope slides	Menzel GmbH
Positively charged nylon membrane	Roche
Reaction tubes 0.5, 1.5ml and 2ml	Eppendorf
Silver wire 0.25mm	WPI Inc.
Sterile filters	Millipore
Sterile filters FP 30/0.2 CA-S	Whatman
Syringes	Braun
Tubes (15 and 50ml)	Falcon

2.2.12 Technical equipment

AxioCam MRC	Carl Zeiss Mikroskopie
Bacterial shaker	GFL GmbH

Beckman Avanti J.25 centrifuge	Beckmann Instruments GmbH
CCD Imago camera	Till Photonics GmbH
Centrifuge Rotixa 50 RS	Hettrich Zentrifugen
CO_2 incubator	Heraeus
Cryostat microm HM560	MICROM International
EPC-10 patch-clamp amplifier	HEKA Electronic
Flaming/Brown Micropipette Puller Model P-97	Sutter Instruments Co.
Fluorescence microscope (Axioskop 2)	Carl Zeiss Mikroskopie
Fluorescence microscope Hal100	Carl Zeiss Mikroskopie
HS18 laminar airflow	Heraeus
Hybridization oven (Hybaid shake'n'stack)	Thermo Electron Corporation
Inverted microscope (Axiovert 200)	Carl Zeiss Mikroskopie
Light-microscope Axiovert 25	Carl Zeiss Mikroskopie
Micro-Forge MF-200	WPI Inc.
Micromanipulator 5171	Eppendorf
Microwave	Galanz Inc.
Mini centrifuge Galaxy mini	VWR International GmbH
Minispin table top centrifuge	Eppendorf
Monitor (CDM 1003)	MONOCAR Int. GmbH
Multifuge 4KR	Heraeus
Objective "A-Plan" 10x/0.25 Ph1	Zeiss
Objective "LD Achroplan" 63x/0.75 Korr. Ph2	Zeiss
Peltier device	E.S.F electronic
Peristaltic pump	WPI Inc.
pH-Meter (MP220)	Mettler-Toledo
Photometer Gene Quant II RNA/DNA Calculator	Pharmacia Biotech
Polychrome manual control	TILL Photonics GmbH
Polychrome V monochromator	TILL Photonics GmbH
Power PAC 300 for electrophoreses	BIO-RAD
PowerLab 4/20	ADInstruments GmbH
SPECTRAmax® Spectrophotometer	Molecular Devices
Table top centrifuge Biofuge fresco	Heraeus
Temperature controller RDTC-1	E.S.F electronic
Thermoblock	Eppendorf
Thermocycler (Mastercycler personal) for PCR	Eppendorf
Thermomixer comfort	Eppendorf
Transilluminator RH-2	Herolab
Ultrapure Water Systems (Direct-Q^{TM} 5)	Millipore
UV Light (Macro Vue UV=25)	Hoefer Inc.
UV-1601 spectrophotometer	Molecular Devices

VC-6 six channel valve controller	Warner Instrument Corporation
Video copy processor (PSI)	Mitsubishi
Vortexmixer (2TM Mixer 7-2020)	Neolab
Water bath SUB14	Grant Instruments

2.2.13 Software

Pulse software	HEKA Electronic
Sigmaplot 10 software	Systat Software Inc.
Sigmastat software	Systat Software Inc.
Tillvision software	TILL Photonics GmbH

2.3 Methods

2.3.1 Induction of inflammation in mice

C57BL/6j mice received an intraplantar injection of 20µl *Complete Freund's Adjuvant* (CFA) solution into the right hind paw under brief isoflurane anesthesia. The inflammation was confined to the right paw throughout the observation period (2-48 h). This inflammatory model is routinely used by our and other research groups (Stein et al., 1988a; Barber and Gottschlich, 1992). Control animals remained untreated.

2.3.2 Tissue preparation for *in situ* hybridization and immunohistochemistry

48 h after CFA injection all mice were deeply anesthetized with isoflurane and the tissue was fixed by transcardial perfusion. Thereby, the animals were sternotomized and a canula was inserted into the left ventricle of the heart. The right ventricle was opened to let the blood wash out with 1x PBS with the help of a pump. It is necessary to free the animal from blood, which may disturb microscopy of the tissue. In order to fixate the tissue, the animals were perfused transcardially with 4% fixation buffer. Approximately 50-100ml of fixation buffer per animal was used. Immediately after perfusion, the whole spine was dissected and after laminectomy, lumbar L3-L6 DRGs were excised and post-fixed for 2-4 h in fixation buffer and cryo-protected overnight in 30% sucrose. The DRGs were then frozen in 2-methylbutane on dry ice, embedded in frozen section medium, and stored at -80℃.

2.3.3 DRG cultures for calcium imaging

48 h after CFA injection, mice were sacrificed by CO_2 inhalation and the lumbar L3-L6 DRGs were quickly dissected as described in chapter 2.3.2. The DRGs were incubated in 1mg/ml collagenase IV and 0.05% trypsin-EDTA solution for 30 min respectively at 37℃. The DRGs were suspended in DRG medium. In order to dissociate the DRGs, they were passed through 18G, 22G, and 25G needles, and were separated from debris by using a 40µm cell strainer. Subsequently, cells were seeded in a droplet of 10µl medium on poly-L-lysine (100µg/ml) coated coverslips. Cells were incubated for 30 min in the incubator to let them adhere to the bottom, and then the coverslips were flooded with DRG medium. After 1-3 hours the DRGs were loaded with Fura-2 and calcium imaging experiments were conducted.

2.3.4 Development of HEK293-Tet-On expression system

Normal transfection of TRPA1 in HEK293 cells was toxic for the cells for unknown reasons (own observation, Story et al., 2003). Cells constitutively expressing TRPA1 appeared unhealthy and TRPA1 expression was down-regulated after several passages of culture. Due to this fact, we used a tetracycline inducible cell expression system (BD Biosciences, Clontech) to express TRPA1 in HEK293 cells. This gave a reliable and robust expression of TRPA1, which we were never able to attain using constitutive expression with promoters such as CMV or SV40.

The BD™ Tet-On gene expression systems were developed by Gossen and colleagues (1995). The expression of the target protein is turned on in the presence of the antibiotic doxycycline and the expression level is high and specific.

The protein “reverse” Tet repressor (rTetR) regulates the expression of the protein rtTA (reverse tetracycline-controlled transactivator) from the pTet-On regulatory plasmid, which also contains a neomycin-resistance gene. A response plasmid (pTRE2 vector) expressed the gene of interest, in our case TRPA1, under control of the tetracycline-response element (TRE). We created a stable Tet cell line, which contains the regulatory plasmid and is resistant to G418. The gene of interest is only expressed upon binding of rtTA protein to TRE that then activates transcription in the presence of doxycycline. The response plasmid (TRPA1 in the pTRE2 vector) was cloned with the standard biological techniques and was transiently transfected into the HEK293 Tet-On cells.

2.3.5 Molecular Cloning

2.3.5.1 Standard molecular cloning techniques

Standard methods of molecular cloning were performed according to Sambrook and Russel (2001) and will not be described in detail (agarose gel electrophoresis, bacterial cultures, ethanol precipitation, phenol-chloroform extraction, restriction digestion, etc). Sequencing of DNA fragments was conducted by the company AGOWA.

2.3.5.2 Determination of concentration and quality of nucleic acids

The amount of double-stranded DNA was measured photometrically at a wavelength of λ=260nm. The nucleic acid concentration was calculated with OD_{260} x 50µm/ml. The purity of DNA was verified by A_{260nm}/A_{280nm} ratios. The ratio lies between 1.75 and 2.0 for pure DNA.

2.3.5.3 DNA amplification with PCR

PCR is an iterative process, consisting of denaturation of the template by heat, annealing of the oligonucleotide primers to the single stranded target sequence and extension of the annealed primers by a thermostable DNA polymerase to dublicate the template DNA. In further cycles, the newly synthesized DNA molecules are denatured and duplicated again, resulting in multiple copies of the original target sequence (Mullis and Faloona, 1987).

Our standard PCR components used to amplify the templates were as follows:

Reaction mix	**Concentration**
template DNA	1µl (10ng or more)
dNTPs (pH 8.0) (200µM)	1µl
10x amplification buffer + $MgCl_2$	5µl
downstream primer 5' (200nM)	2µl (10pM/µl)
upstream primer 3' (200nM)	2µl (10pM/µl)
ddH_2O	37µl
Taq polymerase 5U/µl	2µl
final volume	50µl

Unless otherwise mentioned the following standard temperature and time profile was used for PCR:

Step	Temperature	Time	Cycle
Denaturation	94℃	2 minutes	1x
Denaturation	94℃	30 seconds	35x
Annealing	58-61℃	60 seconds	
Elongation	72℃	30 seconds	
Prolonged elongation	72℃	5 minutes	1x
Pause	4℃	∞	

2.3.5.4 PCR product purification and analysis

PCR products were isolated with agarose gel electrophoresis and extraction of DNA from gels was prepared with the Qiagen '*QIAquick Gel Extraction Kit*' according to the manual or by phenol-chloroform extraction and ethanol precipitation. For details see Sambrook and Russel (2001).

2.3.5.5 Ligation of DNA fragments

The ligation reaction (10µl final volume) was pipetted together on ice and incubated overnight (16 h) at 4℃.

Our standard ligation solution contained:

300ng	DNA fragment
100ng	linearized plasmid vector
1µl	10x ligation buffer
1U/µg DNA	T4 DNA ligase
up to 10µl	ddH_2O

2.3.5.6 Transformation

DNA from ligation reactions was diluted five-fold in 10mM Tris-HCL (pH 7.5) and 1mM EDTA. 1-5µl (1-10ng) of this solution was mixed with 50µl of competent *E. coli* cells in chilled tubes.

The mixture was incubated on ice for 30 min. After a heat shock at 42℃ for the time given in the appropriate manual (30-45 seconds), the mixture was again incubated on ice for 2 minutes. Subsequently, 450µl of S.O.C medium was added and the reaction was incubated at 37℃ for one hour while shaking in a thermoblock. Finally, 250µl of the bacteria reaction was spread on LB-Agar plates containing the appropriate antibiotic for selection and incubated for 16 h at 37℃.

2.3.5.7 Isolation of Plasmids from bacteria

One bacterial clone was incubated in 5ml LB-medium overnight at 37℃ under constant agitation. After 16 h the suspension was centrifuged and plasmid DNA (small scale up to 20µg) was obtained by using the Qiagen '*QIAprep Spin Miniprep Kit*' according to the manual provided. After analysis of mini-DNA, 500µl of the small scale cell suspension was incubated in 100ml LB medium (and antibiotic) overnight at 37℃ while shaking. For isolation of plasmid DNA in a large scale (up to 500µg) the Qiagen '*QIAfilter Plasmid Maxi Kit*' was used. The DNA was stored at -20℃ until furth er experiments were conducted.

2.3.6 Mutagenesis of mouse and human TRPA1 via PCR

Single amino acid substitution mutations of TPRA1 were achieved using the '*QuikChange II XL Site Directed Mutagenesis Kit*' according to the manufacturer's protocol. Primer Design was performed with the help of the 'primerX' software (http://bioinformatics.org/primerx/cgi-bin/protein_2.cgi).

For complete deletion of the EF-hand domain (Δ466-477), we used a modification of the *Stratagene* protocol described by Makarova and colleagues (2000). All mutations were verified by sequencing.

Reaction mix	**Concentration**
template DNA	10ng
dNTPs mix	1µl
10x reaction buffer	5µl
Quik solution	3µl
downstream primer 5' (125ng)	1.25µl
upstream primer 3' (125ng)	1.25µl
Pfu polymerase (2.5U/µl)	1µl
ddH_2O	up to 50µl
final volume	50µl

Step	Temperature	Time	Cycle
Denaturation	95℃	1 minute	1x
Denaturation	95℃	50 seconds	18x
Annealing	60℃	50 seconds	
Elongation	68℃	1min/kb DNA	
Prolonged elongation	68℃	7 minutes	1x
Pause	4℃	∞	

Afterwards, 1µl DpnI (10U/µl) was added to the reaction mix to digest the parental DNA. The solution was thoroughly mixed, spun down and incubated for 1 h at 37℃. Subsequently, 2µl of the reaction mix was transformed in XL-10 Gold ultra component cells.

2.3.7 Transfection

Transfection is the process of bringing foreign DNA into cells and monitoring protein expression. We used FuGENE®6 transfection reagent, a multi-component lipid-based transfection reagent. It forms complexes with the DNA and thereby transports the DNA into the cell. HEK293 cells stably expressing the Tet-On regulator were transiently transfected with 0.8µg wild-type/mutated human or mouse TRPA1 and 0.2µg of a vector with yellow fluorescent protein (pEYFP-C1) or green fluorescent protein (GFP in pTRE2).

For transfection of a culture dish filled with 1-1.5 ml medium, 97µl of Dulbecco's modified eagle's medium and 3µl FuGENE®6 were carefully mixed. After 5 min the appropriate amount of DNA was added and the solution was incubated at RT for 20 min. Subsequently, the mixture was added dropwise to one plate.

24 h later, expression of TRPA1 was initiated with 1µg/ml doxycycline and cells were used for experiments after further 16-24 hours of incubation. All experiments were repeated with at least 3 different transfections. For control cells, the YFP or GFP containing plasmid was transfected alone.

For experiments in which we used the dominant negative $CaM_{1,2,3,4}$ mutant to investigate CaM dependent regulation of TRPA1, we first optimized experimental conditions using TRPM4 expressed in our tetracycline inducible cell expression system. Expressing $CaM_{1,2,3,4}$ under a CMV promoter at a 1:1 transfection ratio with TRPM4 (50ng/cm^2 of each cDNA) produced maximum inhibition of the TRPM4 Ca^{2+} activated

current. These conditions were then applied to TRPA1 and tested with 10 and 100µM intracellular Ca^{2+}.

2.3.8 Fluorescent *in situ* hybridization and immunohistochemistry

2.3.8.1 Principle

In situ hybridization (ISH) is a technique used to detect, visualize and localize RNA at the cellular level. In FISH (Fluorescence *In Situ* Hybridization) fluorophore-labeled probes or reagents are used for detection.

2.3.8.2 Digoxygenin (DIG) labeled RNA probes preparation

For the preparation of each RNA probe the plasmid (pGEM-T-Easy) containing TRPA1 was linearized with the adequate restriction reaction. The linearized plasmid was then purified with the '*High Pure PCR Product Purification Kit*' and used for the following reaction:

1µg linearized DNA template
2µl DIG RNA labeling mix
2µl 10x transcription buffer
2µl RNA polymerase (T7 or SP6 for sense or antisense probes)
add DEPC H_2O up to 20µl

After incubation of the reaction at 37℃ for 2 hours, the template DNA was eliminated by adding 2U of DNase I and incubation at 37℃ for 15 min. Finally, the polymerase reaction was stopped by adding 2µl of 0.2M EDTA (pH 8.0).

In order to yield better penetration of the tissue, the probes were hydrolyzed by adding an equal volume of DEPC-H_2O and two volumes of carbonate buffer and were incubated for 1 hour at 60℃. The reaction was stopped by adding an equal amount of hydrolysis-neutralization buffer. Then, the probe was purified by ethanol precipitation and resuspended in 100µl DEPC-H_2O. The probes were stored at -80℃ until *in situ* hybridization.

2.3.8.3 Estimation of the DIG-labeled RNA probe concentration

An accurate quantification of DIG-labeled RNA is important for optimal results during the *in situ* hybridization. The estimation of the probe's concentration was performed with a dilution series of the DIG-labeled RNA probes and dilutions of an appropriate standard. The following dilution series of both were made in RNA-dilution buffer and spotted on a piece of positively charged nylon membrane:

Sample	Conc. of the control	Dilution of R0	RNA dilution buffer
R0	100 ng/µl		
R1	10 ng/µl	1 : 10	2µl R0 + 18µl
R2	1 ng/µl	1 : 100	2µl R1 + 18µl
R3	100 pg/µl	1 : 1000	2µl R1 + 198µl
R4	10 pg/µl	1 : 10000	2µl R2 + 198µl
R5	5 pg/µl	1 : 20 000	2µl R4 + 10µl
R6	1 pg/µl	1 : 100 000	2µl R4 + 18µl

The RNA spots were fixed to the membrane by crosslinking with UV light for 5 min. The membrane was transferred to a petri dish and washed in 20ml 1x TN buffer for 2 min while shaking. It was then pre-incubated for 30 min in 10ml blocking solution (in TN buffer) and successively incubated for 30 min with anti-DIG alkaline phosphatase diluted 1:5000 in blocking solution (in TN buffer). The membrane was then washed 2x 15 min with TN buffer containing Tween20 (0.03%) and equilibrated for 5 min in detection buffer. Subsequently, the membrane was incubated overnight in the dark in a freshly prepared solution containing 200µl NBT/BCIP solution in 10ml detection buffer. The membrane was finally washed in ddH_2O and the spot intensities of the control and sample dilutions were compared to estimate the concentration of the DIG-labeled RNA probes.

2.3.8.4 In situ hybridization protocol

All materials and solutions were treated with EDTA/NaOH solution or active DEPC to make it RNase free. 10µm thick DRG cryosections were cut and taken up on polysin microscope slides. Sections were airdried for at least 1 hour at RT and then 15 min at 50℃. Sections were washed 2x 5 min in RNase free PBS, treated with 1µg/ml proteinase K (in PK buffer) for 5 min, and washed again. The slides were then shortly pre-incubated in 0.1M triethanolamine for 3 min and succesively acetylated with 0.25%

(v/v) acetic anhydride in 0.1M triethanolamine for 10 min. After 3x 5 min washing in RNase free PBS, the slides were placed in a previously prepared humid box. In order to pepare it, a mix of 50% formamide and 4x SSC was poured into the chamber. The slides were covered with 750µl hybridization buffer and incubated for 3-4 hours at 56℃. For hybridization, 150ng of each DIG-labeled cRNA per ml hybridization buffer were mixed, denatured at 60℃ for 15-20 min and then imm ediately cooled on ice. The pre-hybridization solution was gently poured off the slides and 200µl of hybridization solution containing the probes were added. The slides were carefully covered with parafilm to ensure an even distribution of the probes and to avoid drying. The hybridization proceeded in the humid box at 56℃ ov ernight.

The following day, slides were placed in 2x SSC at 55℃ so that the parafilm could be removed without force. They were then washed 4 times in 2x SSC at 55℃ for 10 min each, once in 2x SSC at 55℃ for 45 min, once in 0. 1x SSC at 55℃ for 1 hour, once in 0.2x SSC at RT for 10 min, and once in maleic acid solution at RT for 10 min.

The detection was started by placing the slides in blocking solution for 30 min at RT. They were then transferred to a humid box with H_2O on the bottom, where they were laid flat and then incubated with anti-DIG antibody conjugated with horseradish peroxidase diluted 1:100 in blocking solution for 30 min at RT. The slides were then washed 3x 5 min in maleic acid solution with Tween20 (0.03%). The signal was visualized using the Tyramide Signal Amplification System according to the manufacturer's instructions. The slides were finally washed 3x 5 min in maleic acid solution (with 0.03% Tween20) and were immunohistochemstrically co-stained with CGRP.

2.3.8.5 Immunohistochemistry

Slides were pre-incubated in the CGRP-blocking buffer for 30 min at RT and succesively incubated overnight at 4℃ with primary antibody in CGRP-blocking buffer (1:2000). The following day, the slides were washed 5x 10 min (washing buffer) and incubated for 1-2 hours at RT with the respective secondary antibody dilution (anti-rabbit) (1:250). Finally, the slides were washed 3x 10 min in maleic acid solution, rinsed with ddH_2O and mounted with mowiol solution.

2.3.8.6 Counting

All cell counts and quantification were conducted by an observer blinded to the experimental conditions.

2.3.9 Electrophysiology

2.3.9.1 Principle

Electrophysiology studies the transport of charged ions across the cell membrane and the proteins that are involved in that transport.

The patch-clamp technique has been developed by Erwin Neher and Bert Sakmann (1976). It is useful to observe and analyze currents flowing through ion channels in different configurations, e.g. currents flowing through ion channels in the whole cell or currents flowing through one single ion channel. The underlying principle of the patch-clamp technique is to avoid a change in the set membrane potential. The membrane potential is 'clamped' to a certain value and can be controlled by a feedback mechanism and if necessary a compensatory current is injected into the cell to counterbalance the difference. The patch-clamp electrode is not injected into the cell but the micropipette forms a tight seal between the cell membrane and micropipette tip (Hamill et al., 1981). Thereby you can pull out a piece of membrane, the 'patch', from which you can record currents flowing through a single ion channel.

2.3.9.2 Whole-cell and single channel recordings

Recordings were obtained with the EPC-10 amplifier using 'Pulse' software. Giga-seals were formed with clean micropipettes that had a resistance between 2 and 5MΩ in standard pipette solutions. Whole-cell membrane currents were monitored using an EPC-10 patch-clamp amplifier at 24℃ unless otherwise specified. All voltages were corrected for a liquid junction potential of 13.5mV. Capacitance and access resistance were continuously monitored and between 50 and 90% of the series resistance was compensated. Data were sampled at 10-20kHz and filtered at 2-5kHz. Single channel data were obtained in the inside-out patch-clamp configuration at RT. Data were sampled at 10kHz and filtered at 2kHz for analysis. Only cells expressing GFP were selected.

2.3.9.3 Voltage ramps

In order to monitor the temporal development of Ca^{2+}-activated currents in whole-cell configuration, cells were held at -20mV, stepped to -120mV for 20ms, ramped to +100mV (0.25 mV/ms) and returned to the holding potential. Such a ramp was applied every 5 seconds for 300 seconds. Current amplitude was determined at -60mV and +80mV for each ramp.

2.3.9.4 Tail current analysis

Series of voltage activated currents were acquired to measure voltage dependent activation of TRPA1 during maximum Ca^{2+} activation or temperature ramps.

The step protocol consisted of 200ms voltage steps from +160mV to -120mV in 20mV steps followed by a 50ms step at -120mV.

Normalized conductance (G/G_{max}) was mostly obtained from tail current amplitudes determined during the first millisecond of the final step. In some cases however, especially in Ca^{2+}-free conditions, tail currents could not be accurately measured, in which case conductance was calculated from the steady-state current.

2.3.10 Calcium Microfluorimetry

2.3.10.1 Principle

With the help of ratiometric calcium microfluorimetry you can indirectly analyze the activity of cells via an increase in the intracellular calcium level. Ion indicators bind selectively to calcium and, upon binding, show altered fluorescent properties. Calcium binding to the fura-2 calcium indicator shifts the fura-2 excitation spectrum towards a shorter wavelength whereas the peak emission does not change. The ratio of the fluorescent intensities measured at the two excitation wavelengths can be used to measure the calcium increase in the cell. The measured ratio rules out effects of dye concentration, cell thickness, excitation light intensity, dye leakage, photobleaching and camera sensitivity (Grynkiewicz et al., 1985; Takahashi et al., 1999).

2.3.10.2 Cell preparation and experimental procedures

Transfected HEK293 Tet-On cells or cultured DRG neurons were distributed on glass coverslips coated with poly-L-lysine. Ratiometric calcium imaging was performed using fura-2 dye and analysed using ‘Tillvision’ software. Cells were loaded with 3µM fura-2

solution in CIB and incubated for 30 min at 37℃ (transefected Hek293 Tet-On cells) or at RT (DRG). After 30 minutes the fura-2 containing solution was replaced with CIB for 15 min. The coverslip with the cells was placed in a recording chamber that allowed direct application of buffer at controlled temperature. Pairs of images were recorded every 1 or 2 sec at alternating exposures of 340nm and 380nm (exposure time 50ms) using a Polychrome V monochromator and a CCD Imago camera. The background fluorescence was subtracted and the ratio of fluorescence at 340nm and 380nm was calculated. The coverslips were constantly superfused with CIB buffer at approximately 2ml/min. Drugs were applied via a gravity-driven perfusion system that also allowed rapid exchange of solutions. Cold stimuli were applied using a peltier device that cooled the CIB buffer at about 1℃/sec from ~31℃ to ~9℃. Temperature changes were monitored with a thermocouple placed within the flow of buffer and close to the cells. All graphs are averaged ratios of 50-200 individual cells. Drugs were washed out for 3-5 min before 40mM KCl was applied for 10 sec to determine the total number of living DRG cells.

A response was designated as a 20% increase in fluorescence ratio from baseline. The numbers of responders was expressed as a percentage of KCl responsive cells (DRG cells or YFP-expressing HEK293 Tet-On cells).

2.3.11 Data analysis

Data was extrapolated using Microsoft Excel 2003 for Windows. ‘Sigmaplot’ software was used for data analysis and plots. Statistical significance was determined by comparison of responses with appropriate controls using the Student’s paired and unpaired t-test, Mann-Whitney rank sum test or one-way ANOVA and Kruskal-Wallis ANOVA on ranks (Dunn’s method) (*P<0.05). In all graphs, grouped data are expressed as means ± s.e.m.

3 Results

3.1 Intracellular calcium activates the ion channel TRPA1

3.1.1 TRPA1 is activated by intracellular calcium

We and other research groups observed that cells, which heterologously express TRPA1, show high spontaneous activity of single-channel currents and whole-cell currents (Nagata, 2007) in the presence of calcium. Moreover, Jordt and colleagues (2004) suggested that calcium released from intracellular stores might activate TRPA1.

In the course of my PhD project we were searching for an endogenous compound that is able to activate TRPA1 and therefore we tested, in collaboration with Brian Yurgionas, whether TRPA1 is activated by intracellular calcium using whole-cell patch clamping. Intracellular calcium concentration was controlled by using buffered intracellular solutions that perfuse the cells after the whole-cell configuration was obtained. Immediately after establishment of the whole-cell configuration the raised intracellular calcium level activated TRPA1-transfected HEK293 Tet-On cells but not GFP-transfected controls. We applied voltage ramps from -120 up to +100mV from a holding potential of -20mV and we measured the activation to 1mM intracellular calcium every 5 sec for 5 min (figure 5). Figure 5 shows averaged whole-cell currents (n=11) activated by calcium depicted against time at -60mV and +80mV. TRPA1 containing cells were almost immediately activated by dialysis of calcium from the pipette solution. The maximal activation is reached within 30 seconds (see time point 1 in figure 5) and the current declined back to baseline levels within 5 min (see time point 2 in figure 5). Depicting the current-voltage (I-V) relationship indicates a strong outward-rectifying behavior of TRPA1 upon calcium stimulation (figure 6). The I-V curves were obtained from voltage-ramps, measured at times indicated by the numbers in figure 5. The outward-rectifying behavior is typical for TRP channel responses to an agonist and might also point towards the existence of a voltage dependence of TRPA1. We can conclude from these experiments that intracellular calcium activates TRPA1 in the whole-cell configuration.

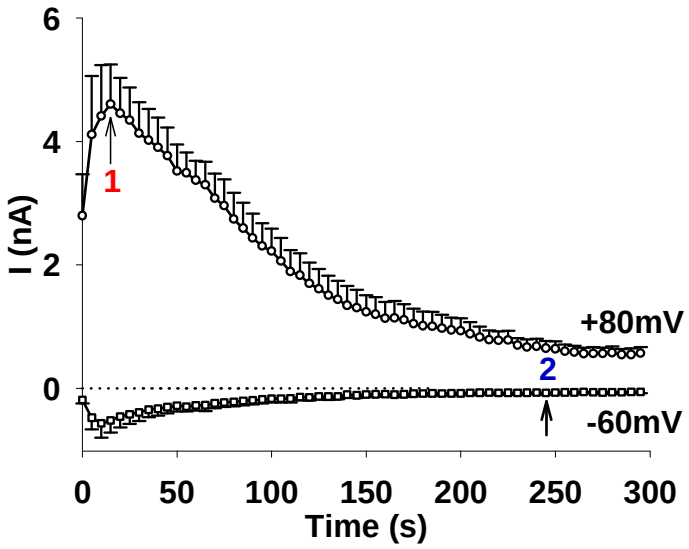

Figure 5 TRPA1 is activated by intracellular calcium. Average TRPA1 whole-cell currents (I) at +80mV and -60mV in HEK293 cells in the presence of 1mM intracellular calcium (n=11). Data are expressed as mean ± s.e.m.

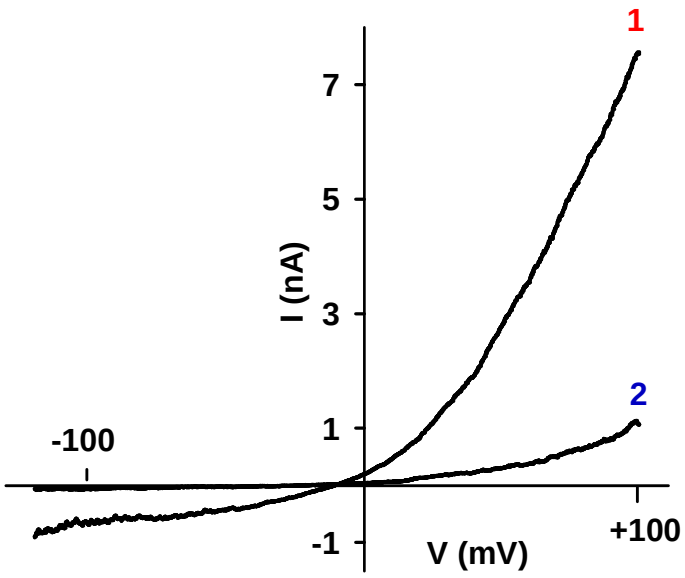

Figure 6 Current-voltage relationship (I-V) of TRPA1 currents measured at time points indicated in figure 5.

3.1.2 TRPA1 is directly activated by intracellular calcium in excised patches

The next question was to determine whether intracellular calcium directly activates TRPA1. In order to test this possibility, inside-out single channel recordings were performed. Intracellular pathways are most likely disrupted when a patch of membrane is taken out of the membrane and thus it can be tested whether an agonist directly regulates a channel. 100µM of calcium was added to the bath solution, which corresponds to the intracellular side of the cell. In the absence of intracellular calcium almost no channel openings were observed, but in the presence of calcium, TRPA1 is activated and pronounced channel openings were recorded (figure 7). With the help of an amplitude histogram, all data points were grouped according to their amplitude. The histogram (figure 7) clearly shows two peaks at the corresponding amplitudes for the open state and the closed state. Current amplitudes at positive potentials were greater than at negative potentials (2.68pA vs. 1.19pA). Moreover, the open probability indicates the fraction of time the channel is in the conducting state. The probability of the channel to be open is slightly higher at positive potentials (+30mV) than at negative potentials (-30mV). This could also be an indicator for a weak voltage dependence of TRPA1. We reasoned from this experiment that TRPA1 is indeed directly activated by intracellular calcium.

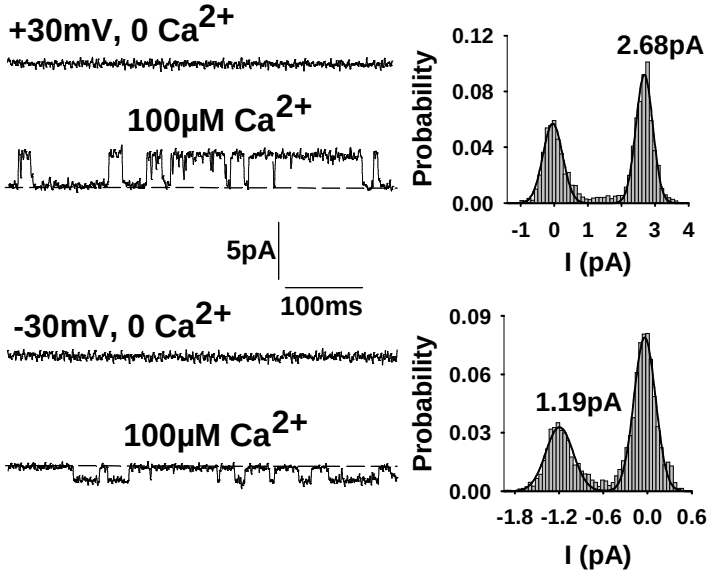

Figure 7 Representative inside-out single channel recordings at +30mV and -30mV in the absence and presence of 100µM intracellular calcium and corresponding amplitude histograms at indicated potentials.

3.1.3 Calcium activates TRPA1 in a concentration-dependent manner

The next step was to assess whether intracellular calcium is able to activate the ion channel TRPA1 in a concentration dependent manner.

We applied voltage ramps from -120mV to +100mV every 5 sec for 5 min and measured the maximal current reached during the experiment at +80mV. Figure 8 shows a dose-response curve for maximum TRPA1 activation at +80mV at different concentrations of intracellular calcium. The magnitude of the currents was dependent on the amount of calcium present in the solution with a half-maximal effector concentration (EC_{50}) of 6µM.

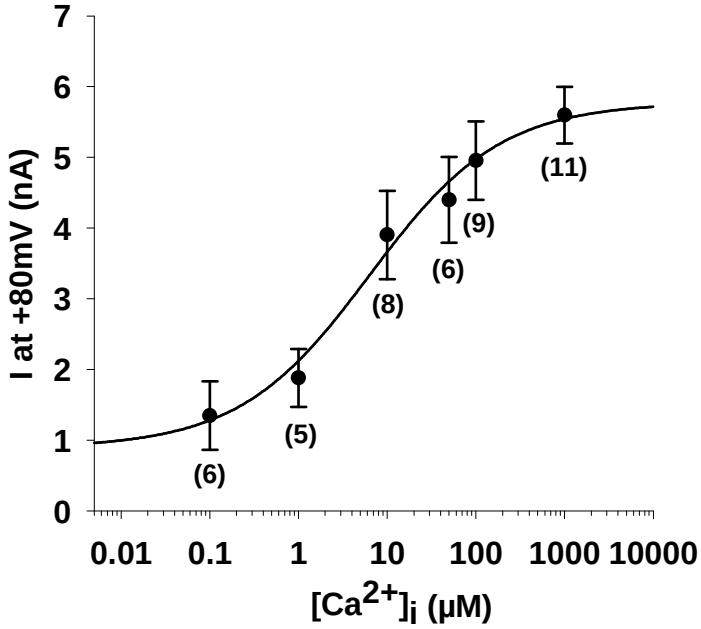

Figure 8 Concentration-response curve of calcium induced whole-cell currents in TRPA1-expressing HEK293 cells. Voltage ramps were applied and the maximal currents at +80mV were extracted. The n numbers of cells recorded at certain concentrations are shown in brackets. Data are expressed as mean ± s.e.m.

3.1.4 Calcium shifts the voltage-dependent activation curve of TRPA1 to physiological potentials

The outward-rectifying behavior of TPRA1 in response to intracellular calcium (figure 6) at positive potentials is a typical phenomenon of TRP channel activation by their agonists. This might also reflect a weak voltage dependence of the channel. TRP channels were originally considered to be voltage independent. However, recently it has

been shown that several TRP channels were slightly voltage dependent with an activation curve extending mainly into the non-physiological range (Nilius B, 2005). These TRP channels are TRPV1, TRPM8, TRPV3, TRPV6, TRPM4, and TRPM5 (Hofmann et al., 2003; Nilius et al., 2003; Voets et al., 2003; Chung et al., 2004a; Voets et al., 2004). Several reports provided evidence that the voltage-dependence of TRP channels can be modulated by application of certain agonists or temperature stimuli in such way that the activation curve is gradually shifted to the left, i.e. to more physiological potentials (Hofmann et al., 2003; Nilius et al., 2003; Voets et al., 2004).

We investigated whether a similar shift exists for the voltage-dependent activation curve of TRPA1 when different concentrations of intracellular calcium were applied. Figure 9 shows the voltage step protocol we used and three representative current traces of TRPA1 containing HEK293 cells at different concentrations of intracellular calcium (0µM, 1µM, and 100µM). The voltage step protocol started at a holding potential of -20mV and 20mV steps were used from -120mV to +160mV.

In calcium free conditions channel currents could only be measured at potentials above +60mV and the voltage for half-maximal activation ($V_{1/2}$) was 155 ± 25mV. Increasing intracellular calcium levels induced a concentration-dependent leftward shift of the activation curve towards negative and thus more physiological potentials. Dialyzing TRPA1-expressing cells with 1µM, 6 µM, and 100µM intracellular calcium changed the $V_{1/2}$ to 35 ± 9mV, 14 ± 5mV and -1 ± 3mV, respectively. The probability of the channel to open is higher in the presence of intracellular calcium. Therefore, we can conclude that voltage dependence and activation by intracellular calcium are closely linked in TRPA1.

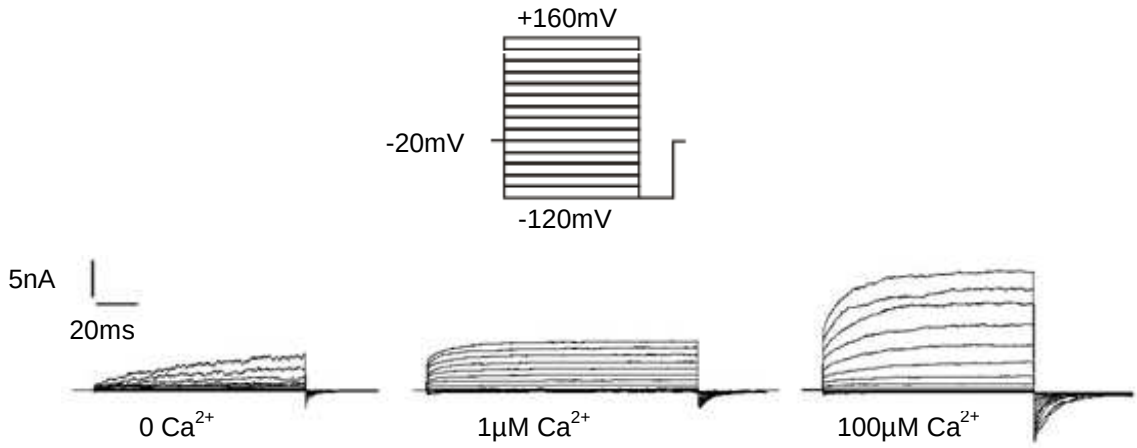

Figure 9 The voltage step protocol and representative current traces at 0µM, 1µM, and 100µM intracellular calcium.

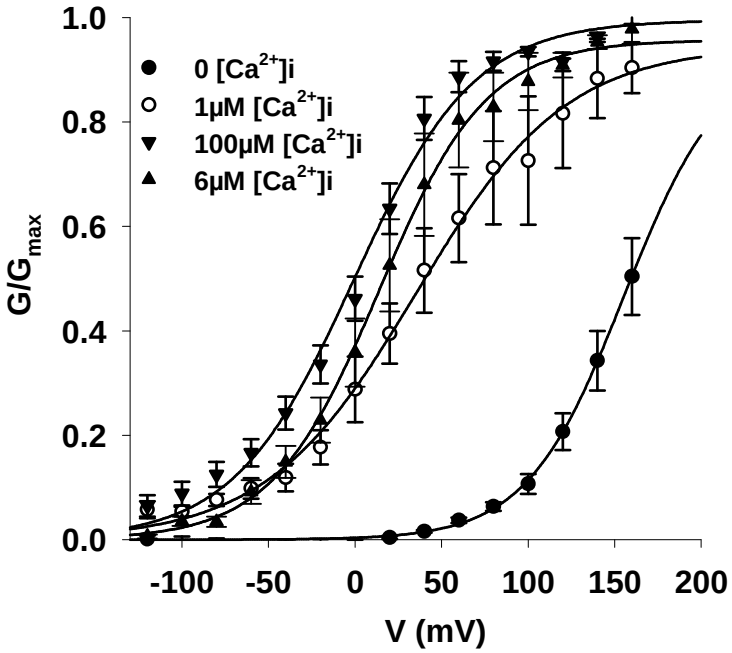

Figure 10 Voltage dependent activation curves of TRPA1 in the presence of different calcium concentrations (0µM 1µM, 6µM, and 100µM) (n=9-11). G/G_{max}, normalized conductance. Solid lines are best fits to Boltzmann functions ($I_{tail}=I_{max}/(1+\exp(-(V-V_{1/2})/s))$). Data are expressed as mean ± s.e.m.

3.1.5 Is TRPA1 also activated by other divalent cations?

TRPA1 is, like most other TRP channels, a non-selective cation channel and calcium is a divalent cation that directly activates TRPA1 in a concentration dependent manner. Therefore, we decided to conduct experiments in order to test whether several other divalent cations can activate TRPA1.

We applied 100µM of strontium (Sr^{2+}), barium (Ba^{2+}), and manganese (Mn^{2+}) in calcium free conditions and measured currents of TRPA1-transfected HEK293 Tet-On cells and GFP-transfected control cells at -60mV and +80mV. The same voltage ramps as in chapter 3.1.1 were used and we also investigated whether the voltage-dependent activation curve of TRPA1 is shifted by these cations. At -60mV, there was no significant activation of TRPA1-transfected cells observable compared to GFP-transfected control cells except for manganese (*P*=0.026; Mann-Whitney rank sum test) (figure 11 A). However at +80mV, TRPA1 is significantly activated all cations in comparison to control cells (Sr^{2+}: *P*=0.005, t-test; Ba^{2+}: *P*=0.004, Mann-Whitney rank sum test; Mn^{2+}: *P*=0.002, Mann-Whitney rank sum test) (figure 11 B). The currents at +80mV were bigger than at -60mV. In general, none of these cations is capable of

shifting the voltage-dependent activation curve as much as calcium. The biggest shift was observed for strontium (figure 12). The voltages of half-maximal activation ($V_{1/2}$) of these cations at 100µM concentration were always above +126mV for TRPA1-transfected cells and above +260mV for GFP-transfected controls. We can conclude from this experiment that other divalent cations such as strontium, barium and manganese do not activate TRPA1 as strong as calcium.

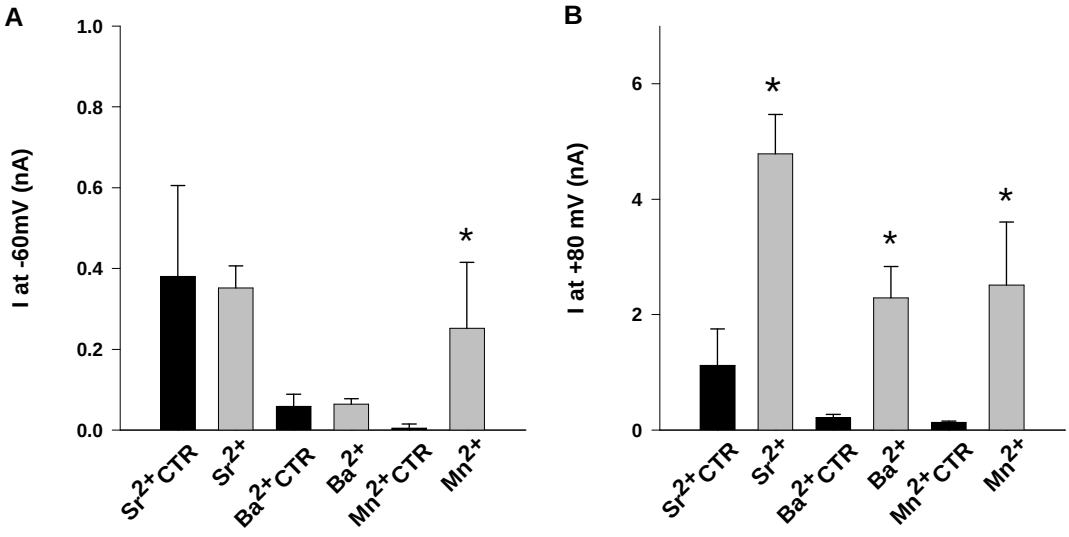

Figure 11 Activation of TRPA1 containing HEK293 Tet-On and GFP-transfected control (CTR) cells to 100µM strontium, barium, and manganese at -60mV (**A**) and +80mV (**B**). Please note the difference in scaling. Data are expressed at mean ± s.e.m. **P*<0.05 in comparison to GFP-transfected cells (t-test and Mann-Witney rank sum test).

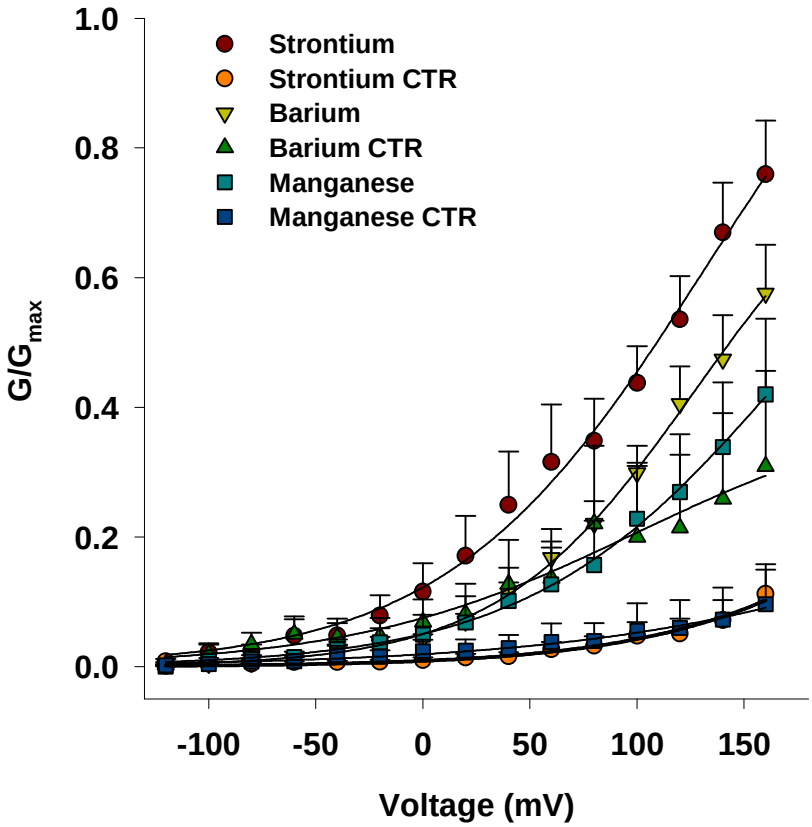

Figure 12 Voltage dependent activation of TRPA1- and GFP-transfected control cells (CTR) in the presence of 100µM strontium, 100µM barium, and 100µM manganese. G/G_{max}, normalized conductance. Solid lines are best fits to Boltzmann functions. Data are expressed as mean ± s.e.m.

3.1.6 TRPA1 agonists activate the channel in the absence of calcium

We tested whether the most commonly used agonists of TRPA1 are able to activate the channel in the absence of calcium. Cinnamaldehyde, mustard oil, and acrolein are relatively specific agonists for TRPA1 (Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2006) and often used pharmacological agents in order to investigate the channel. We tested these agonists at several concentrations (100µM, 200µM, and 400µM for mustard oil; 400µM, 800µM, and 1600µM for cinnamaldehyde; 20µM, 40µM, and 80µM for acrolein) in calcium free conditions with whole-cell patch clamping. The cells were perfused with the agonist for 2 minutes and the currents were recorded at a holding potential of -60mV. The response to these agonists was strongly reduced by about 10-fold (figure 13) in comparison to the response of the channel in the presence of calcium (not shown). Furthermore, very high concentrations were necessary to evoke a response above 1nA. Figure 13 shows the mean whole-cell current at -60mV (n=5-6) for

all concentrations applied. However, the channel can still be activated in a dose dependent manner. In summary, TRPA1 is responsive to cinnamaldehyde, mustard oil, and acrolein in the absence of intracellular calcium but very high concentrations are necessary.

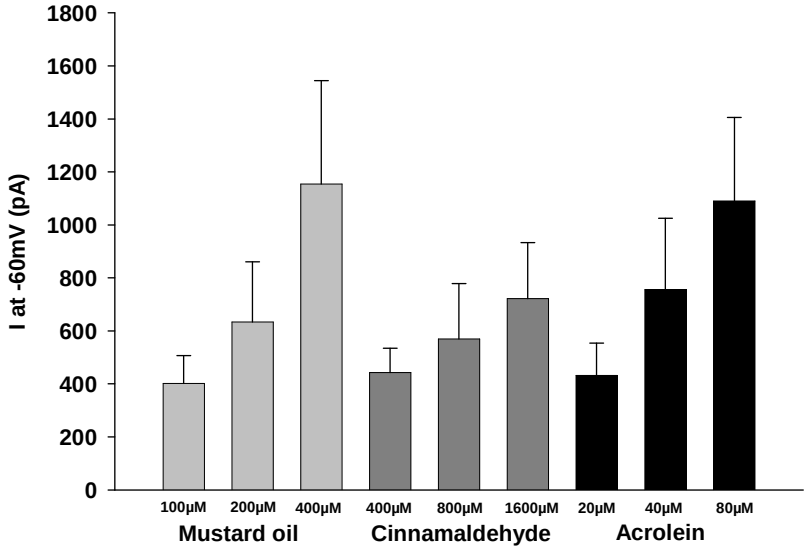

Figure 13 Average peak current of TRPA1 evoked by mustard oil, cinnamaldehyde, and acrolein in calcium free conditions at concentrations indicated in the graph. Data are expressed as mean ± s.e.m.

3.2 Calcium activates TRPA1 via the EF-hand domain

3.2.1 Calmodulin is not required for the calcium sensitivity of TRPA1

The next step was to investigate what the underlying mechanism of the calcium sensitivity of TRPA1 is. Calmodulin (CaM) is ubiquitously expressed in all eukaryotic cells and has been identified as a calcium binding protein with four calcium binding domains. It can regulate many cellular processes and has been shown to be a mediator of calcium dependent regulation of many proteins, including ion channels (Zhu, 2005). Moreover, it regulates also several TRP channels, such as TRPM4, TRPC4, or TRPV1 (Numazaki et al., 2003; Rosenbaum et al., 2004; Nilius et al., 2005a; Zhu, 2005). Therefore, calmodulin seemed to be a likely candidate for the calcium-dependent regulation of TRPA1. In collaboration with Julia A. Jira, we tested whether CaM is necessary for the calcium sensitivity of TRPA1. In order to test a regulatory role of calmodulin, a dominant negative CaM mutant ($CaM_{1,2,3,4}$) was co-transfected (1:1 ratio) with TRPA1 in HEK293 Tet-On cells. This CaM mutant is unable to bind any calcium. As a positive control, we also examined $CaM_{1,2,3,4}$ modulation of the TRP channel TRPM4 because it is known to be regulated by CaM (Nilius et al., 2005a). We analyzed the maximal current flowing at +80mV by means of voltage ramps. Over-expression of $CaM_{1,2,3,4}$ significantly reduced TRPM4 currents elicited by 100µM calcium (figure 14 B). For TRPA1, two concentrations of intracellular calcium were tested (10µM and 100µM). However, $CaM_{1,2,3,4}$ co-transfection had no effect on calcium-activated TRPA1 currents (figure 14 A). In addition to these experiments, we intracellularly applied 500nM wild-type CaM or 100µM of the CaM antagonist W-7 (figure 14 C and 14 D). Neither wild-type CaM nor the antagonist W-7 affected the calcium sensitivity of TRPA1. Thus, even though CaM is essential for many proteins, including TRP channels, it does not appear to regulate the calcium-dependent activation of TRPA1.

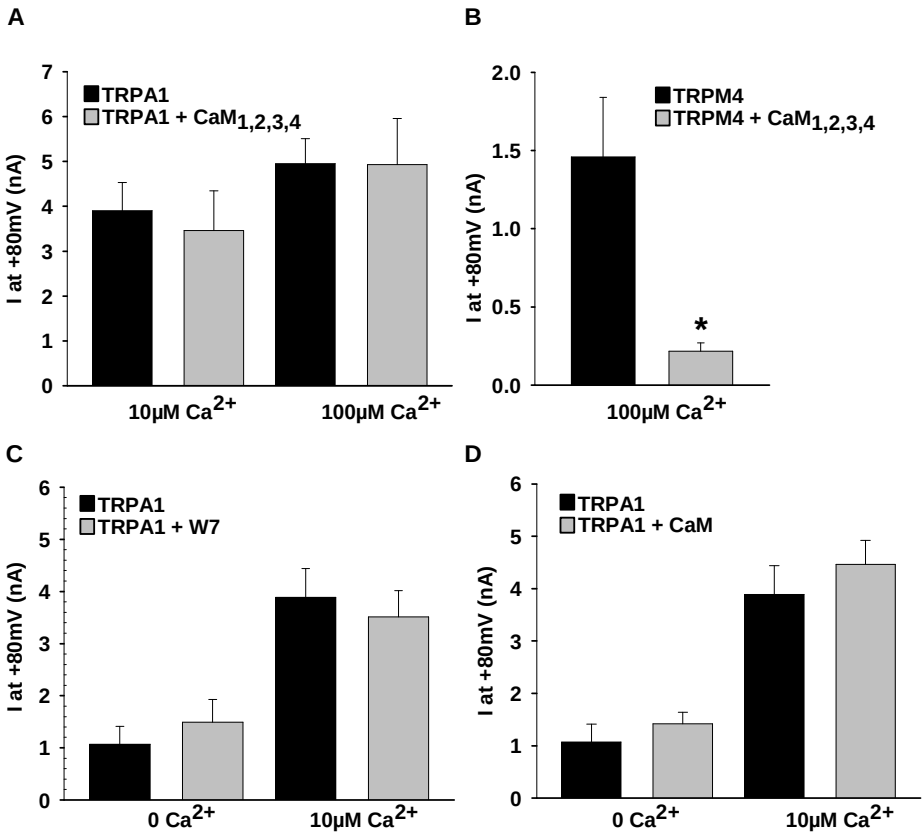

Figure 14 A, Average maximum TRPA1 currents at +80mV are not affected by over-expression of the dominant-negative mutant $CaM_{1,2,3,4}$ (n=6-11). **B**, TRPM4 currents are significantly reduced by over-expression of $CaM_{1,2,3,4}$, *P*=0.03 (Mann-Whitney rank sum test). **C**, The CaM antagonist W-7 (100µM) does not alter calcium activation of TRPA1. **D**, Intracellular application of 500nM wild-type CaM does not modulate calcium-induced TRPA1 currents. Data are shown as mean ± s.e.m. **P*<0.05 in comparison to the normal TRPA1 response to calcium (t-test and Mann-Whitney rank sum test).

3.2.2 TRPA1 contains an EF-hand domain responsible for the calcium sensitivity of the channel

In the last chapter we have demonstrated that calmodulin is not required for the calcium sensitivity of TRPA1. Other options might be further calcium-binding proteins or direct gating by an intrinsic calcium sensor. TRPA1 is annotated as containing a putative calcium-binding EF-hand domain in the N-terminus of the channel. An EF-hand domain has two alpha helical domains and a characteristic 12-residue loop region in between that binds the calcium molecule (Kretsinger and Nockolds, 1973; Ikura, 1996). The calcium ion is coordinated in a pentagonal bipyramidal configuration. Residues 1, 3, 5,

7, 9, and 12 (also denoted by X, Y, Z, -Y, -X, and –Z) of the loop region provide oxygen ligands to the calcium ion necessary for its binding (Gifford et al., 2007).

We wanted to examine whether the EF-hand domain is necessary for the calcium activation of TRPA1. In order to do so, we generated several point mutations at key amino acid residues at position 1, 3, 5, and 12 of the EF-hand loop region and also deleted the entire EF-hand loop region (Δ466-477 = ΔEF). We substituted alanines for key residues at D466, S468, T470, D477, and also made a double mutation for S468 and T470 in hTRPA1 cDNA. We then tested these mutated channels for calcium sensitivity with the help of whole-cell patch clamping and voltage ramps (from -120mV to +100mV). The maximal current at +80mV was extracted and the results were depicted at percent responses to the wild-type current (figure 15). Remarkably, ΔEF, D466A, and D477A TRPA1 mutants were completely insensitive to the two intracellular calcium concentrations tested (1µM and 100µM) (figure 15). S468A, T470A, and the double S468A+T470A mutants showed significantly reduced calcium sensitivity at 1µM but the response to 100µM appeared to be comparable to the wild-type response (red line at 100%) (n=5-13).

We partially repeated these experiments with mTRPA1 as well. We did two substitution mutations in mTRPA1 cDNA. The aspartic acid residues corresponding to the human D466 and D477 were substituted to alanines in the mouse cDNA (D469 and D480). Subsequently, three different concentrations of intracellular calcium were applied to test the response of the mouse channel to calcium (1µM, 10µM, and 100µM). Calcium sensitivity of D469A mutants (n=3-10) were slightly but not significantly reduced but the calcium sensitivity of D480A mutants (n=5-13) were almost completely abolished (figure 16 and 17).

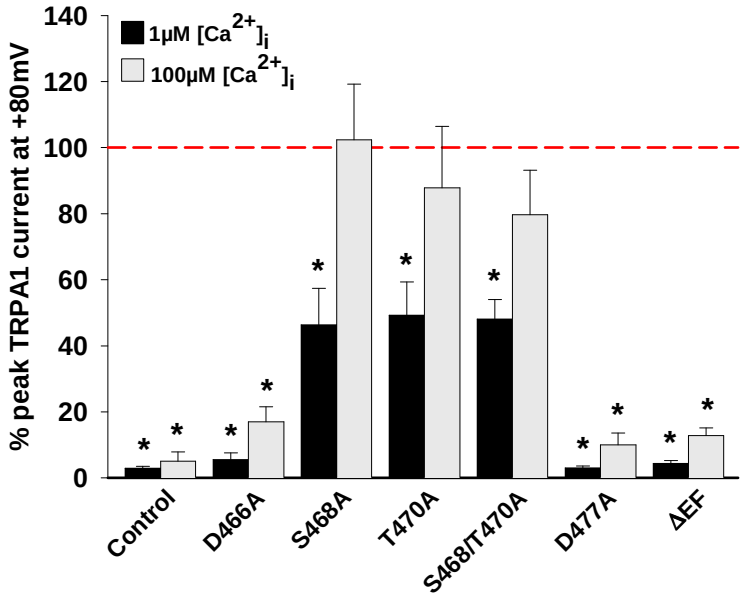

Figure 15 Mutations within the EF-hand domain of hTRPA1 reduce activation by intracellular calcium. Two concentrations were tested (1µM and 100µM). Percentage of wild-type TRPA1 response (dotted red line at 100%) were given at the indicated concentration (n=5-13). Data are expressed as mean ± s.e.m. **P*<0.05 compared to wild-type TRPA1-transfected cells (Kruskal-Wallis ANOVA on ranks, Dunn's method).

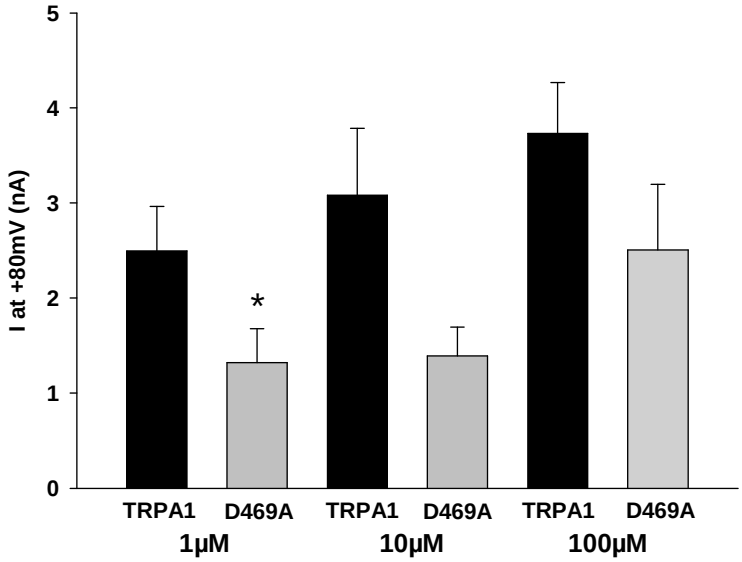

Figure 16 Response of cells transfected with wild-type and mutant mTRPA1 to different calcium concentrations. Substitution mutation at amino acid D469A (corresponding to D466A in human) reduced the response to intracellular calcium (n=3-10). Data are expressed as mean ± s.e.m. **P*<0.05 compared to wild-type mTRPA1-transfected cells (Mann-Whitney rank sum test; *P*=0.014 at 1µM).

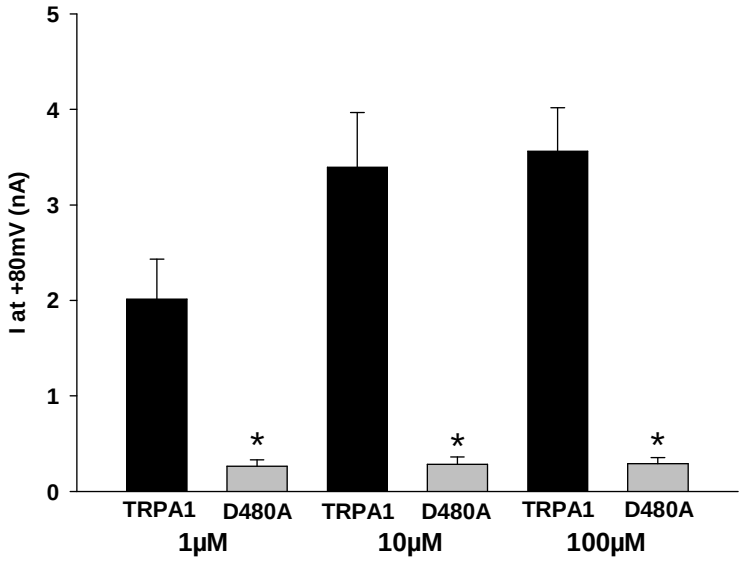

Figure 17 Response of cells transfected with wild-type and mutant mTRPA1 to different calcium concentrations. Substitution mutation at amino acid D480A (corresponding to D477A in human) reduced the response to intracellular calcium significantly (n=4-13). Data are expressed as mean ± s.e.m. **P*<0.05 compared to wild-type mTRPA1-transfected cells (Mann-Whitney rank sum test; *P*=0.005 at 1µM; *P*=0.003 at 10µM; *P*<0.001 at 100µM).

3.2.3 EF-hand mutants are expressed in the membrane and are functional

It might be possible that mutations of the EF-hand domain in TRPA1 leave the channel completely nonfunctional or the expression in the plasma membrane is disturbed. Consequently, yellow fluorescent protein (YFP) was tagged to the calcium-insensitive TRPA1 mutant proteins and used to detect expression in transfected cells.

Figure 18 demonstrates that the wild-type and the calcium-insensitive D477A mutant channel were expressed in the membrane of transiently transfected HEK293 Tet-On cells. Moreover, distribution pattern of the mutant channel was comparable to the wild-type protein expression.

20µM mustard oil induces small currents in wild-type TRPA1 and mutant D477A expressing cells in calcium free conditions. Addition of extracellular calcium strongly potentiates the mustard oil response but only for the wild-type channel. In addition to the proper expression in the plasma membrane, all EF-hand mutants were tested for cinnamaldehyde responsiveness. 100µM cinnamaldehyde was applied in a calcium microfluorimetry experiment. All mutants responded to the TRPA1 agonist whereas YFP-transfected HEK293 Tet-On cells did not. The response of the mutant channels

was slightly reduced and delayed in comparison to the response of the wild-type channel but still prominent (figure 20).

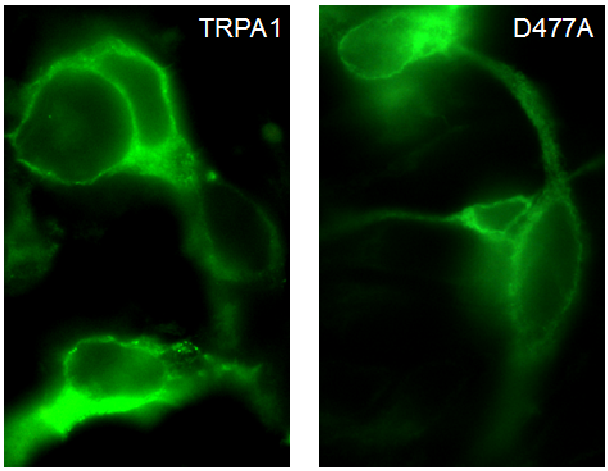

Figure 18 Expression of yellow fluorescent protein-tagged constructs in HEK293 Tet-On cells. The expression pattern of the mutant channel (**D477A**) is similar to the wild-type TRPA1 channel expression (**TRPA1**).

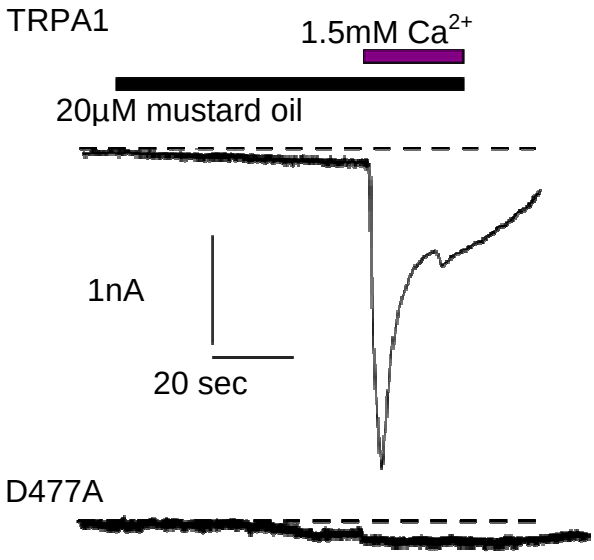

Figure 19 Example of inward current at -60mV evoked by 20µM mustard oil in wild-type hTRPA1 and D477A mutant TRPA1-expressing cells. Note that extracellular calcium strongly potentiates the small mustard oil response of TRPA1 but had no effect on the D477A-TRPA1 current.

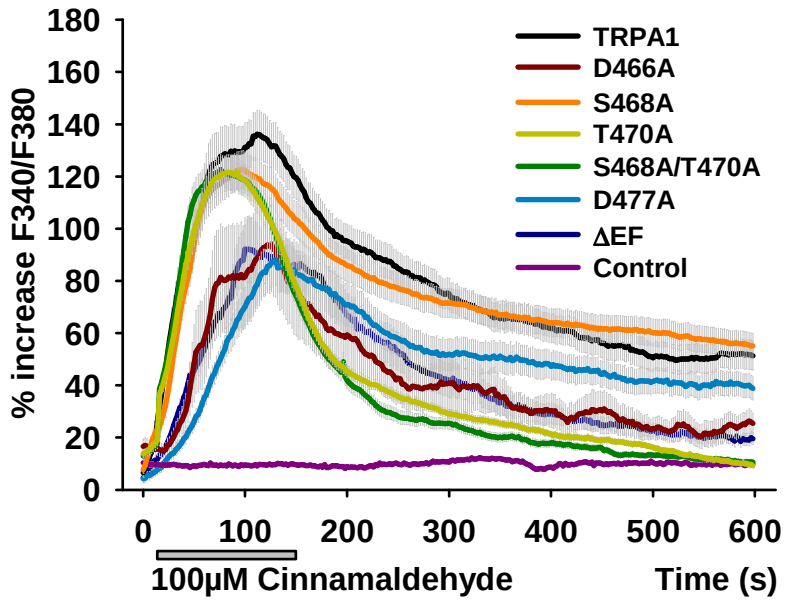

Figure 20 100µM cinnamaldehyde activates wild-type TRPA1 (black) and mutant channels in calcium imaging experiments, whereas YFP-transfected control cells were not activated by cinnamaldehyde (purple).

3.3 Calcium release from intracellular stores can activate TRPA1

3.3.1 Reduced sensitivity of EF-hand mutants to calcium release by thapsigargin, bradykinin, and carbachol

Conceivably, any signal leading to an elevation in intracellular calcium levels in TRPA1-expressing sensory neurons could activate the channel. We tested the role of intracellular calcium in this context with the help of pharmacological agents in calcium imaging experiments.

We applied 1µM thapsigargin, which inhibits sarco-and endoplasmic reticulum Ca^{2+} (SERCA) transport ATPases (Inesi et al., 1998). Thereby it induces a release of Ca^{2+} from intracellular stores and blocks the re-uptake of calcium. We measured the activity of TRPA1 due to application of thapsigargin with calcium imaging. Additionally, we tested our calcium-insensitive EF-hand mutants. Thapsigargin-evoked release of intracellular calcium results in a strong response of the wild-type TRPA1-expressing cells. The response of the full EF-hand mutant (ΔEF =Δ466-477) corresponds to the response of YFP-transfected control cells. There was a ~50% increase in the fluorescence ratio but the response was not as strong as the TRPA1 response. In D466A and D477A TRPA1 mutants the activity of the channel was abolished as well and markedly diminished in S468A, T470A and S468A/T470A expressing cells (figure 21).

Several studies at the cellular and whole animal level have demonstrated a robust coupling between the proinflammatory peptide bradykinin, which produces nociceptor excitation and hypersensitivity via PLC and PKA signaling pathways (by way of GPCR), and the ion channel TRPA1 (Bandell et al., 2004; Bautista et al., 2006; Wang et al., 2008). We tested the role of Ca^{2+} in this context with the help of our calcium-insensitive TRPA1 mutations. Application of bradykinin (1µM) to cells evoked a small increase in intracellular calcium levels (presumably due to release from intracellular stores) that was strongly potentiated in the presence of TRPA1 (figure 22). This response was significantly reduced in cells expressing calcium-insensitive TRPA1 mutant channels, suggesting that Ca^{2+} signaling is probably the predominant means of TRPA1 activation by bradykinin. The response of D466A, D477A, and ΔEF mutant TRPA1 were almost reduced to the response level of YFP-transfected control cells. Unexpectedly, the response of three mutant channels was identical to the wild-type response (S468A) or

even exceeded their activation for unknown reasons (T470A and S468A/T470A) (figure 22).

Moreover, application of the cholinergic agonist carbachol (100µM) evoked a transient increase in intracellular calcium followed by a sustained response phase by binding to endogenous muscarinic acetylcholine receptors. The sustained response to carbachol was potentiated in the presence of TRPA1 in comparison to YFP-transfected control cells. Such an elevation in the response was also observable in S468A, T470A, and S468A/T470 mutant channels but not in the highly calcium-insensitive D466A, D477A, or ΔEF mutants (figure 23).

The fact that the completely calcium-insensitive EF-hand mutants (D466A, D477A, and ΔEF) show a reduced response to carbachol or bradykinin indicates that intracellular calcium is essential for activation of TRPA1 downstream of PLC and that any increase in intracellular calcium level can activate the channel via the EF-hand domain. Thus, calcium is an important and endogenous ligand of TRPA1.

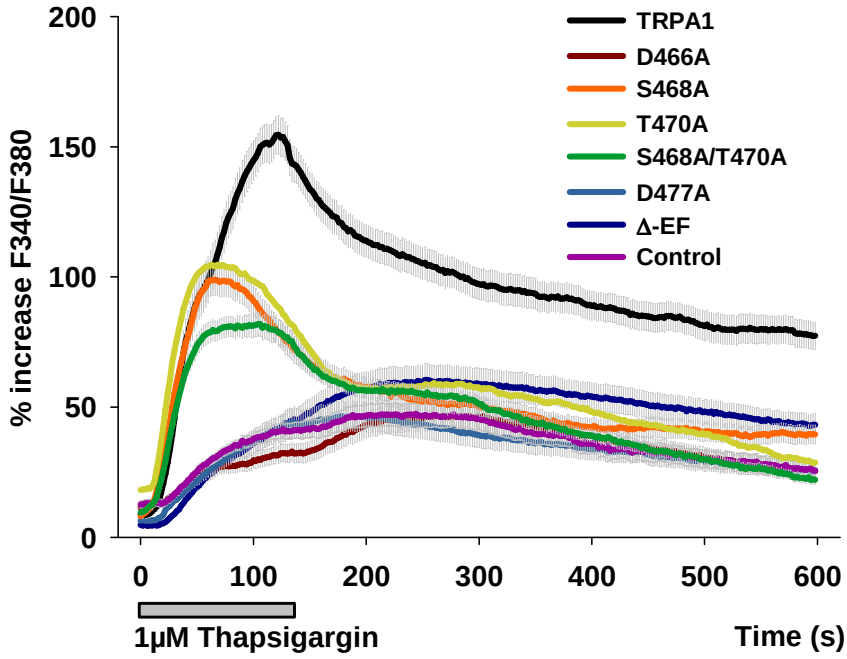

Figure 21 Mutations of the EF-hand domain reduce 1µM thapsigargin-evoked activation of TRPA1. The response of wild-type TRPA1 to thapsigargin is shown in black. Responses of mutations are colored. F340/F380, ratio of fluorescence at 340nm to that at 380nm.

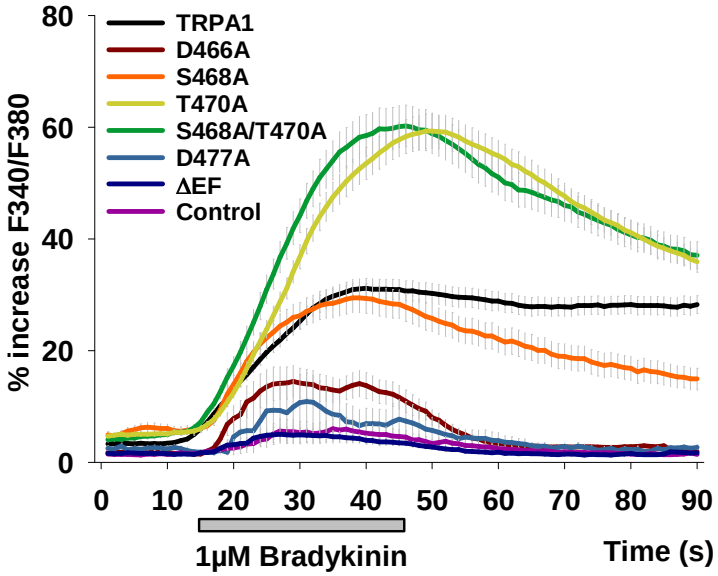

Figure 22 Application of bradykinin (1μM) evokes large $[Ca^{2+}]_i$ responses in cells transfected with TRPA1. This response was strongly reduced in Ca^{2+} insensitive (D466A, D477A, and ΔEF) TRPA1 mutants. F340/F380, ratio of fluorescence at 340nm to that at 380nm.

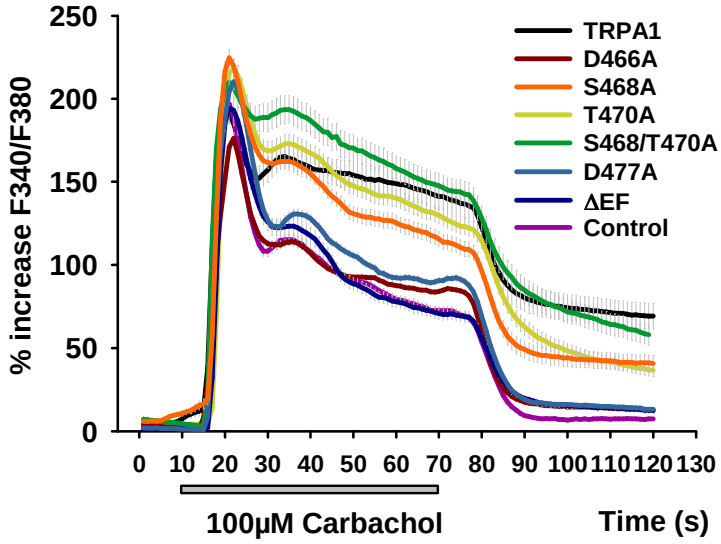

Figure 23 100μM carbachol evokes large sustained intracellular calcium responses in cells transfected with TRPA1. Sustained responses are strongly reduced in calcium insensitive TRPA1 mutants. F340/F380, ratio of fluorescence at 340nm to that at 380nm.

3.4 Is TRPA1 an osmolarity sensing ion channel?

3.4.1 TRPA1 is not activated by osmolarity

It has been suggested that TRPA1 might function as a mechanical activated channel in the inner ear of the mouse and that the channel shares many properties with other mechanotransduction channels (Corey et al., 2004; Nagata et al., 2005).

In one TRPA1 knockout study the mice had deficits in sensing noxious punctuate cutaneous mechanical stimuli but showed no alteration in the transduction properties in hair cells. Thus, TRPA1 might contribute to mechanosensation in the skin (Kwan et al., 2006). Nevertheless, direct evidence of TRPA1 activation by mechanical forces has thus far been missing.

Therefore, we wanted to test whether osmotic changes are able to activate the channel expressed in HEK293 Tet-On cells.

We tested solutions with different osmolarities in calcium imaging and patch-clamp experiments. In order to keep the ion concentration in the solution constant while varying the osmolarity, sodium concentration was reduced to 88mM and the medium osmolarity was adjusted to 300mosmol/l by addition of mannitol. Solutions of lower or higher osmolarity were obtained by reducing or elevating the mannitol concentration, respectively. During calcium imaging recording of the hTRPA1 activity, the cells were superfused with 300mosmol/l buffer for the first 90 seconds. Afterwards, the solution with the appropriate osmolarity was applied for 11 minutes. Finally, the control buffer was again applied (300mosmol/l).

With both, hypotonic solutions (200, 240, and 270mosmol/l) and hypertonic solution (330mosmol/l) we saw an enhanced activation in comparison to the recording at isotonic solution (300mosmol/l). However, only 200 and 240mosmol/l osmotic solutions induced a significant increase (figure 24) in calcium imaging experiments.

In patch-clamp experiments the effects of osmolarity were tested in the absence and presence of intracellular calcium (two concentrations: 100nM and 1µM). The extracellular solution was calcium free and the sodium concentration was reduced to 106mM. Again, the osmolarity was adjusted with mannitol to get specific osmolarity solutions. We applied voltage ramps (from -120 to +100mV) every 5 seconds for 5 minutes and switched from the isotonic control solutuion (300mosmol/l) to 200 and 240mosmol/l solutions after 10 ramps. GFP-transfected control cells did not show a

response to 1µM calcium and/or hypotonic solution. TRPA1-containing cells that were treated with hypotonic solutions in calcium free conditions did not show a more pronounced response. Those cells that were dialysed with calcium containing solutions showed a stronger response than those without calcium (n=5-11) (figure 25). However, there was no elevation in the response to hypotonic solutions (200 and 240mosmol/l) in comparison to the response to 300mosmol/l as seen in the calcium imaging experiments.

Due to the fact that whole-cell patch-clamping is a more direct and more reliable method, we conclude that TRPA1 is not really activated by osmotic pressure although there was a slightly and probably indirect effect in calcium imaging experiments.

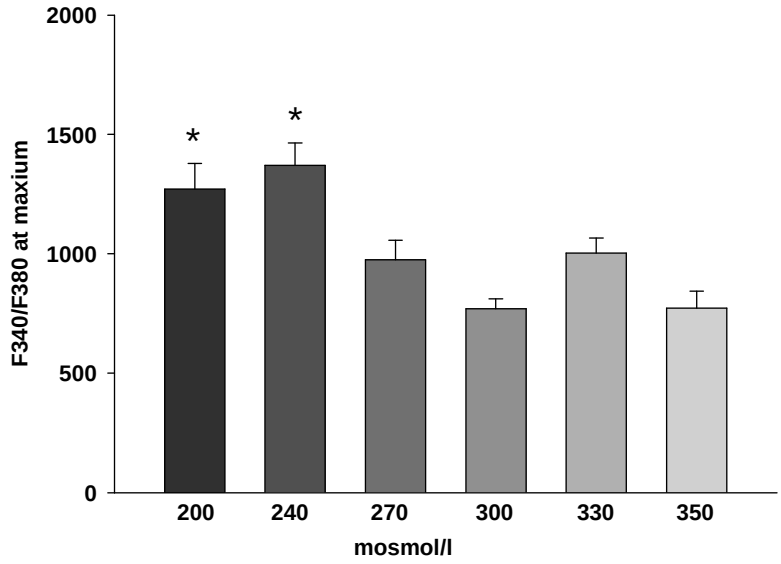

Figure 24 The mean maximum responses of TRPA1 to solutions with different osmolarities. F340/F380, ratio of fluorescence at 340nm to that at 380nm. Data are expressed as mean ± s.e.m. *P<0.05 compared to response at 300mosmol/l (Kruskal-Wallis ANOVA on ranks, Dunn's method).

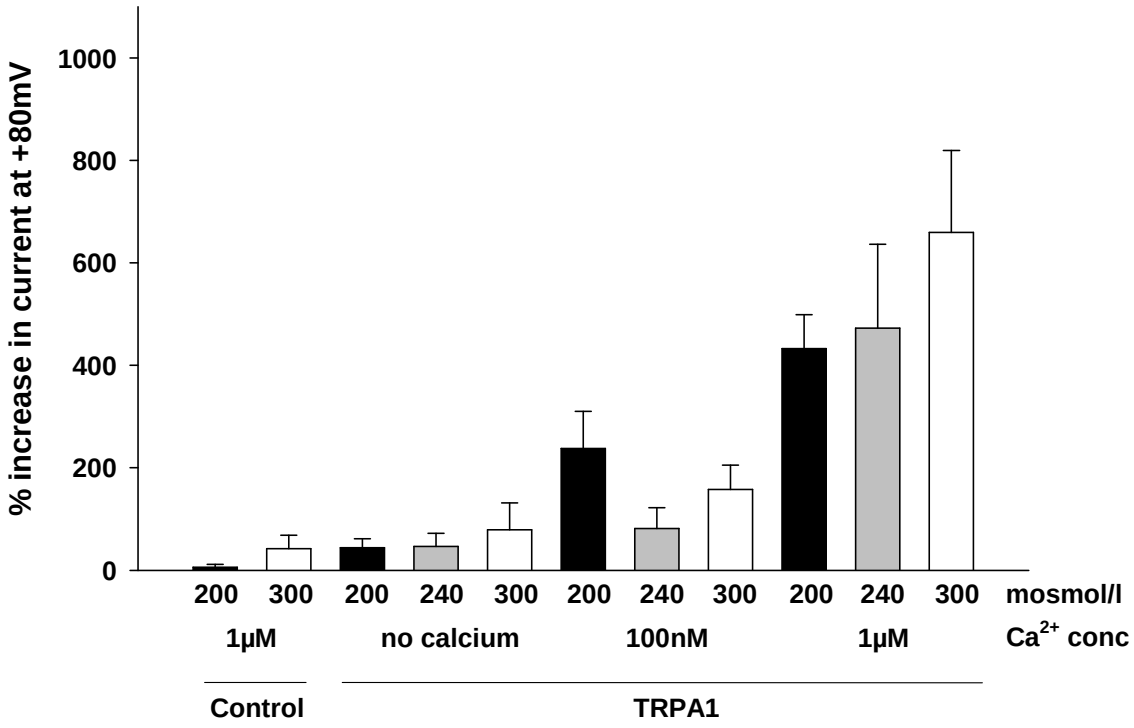

Figure 25 Response of TRPA1- and GFP-transfected control cells to hypotonic solutions in the presence of different calcium concentrations (0, 100nm, 1µM) (n=5-11). Y-axis: the percentage increase from the starting point to the maximal response at +80mV was calculated from voltage-ramp experiments. Data are expressed as mean ± s.e.m.

3.5 Is TRPA1 activated by temperature?

Story and colleagues suggested that TRPA1 is activated by noxious cold. Since then, this result has been strongly debated in the scientific community (Story et al., 2003; Jordt et al., 2004; Kobayashi et al., 2005; Nagata et al., 2005; Bautista et al., 2006; Kwan et al., 2006; Linte et al., 2006; Hill and Schaefer, 2007; Hjerling-Leffler et al., 2007; Sawada et al., 2007). Despite several cellular and knockout studies, there is still no clear consensus as to whether TRPA1 is directly activated by cold.

Moreover in Drosophila, TRPA1 is not activated by cold stimuli but by warm temperatures (Viswanath et al., 2003; Rosenzweig et al., 2005). Therefore, we wanted to look into a putative temperature activation of TRPA1 in more detail and applied cold and warm stimuli.

3.5.1 TRPA1 is not activated by heat

First, we tested whether hTRPA1 is activated by heat. The extracellular solution was calcium free and the intracellular solution contained either 100nM calcium or no calcium. We chose 100nM intracellular calcium because this concentration did not cause any increase during the voltage ramps but calcium might be necessary to induce responses to certain stimuli. We applied voltage ramps from -120 to +100mV in 400ms. This ramp was applied for 100 times every 2 seconds. During the ramp protocol, the solution that superfused the hTRPA1-expressing cells was manually heated from 20℃ to 45℃ immediately after the whole-cell configuration was achieved. The maximum response at +80mV was extracted and analyzed. There was no significant response of TRPA1-expressing cells upon warm temperature stimulation in comparison to control cells (figure 26). We calculated the Q_{10} values (Q_{10}=(Rate2/Rate1)^(10/(T2-T1))) for all groups (n=4-8). GFP-expressing control cells had Q_{10} values of 2.63 and 2.412 in the absence or presence of calcium, respectively, whereas TRPA1-expressing cells had Q_{10} values of 1.327 and 2.814 in the absence and presence of calcium. These values indicate that the channel is not warm temperature sensitive because temperature-insensitive proteins have Q_{10} values between 2-3 (Hille, 2001).

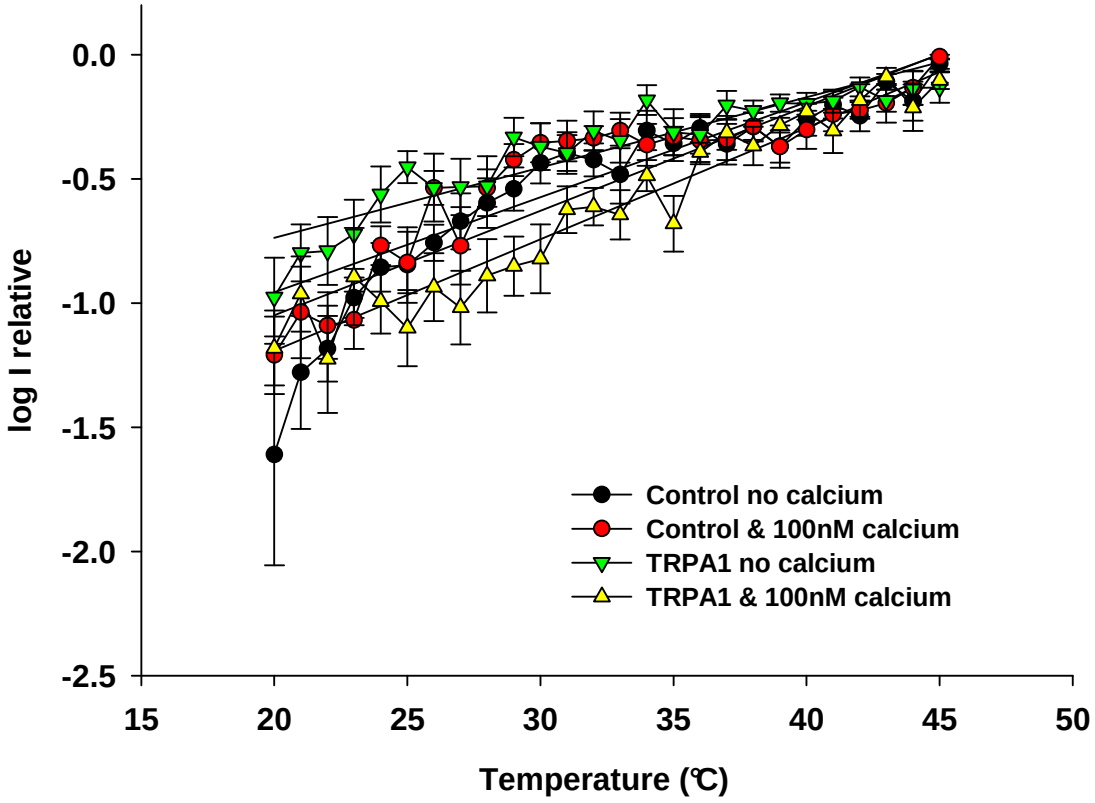

Figure 26 Heat did not activate TRPA1- and GFP-transfected control cells in the presence and absence of calcium (n=4-8). Solid lines are regressions. Data are expressed as mean ± s.e.m.

3.5.2 The response to cold is increased in calcium imaging experiments

With the help of ratiometric calcium imaging we tested whether cold stimuli were able to activate TRPA1-expressing cells. The temperature was reduced from 26℃ down to ~9℃ within 30 seconds (figure 27 A). Indeed, upon cold stimulation there was a substantial elevation in the intracellular calcium concentration in comparison to YFP-transfected control cells. However, we also observed a response in control cells, which did not express TRPA1 (figure 27 B) (everything above a 20% increase in fluorescent ratio was interpreted as a response). The response of control and TRPA1-expressing HEK293 Tet-On cells upon cooling had remarkably similar temperature activation thresholds (TRPA1 17.8±0.6℃ vs. controls 17.2±0.4° C) (figure 28). Furthermore, the response of TRPA1-expressing cells to cold was reduced when calcium free extracellular solutions were applied (figure 27 B). Thus, the cold response is influenced by extracellular calcium.

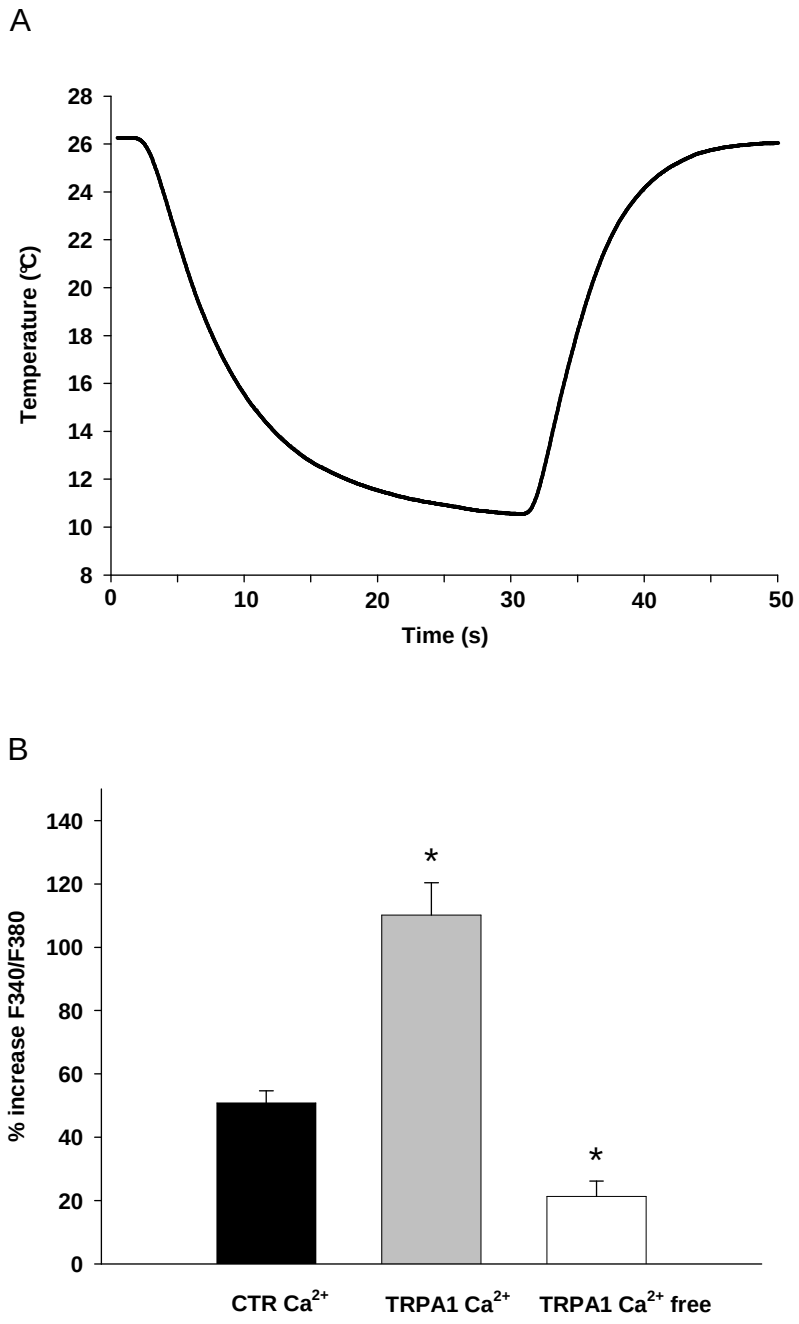

Figure 27 A, temperature activation protocol used to cool the cells in calcium imaging and patch-clamp experiments from RT to ~9°C. **B**, TRPA1-transfected cells are significantly activated by cold stimuli in calcium imaging experiments in comparison to YFP-transfected control cells. Control cells are also activated by cold. The cold response was reduced in calcium free conditions. Data are expressed as mean ± s.e.m. **P*<0.05 compared to control cells (one way ANOVA).

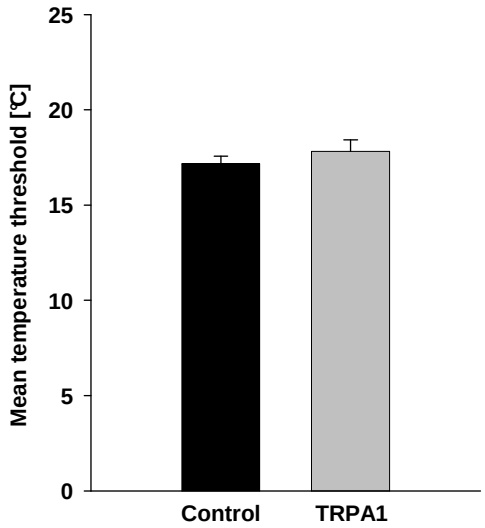

Figure 28 TRPA1-transfected and YFP-transfected control cells have the same mean temperature activation threshold (TRPA1 17.8±0.6℃, control 17. 2±0.4℃). Data are expressed as mean ± s.e.m. There is no significant difference (Mann-Whitney rank sum test)

3.5.3 TRPA1 is not directly gated by cold

The previously described experiment led us to the question whether TRPA1 might be activated by increases in intracellular calcium during cooling rather than directly by cold temperature. We tested this with pharmacological experiments and by examining the response of our calcium-insensitive mutant channels to cold. We found that the non-selective TRP channel blocker ruthenium red, an organometallic dye, partially inhibited cold responses in TRPA1-containing cells (figure 29 B, paired t-test, $P<0.001$) but the response was not completely abolished. The cold response of YFP-transfected control cells was not reduced by ruthenium red (figure 29 A, paired t-test). However, there is a significant difference between the cold responses of TPRA1- and YFP-transfected cells (figure 29 C, Mann-Whitney rank sum test, $P<0.001$). Likewise, cells expressing calcium-insensitive mutants (except mutant T470A) showed reduced cold sensitivity similar to that seen in control cells (figure 30). Therefore, we conclude that TRPA1 was indeed secondarily activated by elevation of intracellular calcium levels during cooling rather than directly by cold stimulation.

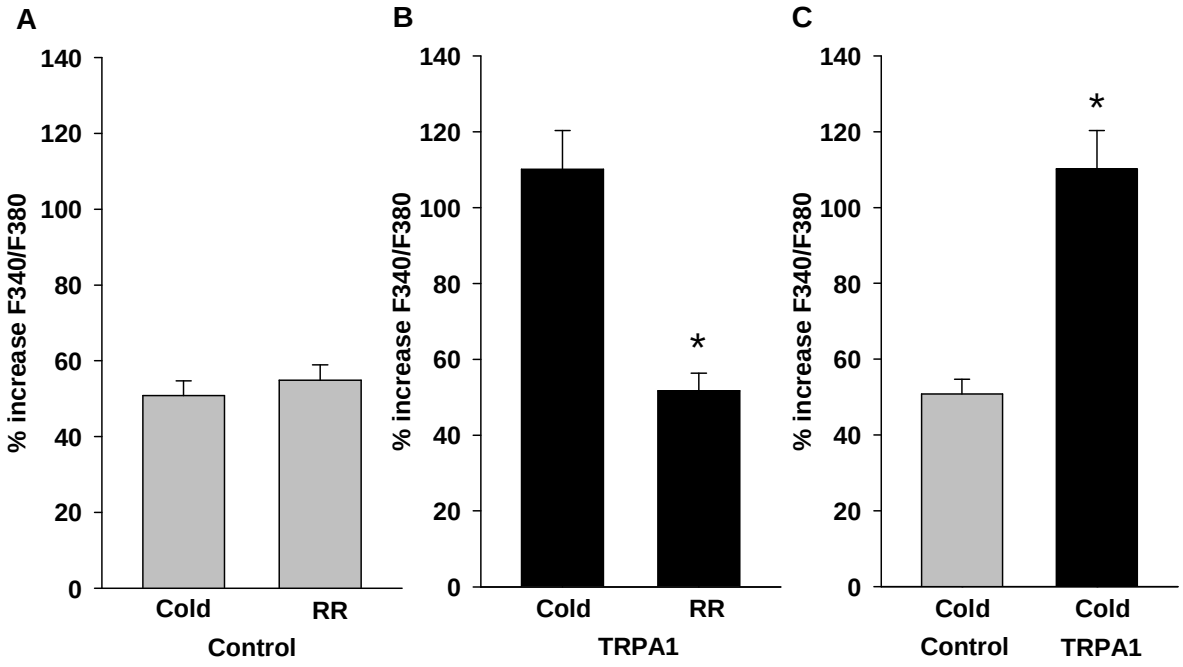

Figure 29 A, response of YFP-transfected HEK293 cells to cold (~10℃) and the response to cold in the presence of ruthenium red (RR) (paired t-test). **B**, response of TRPA1-transfected cells to cold (~10°C). Ruthenium red partially inhibits the response of TRPA1 to cold (paired t-test, *P*<0.001). **C**, there is a significant difference between the cold response of YFP-transfected HEK293 cells and TRPA1-transfected cells (Mann-Whitney rank sum test, *P*<0.001).

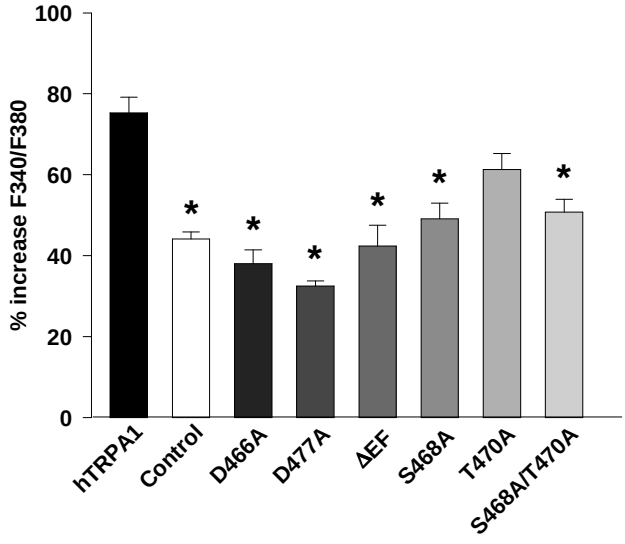

Figure 30 Cold stimuli (~10℃) elicit a rise in intracellular calcium in TRPA1 expressing HEK293 cells. The response to cold is reduced to control levels in TRPA1 EF-hand mutants. Data are expressed as mean ± s.e.m. *P<0.05 compared to hTRPA1-transfected cells (Kruskal-Wallis ANOVA on ranks, Dunn's method).

3.5.4 TRPA1 activation curve is not shifted by cold stimuli

We further investigated the cold activation of TRPA1 by means of whole-cell patch clamping. In calcium free conditions, we never observed currents or changes in the voltage dependence of TRPA1 upon cold stimulation (~15℃). The voltage for half-maximal activation ($V_{1/2}$) was similar for cold and room temperature-induced currents (~155 ± 25mV at 24℃ and ~166 ± 76mV at 15℃) (figu re 31). Nevertheless, calcium might still be necessary for cold sensitivity of TRPA1, as has been reported for heat activation of TRPM5 (Talavera et al., 2005). In order to test this hypothesis I applied cold stimuli in the presence of 10µM intracellular calcium. Cooling did not evoke TRPA1 currents but actually reduced calcium activation of TRPA1, as would be expected for a channel that is not cold sensitive (figure 32). This finding also supports the hypothesis that TRPA1 is not directly gated by cold but instead activated by an increase in basal levels of intracellular calcium levels in HEK293 cells during cold stimulation.

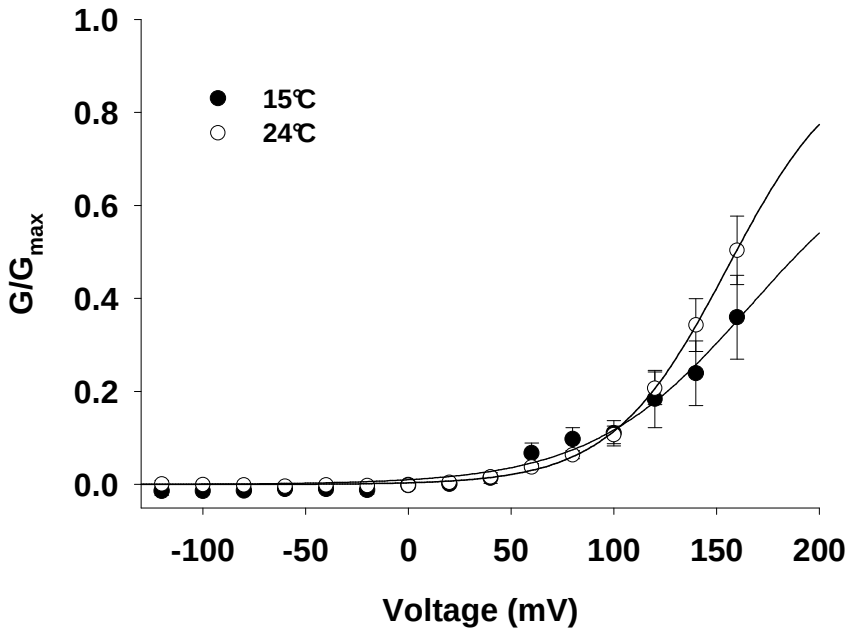

Figure 31 Voltage dependent activation of TRPA1 is not shifted by cooling ($V_{1/2}$ at 24℃ was 155 ± 25mV n=9, $V_{1/2}$ at 15℃ was 167 ± 76 n=8). G/G_{max}, normalized conductance. Solid lines are best fits to Boltzmann functions. Data are expressed as mean ± s.e.m.

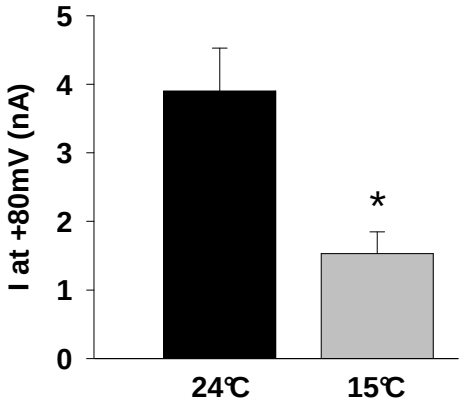

Figure 32 Calcium (10µM) activation of TRPA1 is reduced upon cooling (n=5-8). Data are expressed as mean ± s.e.m. *P*=0.016 compared to 24℃ (t-test).

3.6 TRPA1 expression does not change after inflammation

3.6.1 No change in the number of cells expressing TRPA1

TRPA1 has a function in pain sensation and has also been suggested to play a role in clinical pain states such as neuropathic pain and inflammation. Therefore, we examined the expression pattern of TRPA1 in DRG neurons in a mouse model of inflammation because an increased expression could be an important factor of increased nociceptor excitability or behavioral hypersensitivity to certain stimuli. Mice received an intraplantar injection of CFA into the right hind paw and the DRGs were dissected 48 hours later. Using non-radioactive fluorescent *in situ* hybridization experiments we investigated whether the mRNA transcription level of TRPA1 changed due to inflammation.

We could not detect any changes in the number of cells expressing TRPA1 mRNA after CFA injection (table 2, figure 33A and 34). Moreover, we analyzed the expression of TRPA1 mRNA in the peptidergic subpopulation of nociceptors that contain CGRP. The percentage of TRPA1 and CGRP co-expression did not change upon inflammation. Thus, there is no phenotypic switch in this subpopulation of pain sensing neurons (table 2, figure 33B and 34).

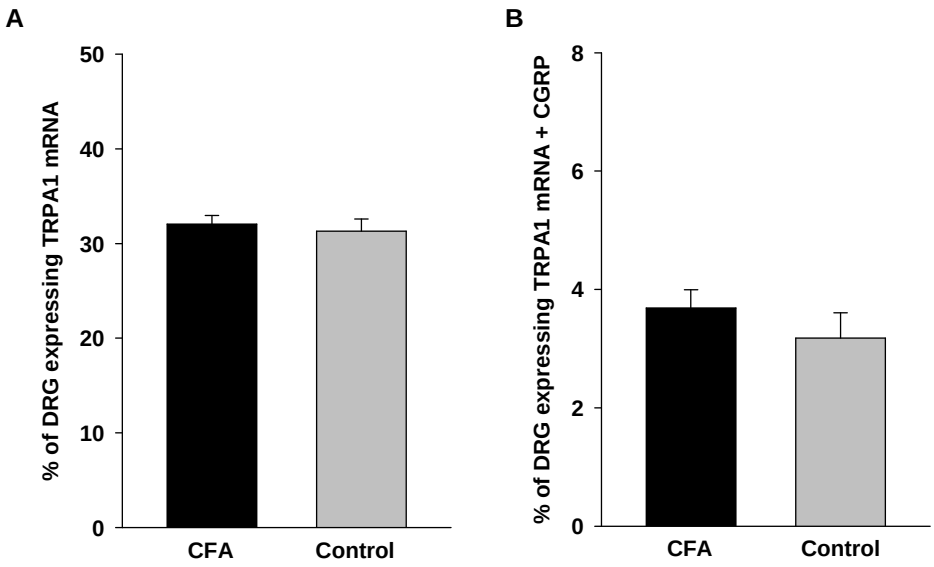

Figure 33 **A**, The percentage of DRG neurons expressing TRPA1 mRNA in control animals and after CFA treatment (ipsilateral) is unchanged. **B**, The number of DRG neurons co-expressing TRPA1 mRNA and CGRP after inflammation remained unchanged. Data are expressed as mean ± s.e.m. (A, t-test; B, Mann-Whitney rank sum test).

	TRPA1	TRPA1 + CGRP
Control	31.3 ± 1.3 (2738/8558)	3.18 ± 0.4 (238/8558)
48h CFA	32.04 ± 0.9 (4071/12601)	3.69 ± 0.31 (433/12601)

Table 2 showing the percentage (± s.e.m, n=3 mice per group) of neurons in the DRG expressing TRPA1 alone and co-localized with CGRP. Numbers in brackets indicate the total number of neurons counted.

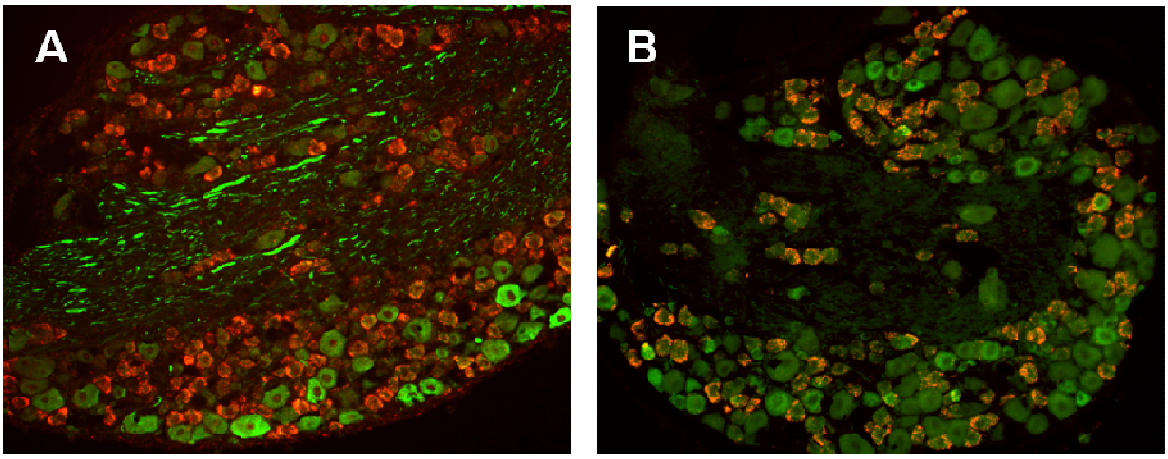

Figure 34 Representative pictures of DRG expressing TRPA1 (red) and CGRP (green). **A**, inflammation after 48h CFA, ipsilateral. **B**, Control (untreated) animals.

3.6.2 The amplitude of cell responses to mustard oil changes

We examined mustard oil responses in the CFA model of inflammation with calcium imaging. In agreement with our *in situ* hybridization experiment, there was no change in the number of DRG neurons responding to the TRPA1 agonist mustard oil (figure 35 A) after 48 hours of CFA. However, we did observe significantly larger mustard oil evoked responses in inflamed mice compared to untreated control mice (figure 35 B). This would suggest that the sensitivity of TRPA1 is increased during inflammation and this could contribute to enhanced nociceptor excitability in inflammatory pain.

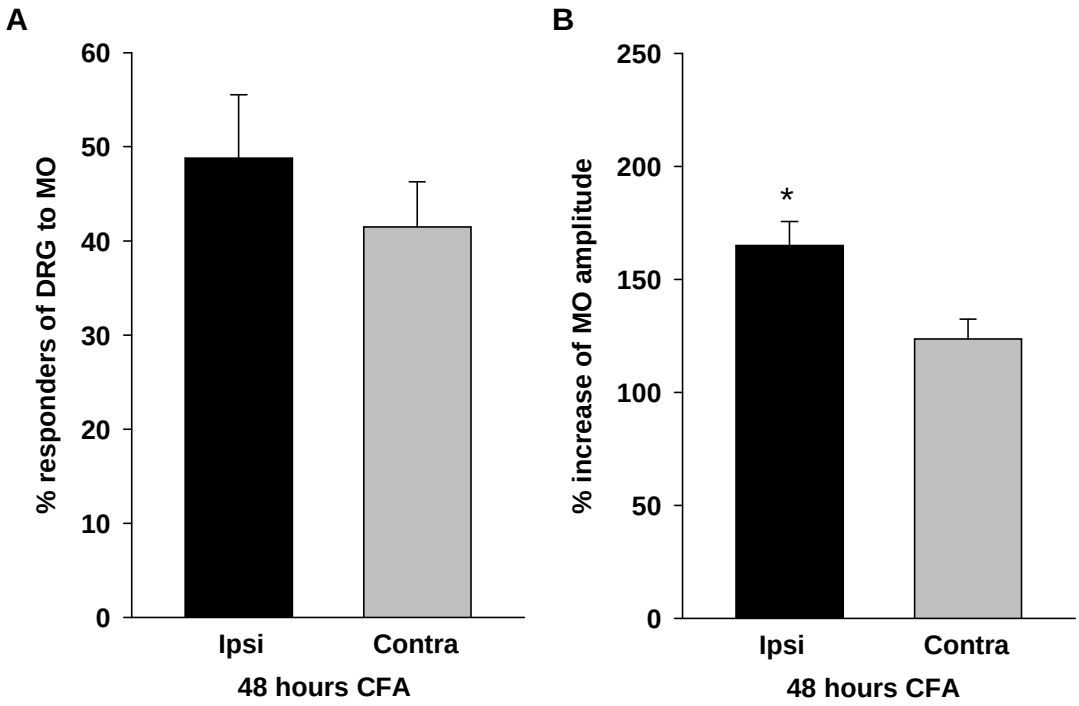

Figure 35 A, The percentage of DRGs responding to mustard oil did not change upon inflammation. Ipsi, ipsilateral site of injected CFA. Contra, contaleteral site of injected CFA. **B,** the amplitude of the response was significantly increased in ipsilateral DRG neurons. Data are expressed as mean ± s.e.m.*P<0.05 compared to the contralateral side (A, T-test; B, Mann-Whitney rank sum test, *P<0.001).

4 Discussion

The major finding of this investigation is the direct and concentration dependent activation of TRPA1 by intracellular calcium. TRPA1 is also a voltage-gated channel and any increase in intracellular calcium concentration activates the channel. We demonstrated that an EF-hand domain in the N-terminus is the molecular basis of the calcium sensitivity of the channel. We did not observe that TRPA1 is activated by changes in osmotic pressure or by warm temperatures. Furthermore, we provided evidence for an indirect activation of TRPA1 upon cold stimulation due to an increase in intracellular calcium levels. Finally, we showed an unchanged expression pattern of TRPA1 mRNA after CFA-induced inflammation but the amplitude to the TRPA1 agonist mustard oil was increased.

The channel functions not as the noxious cold sensor per se but seems to amplify certain stimuli due to activation by intracellular calcium. Additionally, these data suggest that TRPA1 might contribute to an increased excitability of nociceptors during inflammation.

4.1 TRPA1 is activated by intracellular calcium

Until recently, endogenous agonists for TRPA1 have not been identified but several studies pointed towards the importance of calcium for TRPA1 activation. Adding extracellular calcium to TPRA1 channels in the absence of another agonist did not induce activation (Nagata et al., 2005), although recently, another study found that influx of extracellular calcium into the cell is sufficient to activate the channel (Doerner et al., 2007). However, it was clearly demonstrated that the presence of extracellular calcium increases the rate and magnitude of mustard oil-induced currents (Jordt et al., 2004; Nagata et al., 2005). In the absence of calcium, currents were only slowly induced and did not desensitize (Nagata et al., 2005). We confirmed this finding and showed that mustard oil induces only a very small and delayed current in the absence of extracellular calcium. Thus, as has been argued before, external calcium modulates the activity of the channel.

In addition to the modulatory role of extracellular calcium for TRPA1, Jordt and colleagues (2004) showed that a rise of intracellular calcium caused by release from

intracellular stores due to application of thapsigargin is sufficient to activate TRPA1. However, another study could not confirm this finding (Nagata et al., 2005).

We investigated the role of intracellular calcium in TRPA1 gating in more detail and observed that intracellular calcium rapidly activates the channel in a concentration-dependent manner in whole-cell patch-clamp configuration. Recently, two further studies confirmed our result that TRPA1 can be activated by intracellular calcium (Doerner et al., 2007; Hill and Schaefer, 2007). Consistant with our experiments, Doerner and colleagues (2007) reported that intracellular calcium leads to an instantaneously developing inward current. We calculated an EC_{50} of ~6µM, whereas their determined EC_{50} was ~905nM (Doerner et al., 2007). This disparity in the EC_{50} values might be due to methodological differences in both studies.

Similar to previous studies (Jordt et al., 2004; Nagata et al., 2005), we found a delayed activation of the channel by the TRPA1 agonists cinnamaldehyde, mustard oil and acrolein in the absence of intracellular calcium. Moreover, an approximately 10-fold higher concentration was necessary.

TRPA1 is not the only TRP channel that can be activated by intracellular calcium. For example the thermo-TRP channel TRPV4 is modulated by intra- and extracellular calcium (Watanabe et al., 2003) and TRPM4 and TRPM5 are calcium-sensitive channels (Launay et al., 2002; Nilius et al., 2003; Prawitt et al., 2003; Ullrich et al., 2005). One study reported that TRPM4 is endogenously expressed in HEK293 cells and intracellular calcium activates untransfected control cells (Launay et al., 2002). Nevertheless, we and another study did not observe currents induced by intracellular calcium in untransfected HEK293 cells (Nilius et al., 2003). Thus, we conclude that the current induced by calcium in our experiments is not due to an expression of TRPM4.

In addition to calcium, other divalent cations such as strontium, barium and manganese did not activate TRPA1 as strong as calcium did. Although we observed activation of TRPA1-expressing HEK293 cells in patch-clamp recordings at +80mV, we did not detect significant activation at -60mV except with manganese. In comparison, TRPM4, which responds to calcium, was also only partially activated by strontium and not by barium (Nilius et al., 2004).

In summary, TRPA1 is endogenously activated intracellular calcium.

4.2 Calcium directly activates TRPA1 in single-channel recordings

Single-channel openings of TRPA1 in outside-out and inside-out patches have previously been reported (Nagata et al., 2005; Macpherson et al., 2007a). AITC induced single-channel conductance of TRPA1, which is desensitized in the presence of calcium (Nagata et al., 2005).

We could show that calcium applied to inside-out patches containing TRPA1 directly activates the channel in this scenario. Putative signaling pathways are most likely disrupted and therefore the influence of cytosolic factors is reduced. Besides TRPA1, other TRP channels, such as TRPM4, can be activated by calcium in inside-out recordings as well (Launay et al., 2002). Interestingly, others (Kim and Cavanaugh, 2007; Cavanaugh et al., 2008) did not observe that TRPA1 is activated by calcium and thiol-reactive compounds like AITC in excised patches. They claim that TRPA1 requires polyphosphates in order to be activated by calcium and AITC (Cavanaugh et al., 2008). However, they used a concentration of 1 or 5µM calcium to test the activation in inside-out patches. We have shown that the EC_{50} is around 6µM. In our study, we used 100µM and got a significant activation of TRPA1 in excised patches. However, another recently published study confirmed our result that intracellular calcium in the nanomolar range was sufficient to elicit single-channel currents. They showed that the activation was independent of other cytosolic factors but they have also observed endogenous calcium-sensitive channels in untransfected control cells (Doerner et al., 2007). Moreover, the activation of TRPA1 by AITC and other thiol-reactive compounds, such as formaldehyde, in excised patches was observed before as well (Nagata et al., 2005; Macpherson et al., 2007a; Macpherson et al., 2007b). Further studies are necessary to clarify the discrepancies between the studies.

4.3 TRPA1 is weakly voltage-dependent

Non-selective TRP channels were initially considered as voltage-independent cation channels. The fourth transmembrane domain of TRP channels lacks positively charged residues that are common in voltage-gated ion channels (Clapham, 2003). TRPM4b was the first channel that has been identified as a weakly voltage-gated TRP channel. The first hint that TRPM4b is modulated by membrane potential changes came from Launey and colleagues (2002). The voltage-dependence of the channel has been investigated later in more detail by Nilius and colleagues (2003).

We found that TRPA1 shows the typical outward rectifying behavior of TRP channels when intracellular calcium was given as an agonist.

Current rectification can arise for several reasons. It might occur because ions across the membrane are unequally distributed (Hodgkin and Katz, 1949). A second possibility is that rectification is a feature of the channel pore and might occur due to a voltage-dependent block of the channel activity. An example for this possibility is the magnesium block of the NMDA-receptor (Gunthorpe et al., 2000). Third, rectification can occur due to voltage dependence of the probability of channel opening (Gunthorpe et al., 2000).

We observed an increased open probability and a rise in current amplitude in single-channel recordings of TRPA1 upon calcium application. This indicated the TRPA1 might also be voltage-dependent. Application of a voltage-step protocol and tail-current analyses indeed revealed that TRPA1 is weakly voltage-dependent.

So far, several TRP channels have been characterized as voltage-dependent with an activation curve that extends mainly into the non-physiological positive voltage range. Examples are TRPV1, TRPM8, TRPV3, TRPM4, and TRPM5 (Hofmann et al., 2003; Nilius et al., 2003; Chung et al., 2004a; Voets et al., 2004; Chung et al., 2005; Nilius et al., 2005b; Ullrich et al., 2005). Moreover, it has been shown that temperature (TRPV1, TRPM8, TRPV3) or TRP channel ligands (TRPV1, TRPM8, TRPV3, TRPM4), for instance menthol and capsaicin, shifts this voltage dependence to more physiological, i.e. more negative potentials (Hofmann et al., 2003; Nilius et al., 2003; Chung et al., 2004a; Voets et al., 2004; Chung et al., 2005; Nilius et al., 2005b; Ullrich et al., 2005). We could also detect that stimulation of the channel with intracellular calcium shifts the voltage-dependent activation curve to more negative potentials as well, whereas cooling did not.

Other divalent cations such as strontium, barium and manganese only very slightly shifted the voltage-dependent activation curve. Among these cations, strontium induced the strongest shift but the voltages of half-maximal activation were always above +126mV. The calcium activated channel TRPM4 can also be partially activated by strontium but not by barium (Nilius et al., 2004). Other studies confirmed our result that TRPA1 is a voltage-dependent channel (Karashima et al., 2007; Macpherson et al., 2007a). They found inactivation of the channel at higher voltages (Macpherson et al., 2007a) and that low concentration of menthol shifts the voltage dependence towards negative values (Karashima et al., 2007). While voltage-sensitive gating for TRP

channels is poorly understood, it has recently been shown that the transmembrane segment 4 and the S4-S5 linker of TRPM8 seems to be part of the voltage sensor (Voets et al., 2007).

In conclusion, there is a link between calcium activation and voltage-dependent gating of TRPA1.

4.4 Calmodulin does not regulate TRPA1

CaM is a ubiquitously expressed calcium sensor in all eukaryotic cells and has four calcium binding domains. Besides being involved in many cellular processes, CaM can also mediate calcium-dependent regulation of many proteins, calcium pumps, and ion channels (Zhu, 2005) such as potassium channels (Xia et al., 1998; Fanger et al., 1999). Several TRP channels, such as TRPV1, TRPV4, TRPV6, TRPM4, or TRPC4 (Niemeyer et al., 2001; Tang et al., 2001; Numazaki et al., 2003; Strotmann et al., 2003; Lambers et al., 2004; Rosenbaum et al., 2004; Nilius et al., 2005a; Zhu, 2005) are regulated by CaM. It has been shown that CaM associates with the first ankyrin repeat of TRPV1 (Rosenbaum et al., 2004) and TRPA1 has many ankyrin repeats. Therefore, CaM seemed to be a likely candidate for the molecular mechanism of the calcium-dependent regulation of TRPA1. Conversely, we demonstrated that CaM is not responsible for the calcium sensitivity of TRPA1.

TRPM4, like TRPA1, is activated by intracellular calcium and is also weakly voltage-dependent (Launay et al., 2002; Nilius et al., 2003) and previous studies have observed that CaM is the molecular determinant for its calcium sensitivity. Over-expression of the dominant-negative CaM mutant $CaM_{1,2,3,4}$, in which all four EF-hand calcium binding sites are mutated, resulted in a strong decrease of the current amplitude upon calcium stimulation (Nilius et al., 2005a). Calcium can still activate TRPM4, but its sensitivity is reduced by mutating the CaM binding site or by structural changes in the C–terminal part of the channel that confers CaM binding (Nilius et al., 2005a). We also co-expressed TRPA1 with the dominant-negative CaM mutant $CaM_{1,2,3,4}$ and applied wild-type CaM and the CaM antagonist W-7. Nevertheless, the calcium sensitivity of TRPA1 did not change under these conditions whereas we could confirm a reduced calcium sensitivity of TRPM4. Another study confirmed our results (Doerner et al., 2007). In conclusion, calcium sensitivity of TRPA1 is not regulated by the accessory protein CaM.

4.5 The EF-hand domain is required for the calcium sensitivity of TRPA1

Another possibility for the molecular determinant for TRPA1's calcium sensitivity is the existence of an EF-hand domain in the N-terminus of the channel. The EF-hand domain of TRPA1 has been mentioned but not investigated earlier (Sotomayor et al., 2005; Hinman et al., 2006). EF-hand proteins can be calcium sensors or calcium buffers. Calcium sensors, such as calmodulin, activate enzymes upon calcium binding and regulate the activity of many ion channels. Calcium buffers transmit the calcium signal throughout the cells or remove the ions from the cytoplasm (Gifford et al., 2007).

The EF-hand is named after the motif detected between helices E and F in parvalbumin (Kretsinger and Nockolds, 1973). An EF-hand domain consists of two alpha helical domains and a calcium binding loop between those helices. EF-loops are rich in negatively charged amino acids, such as glutamic acid and aspartic acid. In most cases, the loop has 12 residues and binds the calcium molecule (Kretsinger and Nockolds, 1973; Ikura, 1996) by arranging it in a pentagonal bipyramidal configuration. Residue 1, 3, 5, 7, 9, and 12 (also denoted by X, Y, Z, -Y, -X, and –Z) of the loop region provide necessary oxygen ligands for the calcium ion (Gifford et al., 2007).

Using site-directed mutagenesis we could show that deletion of the whole EF-hand domain almost abolished calcium sensitivity of the channel. In addition, we discovered that the aspartic acid residues 1 and 12 of the calcium-binding loop are necessary for the calcium binding. In contrast, mutation of residue 3 and/or 5 did not significantly affect the calcium sensitivity of TRPA1. Another recently published report investigated the EF-hand domain of hTRPA1 in HEK293 cells as well. Application of CALP2, a 12-mer calcium like peptide that functions as an antagonist at EF-hands of CaM, resulted in a reduced response to calcium in single-channel recordings. They substituted all six mentioned residues of the interhelical loop with alanines. The expression of the mutated channel was determined with western blot analysis (Doerner et al., 2007) whereas we used YFP-tagged constructs and showed that the mutated channels are still activated by cinnamaldehyde. Interestingly, their data are different from our results. The mutation of residue 5, 9, and 12 resulted in a non-functional channel, whereas we could show that mutation of residue 12 was still responsive to cinnamaldehyde. Functional mutants were those with a substitution mutation in residue 1, 3, or 7 but only a mutation in the leucine residue (residue 7) led to a calcium insensitive channel that is still sensitive to AITC. This discrepancy between their and our study cannot easily be explained and

further experiments are necessary to clarify the issue. However, we also confirmed the importance of residue 1 and 12 for calcium binding in mouse TRPA1.

Whether the annotated EF-hand in TRPA1 binds calcium in the same way as a classical EF-hand domain is not clear and needs further investigation. Normally EF-hand domains are found pair wise (Nelson and Chazin, 1998) but there is not another obvious EF-hand domain in TRPA1. Due to the fact that TRP channels are believed to form tetrameric structures (Clapham, 2003), EF-hands might pair by oligomerization, as has been proposed for the protein calpain (Nelson and Chazin, 1998). This protease consists of a large and a small subunit with five EF-hands in the latter one. The fifth EF-hand is left unpaired in the monomer but dimerization was observed in the crystals of the large and small subunits (Blanchard et al., 1997; Lin et al., 1997). Examples of EF-hand proteins that contain an odd number of EF-hands are rare. For instance, a single functional EF-hand is found in the C-terminal domain of the voltage-gated calcium channel $Ca_V1.2$ (Brunet et al., 2005).

The structural information for TRPA1 is still lacking, and therefore it is impossible to determine the details of calcium binding. Nonetheless, our functional data demonstrates that disruption of key residues in the region strongly decreases calcium sensitivity of TRPA1.

4.6 Any increase in intracellular calcium leads to an activation of TRPA1

TRPA1 has been proposed to function as a receptor-operated channel that is activated via G-protein-coupled receptors (GPCR) and certain signaling pathways (Bandell et al., 2004; Jordt et al., 2004). Our data provides evidence that any stimuli that lead to an increase in intracellular calcium levels in TRPA1-expressing cells could activate the channel.

Thapsigargin inhibits sarco- and endoplasmic reticulum Ca^{2+} (SERCA) transport ATPases (Thastrup, 1990; Inesi et al., 1998) and thus leads to a receptor-independent increase of cytosolic calcium levels because it blocks the re-uptake of calcium. Bandell and colleagues (2004) demonstrated that release of calcium from stores through the application of thapsigargin does not activate TRPA1 but this experiment disagrees with the results of Jordt and colleagues (2004). They showed that the increase of intracellular calcium by thapsigargin is sufficient to activate TRPA1 and this has been reported by other groups as well (Jordt et al., 2004; Hill and Schaefer, 2007). We

applied thapsigargin and measured the activation of wild-type TRPA1 and all TRPA1 EF-hand mutants. Thapsigargin-evoked release of intracellular calcium results in a strong response of wild-type TRPA1-expressing cells and so we could confirm the finding of Jordt and colleagues (2004). However, the response of the calcium-insensitive mutants to thapsigargin was diminished.

It has been demonstrated previously that the proinflammatory peptide bradykinin is able to activate TRPA1 via a GPCR signaling cascade (Bandell et al., 2004; Bautista et al., 2006; Wang et al., 2008). Furthermore, TRPA1 knockout mice showed an attenuation of the bradykinin-induced hyperalgesia (Bautista et al., 2006; Kwan et al., 2006). In sensory neurons, bradykinin binds to the GPCR B2. Subsequently, PLC is activated and this leads to a breakdown of PIP_2 into DAG and IP_3. IP_3 releases calcium from intracellular stores and DAG activates PKC or is converted into arachidonic acid. PKC activation does not play a role in the activation of TRPA1 (Wang et al., 2008), but does activate TRPV1 in DRGs. The PLC-induced increase in intracellular calcium levels can activate TRPA1. Bradykinin stimulation also activates the PKA pathway that has been proposed to be involved in sensitization of TRPV1 (Amadesi et al., 2006) and TRPA1 (Wang et al., 2008).

The B2-receptor is ubiquitously and constitutively expressed in many tissues and cells (Hall, 1992; Campos et al., 2006), therefore we did not co-transfect the B2-receptor with TRPA1 in HEK293 cells. We observed that wild-type TRPA1 is strongly activated by bradykinin as has been shown before by several other groups (Bandell et al., 2004; Bautista et al., 2006; Wang et al., 2008). The response was significantly reduced in cells expressing Ca^{2+}-insensitive TRPA1 mutant channels, suggesting that Ca^{2+} signaling via the EF-hand domain is probably the predominant means by which TRPA1 mediates the potentiated response to bradykinin. Unexpectedly, the response of some mutant channels was identical to the wild-type response (S468A) or even exceeded their activation for unknown reasons (T470A and S468A/T470A). One explanation for this might be that the phosphorylation state of the mutated channel is somehow altered.

Moreover, the cholinergic agonist carbachol increases intracellular calcium by the activation of PLC through an endogenous muscarinic receptor in HEK293 cells that leads to increased IP_3 levels (Zhu et al., 1998). This results in a transient peak response due to an increase in intracellular calcium followed by a sustained response phase. HEK293 cells possess a carbachol-sensitive intracellular calcium store (Tong et al., 1999) and it has been shown that the sustained response phase to carbachol was

potentiated in cells expressing TRPA1 (Jordt et al., 2004). We could confirm this finding. In addition, the elevated response was also detectable in S468A, T470A, and S468A/T470 mutant channels but not in the calcium-insensitive mutants D466A, D477A, or ΔEF.

More recent experiments demonstrated that histamine, binding to endogenous histamine receptors in HeLa cells, activates the Gq-IP_3 pathway resulting in a rapid increase in cytosolic calcium (Raymond et al., 1991) and also activates TRPA1 in cell-attached patches (Cavanaugh et al., 2008).

The fact that the completely Ca^{2+}-insensitive EF-hand mutants (D466A, D477A, and ΔEF) show a reduced response to carbachol, thapsigargin or bradykinin, indicates that intracellular calcium is essential for activation of TRPA1 downstream of PLC and that any increase in intracellular calcium level, either from release from intracellular stores or by calcium influx, seem to activate the channel via the EF-hand domain. Thus, calcium is an important and endogenous ligand of TRPA1 and the channel functions also as a receptor-operated channel and potentiates responses to certain stimuli. Figure 36 illustrates our finding in a simplified model.

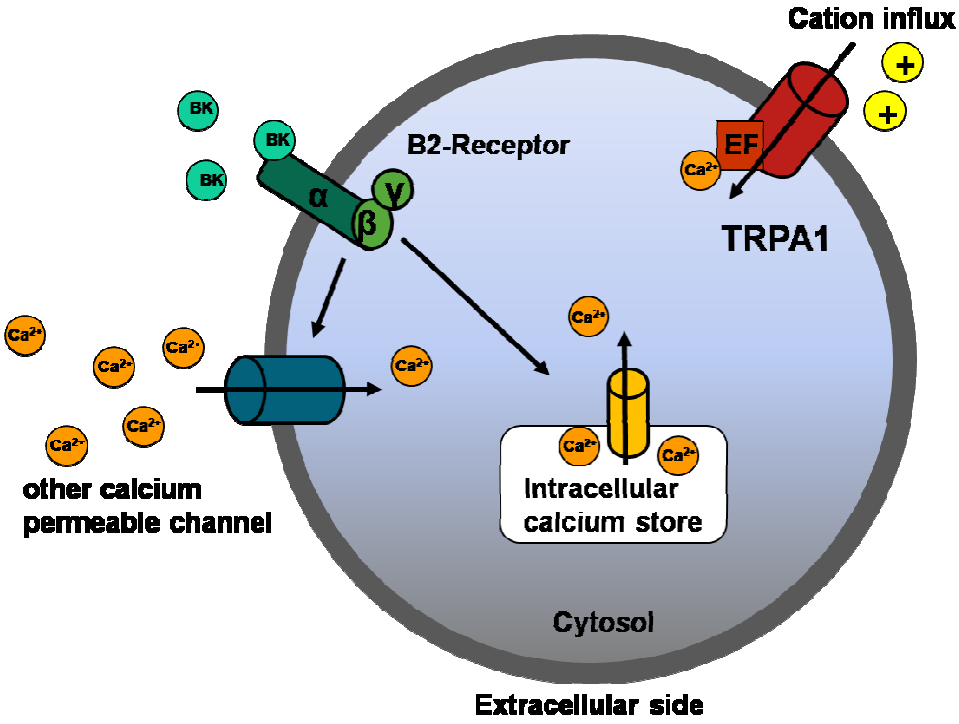

Figure 36 Hypothetical model of calcium activation of TRPA1. Any increase in intracellular calcium levels, either by calcium influx or by release from intracellular stores, is able to activate TRPA1 via the EF-hand domain and leads to a potentiation of the response to the stimulus. BK, bradykinin; Ca^{2+}, calcium ions; EF, EF-hand domain.

4.7 TRPA1 is not activated by osmotic pressure

TRP channels and degenerin/epithelial sodium channel (DEG/ENaC) channels have been proposed to function as putative mechanosensors. The first evidence for an involvement of TRP channels in mechanosensation came from a mutation in the osm-9 gene of *C. elegans*, which encodes a TRPV-like channel. Mutant worms had deficits in the avoidance reaction to osmolality and nose touch (Colbert et al., 1997). Besides osm-9, members of the DEG/ENaC family such as MEC-4 and MEC-10 and the TRP channels NompC and TRPA1 respond to mechanical stimuli in *C. elegans* (Gu et al., 1996; Lai et al., 1996; Colbert et al., 1997; Walker et al., 2000; Kindt et al., 2007). The yeast TRP channel TRPY1 and the *Drosophila* TRP channels TRPN1, Inactive, and Nanchung have also been suggested to play a mechanosensitive function (Walker et al., 2000; Kim et al., 2003; Zhou et al., 2003; Gong et al., 2004). Moreover, there is genetic evidence that the vertebrate DEG/ENaC family homologous, ASIC2 and ASIC3, are involved in somatic mechanosensation (Price et al., 2000; Price et al., 2001).

In 2004, TRPA1 was proposed to play a role in the transduction mechanism of mechanosensation especially in inner hair cells of the ear (Corey et al., 2004). Corey and colleagues (2004) found that TRPA1 is present in mouse vestibular and cochlear hair cells and that expression coincides with the onset of mechanotransduction. Disruption of the transduction apparatus results in a loss of TRPA1 immunoreactivity (Corey et al., 2004). Furthermore, TRPA1 seemed to be a likely candidate for a mechanosensitive channel in mammals because the related channel TRPN1 participates in hair cell transduction in lower vertebrates and invertebrates but is not expressed in mammals. Both channels have many ankyrin repeats that could serve as gating spring (Nagata et al., 2005; Sotomayor et al., 2005). Unexpectedly, TRPA1 knockout studies revealed that the channel does not contribute to vestibular or auditory transduction (Bautista et al., 2006; Kwan et al., 2006). However, one study suggested that TRPA1 might be important in somatic mechanosensation instead because knockout mice display deficits to tactile stimuli (Kwan et al., 2006). Although TRPA1 orthologues plays a mechanosensitive role in *Drosophila* and *C. elegans* (Tracey et al., 2003; Kindt et al., 2007), the function of mammalian TRPA1 in mechanosensation is still controversial. Evidence for a direct activation of TRPA1 by mechanical force is still lacking.

Osmosensory transduction is also a mechanical process. Application of hypo- or hyperosmotic solutions results in cell swelling or in a decrease of cell volume,

respectively, and thus might stimulate osmotic- and mechanosensitive channels (Oliet and Bourque, 1993). Thus far, several TRP channels were shown to be activated by osmotic stimuli, such as TRPV4, TRPV2, TRPM3, and possibly TRPM7 and TRPP2 (Liedtke et al., 2000; Strotmann et al., 2000; Grimm et al., 2003; Muraki et al., 2003; Suzuki et al., 2003a; Beech et al., 2004; Kraft and Harteneck, 2005; Montalbetti et al., 2005; Numata et al., 2007; Pedersen and Nilius, 2007).

TRPA1-expressing HEK293 TetOn cells were treated with hypotonic solutions in order to test the effect of cell swelling on the channel. In calcium imaging experiments, we could detect a significant response to hypotonic solutions whereas in whole-cell patch-clamping under controlled calcium concentrations the effect was not reproducible.

Nevertheless, another recent investigation by Zhang and colleagues (2008) proposed that TRPA1 is not activated by hypotonic solutions but by hypertonic solutions in a concentration-dependent manner. In our experiments, we used hTRPA1 instead of rTRPA1 and we never tried hypertonic solutions higher than +50mosmol/l. Zhang and colleagues (2008) showed only very tiny effects with such a solution and only at +100mosmol/l they could detect a more prominent effect. The whole-cell currents induced by hypertonic solutions are much smaller than the AITC-induced currents. They also reported that hypotonic solutions did not produce a pronounced increase in fluorescence in calcium imaging experiments (Zhang et al., 2008).

Hill and Schaefer (2007) applied mechanical stress to the lipid bilayer by using the two amphipathic molecules, trinitrophenol and chlorpromazine, that also influence other mechanosensitive channels. Trinitrophenol leads to activation of TRPA1, whereas chlorpromazine inhibited TRPA1 at positive potentials but increased the open probability of TRPA1 at negative potentials. Thus, they argue that TRPA1 might be sensitive to membrane deformation (Hill and Schaefer, 2007).

However, another study argues against a role of TRPA1 in hair cell mechanotransduction. Mechanically activated currents in DRG neurons and mechanosensitive channels in cochlear hair cells were blocked by the conopeptide analogue ‘noxious mechanosation blocker-1’ (NMB-1). However, mustard oil-induced activation of TRPA1 expressed in CHO-K1 cells could not be blocked by NMB-1 (Drew et al., 2007).

Due to many inconsistencies between the knockout studies and other experiments, we think that TRPA1 is unlikely to be a mechanotransducing channel as such but we cannot exclude the possibility that it is involved in the process of mechanosensation.

This might be via an increase in intracellular calcium ions or through interaction with another pathway.

4.8 Is TRPA1 a temperature-activated channel?

4.8.1 Heating

Mammalian TRPA1 is an orthologue of four TRPA channels in *Drosophila* (Painless, Pyrexia, dTRPA1, and Waterwitch) (McKemy, 2007). One *Drosophila* orthologue, dTRPA1, is activated by warm temperature around 27°C in heterologous expression studies and not by cooling as was originally proposed for mouse TRPA1 (Viswanath et al., 2003). Recently, it could be shown that knockout of dTRPA1 eliminated thermotaxis in vivo (Kwon et al., 2008). Additionally, Painless mutants have impaired avoidance to noxious heat and mechanical stimulation (Tracey et al., 2003). By means of a RNA interference strategy, it has been determined that dTRPA1 is also responsible for thermotactic behavior in fruit flies, but knockdown of the other three TRPAs had no effect on thermotaxis (Rosenzweig et al., 2005). Temperatures higher than 40°C activate Pyrexia in heterologous expression systems but might hyperpolarize the neuron because it leads to an outflow of potassium. Pyrexia mutant flies show a paralysis at temperatures above 40°C. Thus, it was suggested that the channel might protect flies from high temperatures (Lee et al., 2005b).

In summary, a function in temperature sensation for TRPA1 might be conserved during evolution, and we investigated this in more detail.

We found that hTRPA1 is not activated by hot temperatures and the Q_{10} values were <3. Story and colleagues did also not detect a response to hot temperature of mTRPA1 (Story et al., 2003).

Extreme hot temperatures must be avoided in mammals. TRPA1 does not seem to be involved in that process although in invertebrates it is. In mammals, other TRP channels, such as TRPV1 and TRPV2 for instance, have been suggested to be involved in transduction of hot temperatures (Caterina et al., 1997; Caterina et al., 1999; Caterina et al., 2000; Davis et al., 2000; Rutter et al., 2005). However, until now the molecular identity of the heat transduction channel has not been clearly shown *in vivo*.

4.8.2 Cooling

A major unresolved question is whether TRPA1 is a mediator of noxious cold transduction as was suggested in 2003 (Story et al., 2003). Several groups have explored this issue. However, after five years of research the evidence that TRPA1 functions as a cold transducer is still conflicting. Whereas some groups could confirm the activation of TRPA1 by noxious cold (Story et al., 2003; Bandell et al., 2004; Smith et al., 2004; Elitt et al., 2006; Kwan et al., 2006; Klionsky et al., 2007; Sawada et al., 2007), other groups did not find any cold activation in *in vitro* and *in vivo* experiments (Babes et al., 2004; Jordt et al., 2004; Nagata et al., 2005; Bautista et al., 2006; Hill and Schaefer, 2007).

There are several indications that TPRA1 is important for cold sensation. In the original report on cold activation of TRPA1 in 2003, TRPA1-expressing HEK293 cells were used and were shown to be activated by noxious cold temperatures at approximately 17℃. Another group suggested a role in cold sensation after they reported that artemin over-expression in mice results in an upregulation of the channels TRPV1 and TRPA1. These mice showed an enhanced behavioral response to heat and cold stimulation (Elitt et al., 2006). A more recent publication showed that TRPA1 is activated by very cold temperature in inside-out recordings and that 88% of AITC-sensitive mouse DRG neurons were also activated by deep cooling. Four years after the first report that TRPA1 might function as noxious cold sensor, the long awaited TRPA1 knockout studies were published. One of the two independent studies reported deficits in assays for noxious cold sensation (ice-cold plate withdrawal latency, acetone cooling). However, this finding was only significant in female mice (Kwan et al., 2007).
Additionally, these knockout mice were used in a recent investigation to investigate the function of TRPA1 in visceral sensory fibers. They reported that cold-evoked responses in nodose ganglia of these animals were reduced. Moreover, they showed that the response of cultured cold-sensitive rat nodose neurons overlaps with a response to specific TRPA1 agonists and that this response can be blocked by TRPA1 antagonists. However, a small fraction of cold-sensitive neurons remained to be independent of TRPA1 and they observed many differences in the pharmacological profile of somatic and visceral cold-sensitive neurons (Fajardo et al., 2008).
Other reports could not support the finding of an intrinsic cold activation of TRPA1. Several attempts using calcium imaging and electrophysiological studies in the last five

years failed to see an activation of TRPA1 by noxious cold at all (Babes et al., 2004; Jordt et al., 2004; Nagata et al., 2005; Hill and Schaefer, 2007).

Additionally, numerous expression experiments showed a discrepancy between the number of TRPA1-expressing TG and DRG neurons and neurons that are activated by cold. This inconsistency appeared for the first time when Jordt and colleagues (2004) showed that among mustard oil-sensitive rat TG neurons, 96% did not respond to noxious cold stimuli (Jordt et al., 2004). Moreover, Babes and colleagues (2004) classified cold-sensitive DRG neurons in a TRPM8-positive/menthol-sensitive group and in a menthol-insensitive group. Due to the rare co-expression of TRPA1 and TRPM8 (Kobayashi et al., 2005), TRPA1 should belong to the menthol-insensitive group if it functions as a cold sensor. However, these neurons did not respond to icilin and mustard oil. More recently, it was reported that 19% of cultured DRG neurons were cold-sensitive. Many of those were also sensitive to the agonists menthol and mustard oil but interestingly, 48% of cold-sensitive DRGs did not respond to either stimulus (Munns et al., 2007).

Interestingly, the second knockout study observed no cold deficits between wild-type and knockout cultured neurons and behavioral experiments (acetone test and the cold-plate paradigm) (Bautista et al., 2006).

Furthermore, in an investigation examining the expression of TRP channels during embryonic development, cold responses were present in neurons from E12.5. However, TRPM8-positive neurons occur at E18.5, whereas TRPA1 is expressed postnatally for the first time (Hjerling-Leffler et al., 2007).

We could show that in calcium imaging experiments TRPA1-expressing HEK293 cells responded to a temperature decrease. Moreover, the mean temperature threshold was 17℃ as has been reported previously (Story et al., 2003). Interestingly, YFP-transfected HEK293 cells also showed a response to a temperature decrease, although not quite as strong as TRPA1-expressing HEK293 cells. Moreover, the temperature threshold was almost the same as for TRPA1-expressing cells (~17°C). Until recently, activation of native HEK293 cells by cold has been ignored. However, several months ago, in line with our data, another group demonstrated a minimal induction of calcium uptake by untransfected CHO cells at noxious cold temperatures, but cells expressing the channel showed a stronger activation (Klionsky et al., 2007). Untransfected *Xenopus* oocytes also show a strong current upon cold stimulation in the presence of calcium (Hamada et

al., 2008). Moreover, as has been shown before (Story et al., 2003), we could confirm the finding that cold-induced activation depends on the presence of extracellular calcium. Our major finding is that TRPA1 is not directly activated by cold stimulation. As already described in our model (figure 36), we think that an elevation of intracellular calcium levels during cooling is responsible for the activation of TRPA1 via the EF-hand domain because our calcium-insensitive mutant channels show a reduced response to cold that is similar to the response of control cells. By means of whole-cell recordings, which are a more direct method to show a putative activation upon cold stimulation, we were not able to detect cold-induced shifts of the voltage-dependent activation curve and the response to calcium was reduced during cold stimulation. Therefore, we argue that TRPA1 does not function as cold transducer *per se* but is only secondarily activated by increased calcium levels.

Recently, another report suggested that *Drosophila* dTRPA1 functions as direct thermosensor for hot temperatures but is indirectly activated by a GPCR/PLC pathway during temperature sensation in the non-noxious range (Kwon et al., 2008).

Our data is in apparent contrast to a recent study by Sawada and colleagues (2007) who demonstrated that TRPA1 is activated by cold independently of changes in intracellular calcium (Sawada et al., 2007). There is not an obvious explanation for the differences in results and the controversy whether TRPA1 has a role in noxious cold sensation persists. As described above, many expression studies did not observe a strong overlap between TRPA1 expression and cold sensitivity. It seems likely that other TRPA1-independent mechanisms that mediate cold sensation and lead to an influx of extracellular calcium exist. However, the molecular target and the mechanisms mediating cold sensation are still poorly understood.

Previously, several channels have been suggested to play a role in cold sensation. It has been suggested that cold-induced inhibition of background potassium channels or of the Na^+/K^+-ATPase leads to membrane depolarization of cold-sensitive afferents (Pierau et al., 1974; Spray, 1986; Maingret et al., 2000; Reid and Flonta, 2001a; Viana et al., 2002). Moreover, an involvement of potassium channels might be possible because application of the voltage-gated potassium channel blocker 4-AP induced cold-sensitivity in previously unresponsive DRG and SCG neurons (Munns et al., 2007). TREK-1, a two-pore-domain potassium channel, has been considered as a good candidate that mediates cold sensation because it is thermo-sensitive, inhibited by cold, and expressed in one-third of cold-sensitive neurons (Maingret et al., 2000; Nealen et

al., 2003). However, TREK-1 knockout mice did not show changes of cold sensitivity even though nociceptors appeared to be more sensitive to heat (Alloui et al., 2006).

Blockade of the Na^+/K^+-ATPase with the antagonist ouabain elicits only a 10-50% depolarization in comparison to that induced by cold stimulation. Thus, inhibition of Na^+/K^+-ATPase cannot be the only mechanism by which cold leads to excitation of neurons (Reid and Flonta, 2001a).

Other examples were members of the degenerin family of epithelial sodium channels (DEG/ENaC), which are potentiated by cold temperatures in the presence of an agonist. However, cold temperature alone did not elicit a current (Askwith et al., 2001) and knockout studies of DEG channels in mice have not indicated a function in cold sensation (Price et al., 2000; Price et al., 2001).

It has been widely accepted that TRPM8 plays a role in non-noxious cool sensation (McKemy et al., 2002; Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007) but moreover, Colburn and colleagues (2007) proposed a role of TRPM8 in sensing also unpleasant and noxious cold. TRPM8 knockout mice showed prolonged cold plate paw flick latencies but still the stimulus caused some pain behavior and the animals responded to cold (Dhaka et al., 2007). A double knockout for TRPM8 and TRPA1 is awaited in order to provide more insights. In summary, the evidence for the above mechanisms to mediate cold transduction is not very strong and it is likely that yet unknown molecules might play a role.

More recent experiments have demonstrated that the inactivation of the sodium channel $Na_v1.8$ is cold-resistant and thus the channel is responsible for the sustained excitability of nociceptors upon cold stimulation. $Na_v1.8$ knockout mice do not show the typical foot-lifting or jumping behavior in the cold-plate test at 0℃ (Zimmermann et al., 2007). Additionally, a recent publication could demonstrate that killing of all neurons that express $Na_v1.8$ by using diphtheria toxin, leads to reduced responses to noxious mechanical pressure and cold (Abrahamsen et al., 2008).

Cold sensitivity seems to be a rather complex entity that might be not associated to a specific transduction molecule but instead results from an involvement of many channels and factors in nociceptors and TRPA1 is not the noxious cold transducer *per se*.

4.9 The response to mustard oil is increased after inflammation

Inflammation, which occurs due to tissue damage or infection, has specific symptoms such as hyperalgesia and allodynia (see introduction) (Scholz and Woolf, 2002). During this process nociceptors are sensitized by several mediators (e.g. CGRP and SP) (Schaible and Richter, 2004). It is accepted that TRPA1 has a function in pain sensation although the specific involvement is still poorly understood. There is evidence that the channel functions as a downstream target of inflammatory agents, such as bradykinin (Bandell et al., 2004; Bautista et al., 2006). Moreover, mice treated with the TRPA1 antagonist AP18 showed reduced bradykinin-induced mechanical hyperalgesia (Petrus et al., 2007).

Changes in ion channel expression might contribute to an increased electrical excitability in pain-sensing neurons and hypersensitivity to certain stimuli in animals. It has been demonstrated that altered gene expression occurs during inflammatory and neuropathic pain (Black et al., 2004; Morales-Aza et al., 2004; Ueda, 2006; Chen et al., 2008). One example is the up-regulation of the voltage-gated sodium channels $Na_v1.3$ and $Na_v1.7$ at mRNA and protein levels after inflammation (Black et al., 2004). Therefore, we tested whether an increased expression of TRPA1 after CFA-induced inflammation might contribute to an enhanced excitability and sensitization of DRG nociceptors as well.

We used an *in situ* hybridization paradigm to assess mRNA expression level changes after 48 hours of CFA injection. Unexpectedly, we could not observe a difference between TRPA1 expression levels of ipsilateral DRG neurons of CFA injected mice and DRGs of untreated control mice. In both groups approximately 32% of neurons express TRPA1 mRNA. Several studies reported similar numbers of TRPA1-positive neurons in DRG neurons (Kobayashi et al., 2005; Obata et al., 2005; Sawada et al., 2007). However, the reported numbers of TRPA1-positive neurons detected by using *in situ* hybridization or AITC-responsiveness varied among several studies from 3.6% to 56.5% (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2005; Nagata et al., 2005; Bautista et al., 2006; Kwan et al., 2006), which might occur because of differences in experimental procedures, in species, and in detection sensitivity.

A “phenotypic switch” of molecules has been previously reported in animal models of inflammatory and neuropathic pain. For instance, the number of larger diameter A-fibers in DRG, which express SP, CGRP, or brain-derived neurotrophic factor (BDNF) was increased in various models of inflammation (Neumann et al., 1996; Ohtori et al., 2001;

Ohtori et al., 2002). Moreover in neuropathic pain models, the ion channel TRPV1 was up-regulated in the A-fiber subpopulation of nociceptors (Rashid et al., 2003).
Therefore, this “phenotypic switch” might contribute to the altered sensitivity observed in cultured DRG neurons or behavioral response of animals (Rashid et al., 2003; Ueda, 2006).
We investigated whether the co-localization of TRPA1 and CGRP in peptidergic C-fibers has changed after 48 hours of inflammation. Therefore, we combined *in situ* hybridization and immuno-histochemistry. We observed that only approximately 3% of total DRG neurons express both, TRPA1 and CGRP. Additionally, the percentage of TRPA1 and CGRP co-localization did not change after 48 hours of CFA-induced inflammation.
Originally, TRPA1 expression was reported to almost completely overlap with CGRP. However, since the authors of the study detected only 3% neurons in the DRG as TRPA1-positive, it might be possible that the techniques they used was unable detect TRPA1 in non-peptidergic neurons for unknown reasons (Story et al., 2003). Other studies reported the expression of CGRP in a subpopulation of TRPA1-positive neurons in DRGs (Bautista et al., 2005; Obata et al., 2005; Du et al., 2007) and a population of non-peptidergic neurons that express both, TRPA1 and IB4 (Hjerling-Leffler et al., 2007).
We also indirectly investigated putative functional changes at the protein level by application of the TRPA1 agonist mustard oil in dissociated DRG neurons. The percentage of neurons responding to mustard oil was higher (~41 vs. ~48% contralateral and ipsilateral to CFA-injection, respectively) than in my *in situ* hybridization experiments. This might be explained by the fact that mustard oil is not entirely specific to TRPA1 (Kwan et al., 2006), although another study reported that TRPA1 is the sole mediator of mustard oil sensitivity (Bautista et al., 2006). However, the range of TRPA1 expression varies from study to study up to 56.5% (Nagata et al., 2005). The percentage of ipsilateral and contralateral mustard oil-responding cells did not differ significantly. Interestingly, the amplitude of the mustard oil response was increased in ipsilateral DRGs in comparison to contralateral DRGs. This might contribute to the increased excitability of nociceptors after inflammation and hypersensitivity of animals in behavioral experiments.
In contrast to our observation that TRPA1 expression is unchanged after inflammation, other studies found an up-regulation of TRPA1 after CFA injection and a putative role in

hyperalgesia (Obata et al., 2005; Katsura et al., 2007; Petrus et al., 2007). Obata and colleagues (2005) injected CFA that resulted in an increased number of paw lifts of the ipsilateral foot during a cold plate test at 5℃ at day 1 and 3. Moreover, they found up-regulation in the percentage of TRPA1 mRNA-positive neurons in the ipsilateral DRGs at day 1 and 3 from ~32.4% to ~44.1%. The discrepancies between their and our study might be due to species differences (rats vs. mice) or methodological differences (DIG-labeled vs. radioisotope-labeled probes, quantification) (Obata et al., 2005). Additionally, they showed that antisense-oligodeoxynucleotide (AS-ODN) knockdown before CFA treatment reversed the inflammation-induced cold allodynia (Obata et al., 2005). Recently, Dunham and colleagues (2008) also reported that CFA injection in rats leads to an upregulation of TRPA1 protein in L4 DRGs 3 days after CFA injection. We used mice instead and only investigated TRPA1 mRNA expression after 2 days.

In summary, TRPA1 seems to be involved in the mediation of CFA-induced inflammation and increased nociceptor excitability because we observed an enhanced response to stimulation. However, the exact mechanisms are not completely understood and further investigations are necessary to understand the role of TRPA1 in inflammation. Inflammation is a complex process and of course TRPA1 is not the sole mediator of inflammatory pain symptoms and many neuroplastic changes are induced.

4.10 Conclusion and outlook

Thus far, the key endogenous function of TRPA1 is still unclear. Knock-out studies have shown that a role in hearing and balance can be rejected. Moreover, a major function of the channel in somatic mechanotransduction is unlikely, although a contribution to mechanosensation cannot be fully excluded.

Additonally, we and others could show that TRPA1 seems to contribute to an increased excitability of inflammatory pain.

Is TRPA1 the noxious cold sensor *per se*? We can conclude that TRPA1 is not directly activated by painful cold stimuli and it seems likely that other yet unknown molecules are responsible for cold sensation. However, TRPA1 can function as a ‘potentiator’ of noxious cold via indirect mechanisms. Cold stimulation leads to a rise in intracellular calcium levels that activates TRPA1 secondarily via the EF-hand domain. Indeed, since any increase in intracellular calcium levels can gate the channel, it could also function as an ‘amplifier’ for many kinds of painful stimuli.

Definitely, further investigations are necessary to describe the endogenous function of TRPA1 in more detail. For example it has not been determined whether TRPA1 subunits form heteromultimeric channels with other TRP channel subunits *in vivo*. One can also ask the question whether TRPA1 is a transducer for one specific modality at all because of the multitude of stimuli that activate the channel. One can speculate that it might function as a coincidence detector, accelerator, and or amplifier of painful stimuli. For this reason and due to the fact that TRPA1 is activated by many pungent compounds and its involvement in clinical pain states, specific antagonists to TRPA1 might serve as potential therapeutic agents.

Activation of TRPA1 by calcium via the EF-hand domain might be an important mechanism *in vivo*. Therefore, I have started to create a transgenic mouse that has a point mutation in the EF-hand domain of TRPA1. I will systematically analyze this mouse with the help of behavioral studies, *ex vivo* electrophysiology and DRG cultures in a future project in order to study the functional impact of this domain *in vivo*.

5 Summary and ‘Zusammenfassung’

5.1 Summary

TRPA1 is a non-selective ion channel belonging to the transient receptor potential (TRP) family. The channel is expressed by a subset of small pain sensing neurons in the peripheral nervous system and can be activated by many pain eliciting chemicals, e.g. acrolein and mustard oil. Its endogenous function remains unclear, although it is generally accepted that TRPA1 is important in the pain pathway.

In my PhD project, I systematically investigated the endogenous function of TRPA1 in vitro with the help of calcium imaging and patch clamping.

In initial studies, we demonstrated that TRPA1 is voltage sensitive and directly gated by intracellular calcium. Interestingly, it can be activated by several stimuli, which raise intracellular calcium levels in sensory neurons. We identified, by means of mutagenesis, an EF-hand domain in the N-terminus of TRPA1 and demonstrated that it is responsible for the calcium sensitivity of the channel. Osmotic pressure and warm temperatures did not activate the channel, but TRPA1 was secondarily activated by cold stimuli due to an increase in intracellular calcium. Thus, TRPA1 is not the cold sensor *per se* but potentiates the response to painful stimuli in nociceptors.

In addition, we have investigated the expression and functional response of TRPA1 in mouse models of inflammatory pain because changes might modulate the excitability of pain sensing neurons after inflammation. Thus, we injected Complete Freund's Adjuvant (CFA) into the hind paw of mice. We assessed TRPA1 expression by means of calcium imaging of dissociated dorsal root ganglia (DRG) neurons and *in situ* hybridization of DRG sections after inflammation. The expression pattern of TRPA1 was unchanged when we compared untreated mice and inflamed animals. However, mustard oil evoked larger responses in CFA-treated mice compared to control mice. This suggests that TRPA1 might contribute to enhanced excitability and hypersensitivity during inflammation.

Currently, we are generating transgenic mice with a point mutation in the EF-hand domain of TRPA1 and we will systematically analyze the phenotype of these mice with behavioral assays, patch clamping and skin-nerve preparations.

5.2 Zusammenfassung

TRPA1 ist ein nicht-selektiver Ionenkanal der ‚*Transient Receptor Potential‘* (TRP) Protein Familie. Dieser Kanal wird überwiegend in einem Teil von kleineren Schmerzfasern des peripheren Nervensystems exprimiert und kann durch diverse schmerzauslösende Substanzen aktiviert werden, wie zum Beispiel durch Akrolein und Senföl. Seine endogene Funktion ist noch immer ungeklärt, obwohl TRPA1 mittlerweile eine Rolle in der Schmerzbahn zugesprochen wird.

Während meines PhD Projektes, habe ich die endogene Funktion von TRPA1 systematisch *in vitro* mit Hilfe von Kalzium Mikrofluorimetrie und der Patch-Clamp Technik untersucht.

Erste Untersuchungen ergaben, dass TRPA1 ein leicht spannungssensitiver Kanal ist und direkt durch intrazelluläres Kalzium aktiviert wird. Interessanterweise wurde er durch mehrere Stimuli, die die intrazelluläre Kalziumkonzentration erhöhen, indirekt aktiviert. Mittels Mutagenese identifizierten wir eine EF-Hand Domäne im N-Terminus des Kanals, die für die Kalziumsensitivität des Kanals verantwortlich ist. Osmotischer Druck und warme Temperaturen öffneten den Kanal nicht, aber kalte Temperaturen aktivierte TRPA1 indirekt über einen Anstieg der intrazellulären Kalziumkonzentration. Wir schließen daraus, dass TRPA1 nicht der Kältesensor *per se* ist, aber die Antworten auf schmerzhafte Stimuli in Schmerzneuronen verstärkt.

Des Weiteren, haben wir die Expression und funktionalen Antworten von TRPA1 in einem Entzündungsmodell für Mäuse untersucht, da Veränderungen zu einer gesteigerten Erregbarkeit der Schmerzneurone während einer Entzündung führen könnten. Aus diesem Grund wurde ‚Complete Freund’s Adjuvant‘ (CFA) in die Hinterpfote von Mäusen injiziert. Die Kanalexpression in Wurzelganglienzellen nach der Entzündung wurde mittels Kalzium Mikrofluorimetrie und *in situ* Hybridisierung geprüft. Das Expressionsmuster von TRPA1 blieb unverändert im Vergleich von gesunden und CFA-behandelten Tieren. Senföl führt aber zu einer erhöhten Zellantwort in CFA-behandelten Mäusen im Vergleich zu unbehandelten Mäusen. Dieses Ergebnis weist darauf hin, dass TRPA1 möglicherweise bei der erhöhten Erregbarkeit und Überempfindlichkeit während des Entzündungsprozesses eine Rolle spielt.

Gegenwärtig, generieren wir transgene Mäuse mit einer Punktmutation in der EF-Hand Domäne von TRPA1 und werden den Phänotyp dieser Mäuse systematisch mit Verhaltensexperimenten, der Patch-Clamp Technik und Haut-Nerve Präparationen untersuchen.

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7 Appendix A: Details of companies and distributers

Table A1: Companies and distributers

Company	City	Country
ADInstruments GmbH	Spechbach	Germany
Agowa	Berlin	Germany
Ambion	Huntingdon	UK
Angewandte Gentechnologie Systeme GmbH AGS	Heidelberg	Germany
Applied Biosystems	Foster City, CA	USA
BD Biosciences Discovery Labware	Franklin Lakes, NJ	USA
Beckmann Coulter	Krefeld	Germany
Beckmann Instruments GmbH	Heidelberg	Germany
Becton-Dickinson GmbH	Heidelberg	Germany
Biochrom AG	Berlin	Germany
Bioline	Luckenwalde	Germany
Biometra	Göttingen	Germany
BIO-RAD Laboratories	Munich	Germany
BioTeZ Berlin-Buch GmbH	Berlin	Germany
Braun	Melsungen	Germany
Calbiochem	San Diego, CA	USA
Carl Roth GmbH	Karlsruhe	Germany
Carl Zeiss Mikroskopie	Göttingen	Germany
Clontech	Saint-Germain-en-Laye	France
Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)	Braunschweig	Germany
Difco Laboratories	Detroit	USA
E.S.F electronic	Göttingen	Germany
Eppendorf	Hamburg	Germany
Falcon	Heidelberg	Germany
Fermentas	St. Leon-Rot	Germany
Galanz Inc.	Ontario	Canada
GE Healthcare	Munich	Germany
Geneservice Ltd.	Cambridge	UK
GFL Gesellschaft für Labortechnik GmbH	Burgwedel	Germany
GIBCO Invitrogen Corporation	Karlsruhe	Germany
Grant Instruments Cambridge Ltd	Shepreth	UK
Heidolph Elektro GmbH	Kelheim	Germany
HEKA Electronic	Lamprecht	Germany

Heraeus	Hanau	Germany
Herolab GmbH	Wiesloch	Germany
Hettrich Zentrifugen	Königs-Wusterhausen	Germany
Hilgenberg	Maisfeld	Germany
Hoefer Inc.	San Francisco, CA	USA
Infors HT	Bottmingen	Switzerland
Intas	Göttingen	Germany
Integra Biosciences IBS AG	Chur	Switzerland
Invitek	Berlin	Germany
Invitrogen Corporation	Karlsruhe	Germany
Knick	Berlin	Germany
Kodak	Stuttgart	Germany
L. Fischer	Heidelberg	Germany
Lab4you GmbH	Berlin	Germany
Machery-Nagel	Düren	Germany
Medingen	Rochester, NY	USA
Menzel GmbH	Braunschweig	Germany
Merck AG	Darmstadt	Germany
Mettler-Toledo	Schwerzenbach	Switzerland
Miles Inc.	Elkhart	USA
Millipore GmbH	Eschborn	Germany
Mitsubishi		Japan
Molecular Devices	Sunnyvale	USA
Monocar International GmbH	Bremen	Germany
MWG Biotech AG	Martinsried	Germany
Neolab	Heidelberg	Germany
New England BioLabs (NEB) Inc.	Ipswich, MA	USA
Nunc GmbH	Wiesbaden	Germany
PAN Biotech GmbH	Aidenbach	Germany
PerkinElmer	Boston, MA	USA
Pharmacia Biotech	Piscataway	USA
Promega	Mannheim	Germany
Qiagen	Hilden	Germany
Rhodia Organic Fine Limited	Bristol	UK
Richard Allan Scientific	Kalamazoo, MI	USA
Roche Diagnostics	Mannheim	Germany
Sarstedt AG & Co.	Nümbrecht	Germany
Serva	Heidelberg	Germany
Sigma-Aldrich	Munich	Germany
Stratagene	La Jolla, CA	Germany

Sutter Instruments Co.	Novatar	USA
Systat Software Inc.	Erkrath	Germany
Thermo Electron Corporation	Waltham, MA	USA
TIB MOLBIOL	Berlin	Germany
TILL Photonics GmbH	Gräfelfing	Germany
TPP AG	Trasadingen	Switzerland
Vector Laboratories Inc.	Burlingame, CA	USA
VWR International GmbH	Darmstadt	Germany
Warner Instrument Corporation	Hamden, CT	USA
Whatman GmbH	Dassel	Germany
WPI Inc.	Sarasota	USA

8 Appendix B: Generation of the EF-hand point mutation *in vivo*

My current project is to create a transgenic mouse with a point mutation in the TRPA1 EF-hand domain. The aspartic acid residue at D480 should be changed into an alanine residue. In order to realize this project, I started to work in collaboration with the laboratory of Prof. Dr. Carmen Birchmeier-Kohler under the supervision of Dr. Hagen Wende at the Max-Delbrück-Center for molecular medicine (MDC) in Berlin-Buch. In this chapter I describe the materials and methods I have used so far to realize the project.

8.1 Principle

We used ‘recombineering’ in order to create the target vector. The term ‘recombineering’ means *in vivo* genetic engineering using bacteriophage lambda (λ) recombination proteins and short DNA homologies (recombination-mediated genetic engineering) (Ellis et al., 2001). Homologous recombination is the process of exchanging DNA between two molecules through regions of identical sequence (Court et al., 2002). It is a simple, precise, and very efficient method. The lambda proteins can efficiently catalyze recombination between short homologies (Sawitzke et al., 2007). In order to use recombineering, a PCR cassette with flanking regions of homology is amplified and the lambda phage recombination proteins are introduced into a BAC-containing bacterial strain. The PCR cassette is transformed into the latter strain and recombination is induced (Copeland et al., 2001).

8.2 Target vector

The first step was to clone homology arms (A and B) into the pDTA vector (see 8.3.3). Afterwards, a “genomic subclone” was created by recombining the vector with a BAC containing the TRPA1 gene. Second, the genomic subclone (TRPA1 in pDTA) was then recombined with a sequence containing a so-called floxxed ‘neomycin/kanamycin self-excision cassette’ from the pBS-Cre-Neo/Kan plasmid (see 8.3.3) and the point-mutated TRPA1 gene (homology arms C and D). The testes specific promoter from the angiotensin-converting enzyme gene will drive expression of the Cre-recombinase

gene. The marker gene Neo is located 3' of the Cre gene to avoid transcriptional read-through of Cre when the Neo gene is transcribed. Moreover, the Cre gene has an intron to prevent in-frame translation and subsequent self-excision in bacteria. The cassette will be excised with Cre as soon as it passes through the male germ line and a single loxP element remains at the chromosomal locus (Bunting et al., 1999). The sequence next to the cassette contains exon 11 and exon 12 of TRPA1. The point-mutation of the EF-hand domain is induced in exon 12 beforehand and then the sequence is recombined with the pDTA vector containing the genomic subclone of TRPA1.

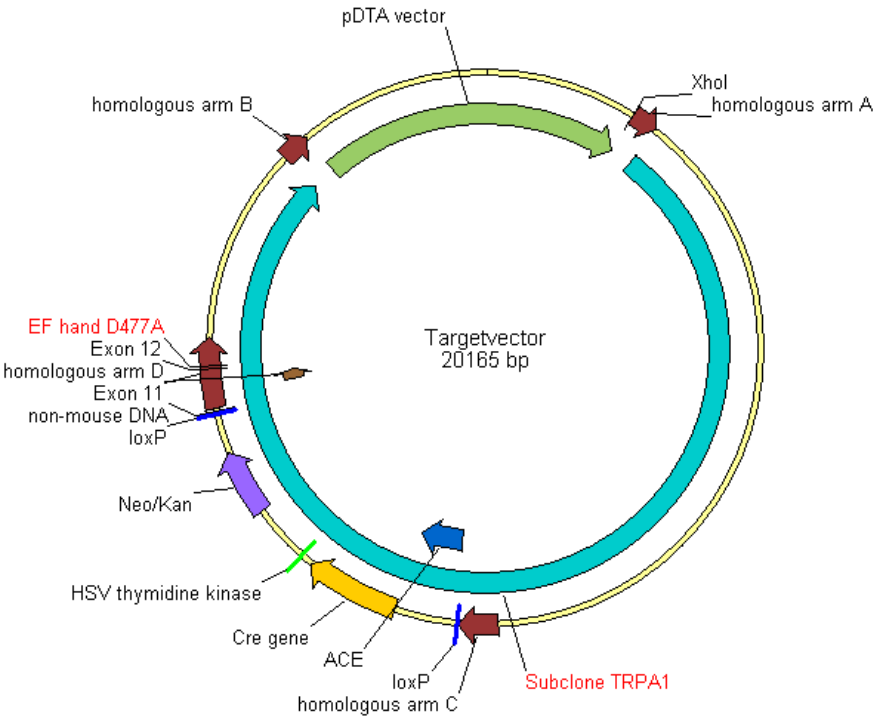

Figure 37 the target vector was constructed by means of homologous recombineering.

8.3 Materials

In the following chapter the materials are listed in alphabetical order and the distributors' names are given. For more details on distributors (city, country) please see Appendix A.

8.3.1 Oligonucleotides

All used oligonucleotides were ordered from MWG Biotech, TIB MOLBIOL, or BioTeZ. The name, sequence and application are listed in table B1. Usually, they were diluted to 10pmol/µl.

Table B1

Name	5' -3' sequence	Application
TRPA1_3_ext_up	TTATCTCACTGTCTAACATCCGA	DNA probe 3', Identification of positive clones (southern hybridization
TRPA1_3_ext_lw	TGATTTCATGACTTTTTATGGC	
5_neu_hagen_lw	CAACAGTTTCCTCCTAACGC	DNA probe 5', Identification of positive clones (southern hybridization
5'probe_short_up	GGGCAGTGGTGAAGGTTTGT	
EF-hand_up	CCAGACTCCCAATATTTGAGCG	to detect point mutation or wild type sequence
EF_hand_lw_varC_2	AGAGGGGTCATCCCATGGAGAG	
EF_hand_lw_varA_2	AGAGGGGTCATCCCATGGAGAT	
mEF-SUB_A_up5	GACCTCGAGGTCGACATGAGA TCCAGATGGGTG	Homologous 5' arm for genomic subclone
mEF_SUB_A_low3	GACCCCGGGATCAAGGTGAAT TGAGACTATAGG	
mEF_SUB_B_up5	GACCCCGGGCCCCCATCAGAT CCTG	Homologous 3' arm for genomic subclone
mEF_SUB_B_low3	GACACTAGTGATCCAGTTCTTC TCTGTCTTATTTAGG	
mEF_MUT_C_up5	GACCTCGAGAATCAGTCATTTT CCTTC	Homologous 5' arm to clone self excision cassette
mEF_MUT_C_low3	GACAAGCTTTCTAGAACATGTC CTCCCTTCTGC	
mEF_MUT_D_up5	GACGAATTCGGATCCTCAGACC CGAGTGACCCACTGCTCAAATG CTGCCTTGG	Homologous 3' arm to clone self excision cassette
mEF_MUT_D_low3	GACGCGGCCGCCCGCACTTAC TCTGCAGCAG	
KO_D477A_5'_2	CTTTTGAATGAAGGGGCTCTCC ATGGGATGAC	Mutagenesis of D477A region in mouse TRPA1
KO_D477A_3'_2	GTCATCCCATGGAGAGCCCCTT CATTCAAAAG	
TV_loxP1	CAAGACCCCCATCTGTTTTG	Sequencing primer for loxP

TV_loxP2	TGTGCATCCGTCATTGTCTT	sites

8.3.2 Bacteria

The name, genotype, and reference of the bacteria used are listed in table B2.

Table B2

Name	Insert	Reference
Escherichia coli DH10B	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G	Invitrogen
Escherichia coli DY380	DH10B [l*cl*857 (*cro–bio*A) <> *tet*]	(Lee et al., 2001)
Escherichia coli EL350	DH10B [l*cl*857 (*cro–bio*A) <> *ara*C–PBAD*cre*]	(Lee et al., 2001)

8.3.3 Plasmids

The plasmids used are listed in table B3. The name, insert of interest and the reference are given.

Table B3

Name	Insert	Reference
pBluescript SKII (+)		Stratagene
pKS-DTA	DTA cassette	Mathias Treier, EMBL, Heidelberg
pBS-Cre-Neo/Kan	5'- loxP-tACE-Cre-dual-Neo/Kan-loxP -3' in pBS	Mathias Gierl, MDC, Berlin
pDTA	5'- rare cutters – amplified DTA-cassette DTA.1.3 (from pKS-DTA) – multiple cloning sites – rare cutters – 3' in pBS	Mathias Gierl, MDC, Berlin

8.3.4 BAC clones

In this project, BAC clones containing the genomic locus of the mouse TRPA1 gene were identified and used. The bMQ BAC library was selected. The library was generated from AB2.2 ES cell DNA (129S7/SvEv Brd-Hprt b-m2). The host was *E. coli* DH10B. The bMQ-302J15 clone (Geneservice Ltd.) was used for the cloning of the targeting vector. It was transferred to LB broth containing chloramphenicol and 8% glycerol and incubated overnight at 37℃.

8.3.5 Eukaryotic stem cell lines

For culture and transfection of embryonic stem cells for the development of transgenic mice, the embryonic stem cell line E14.1 from the stem 129/OlaHsd was used (Kuhn et al., 1991). For this project one aliquot of passaged embryonic stem cells from the lab of Prof. Dr. Carmen Birchmeier-Kohler was used.

8.3.6 Enzymes

PfuUltra polymerase	Stratagene
Proteinase K	Sigma-Aldrich
Restriction enzymes with buffers	NEB
RNase A	Roche
Shrimp alkaline phosphatase SAP (1U/µl)	Roche
T4 DNA ligase	Promega, NEB
Taq polymerase	Roche

8.3.7 Antibiotics

Ampicillin	Roth
Chloramphenicol	Sigma-Aldrich
Geneticin G418	Invitrogen
Kanamycin	Sigma-Aldrich
Penicillin/streptomycin solution	Invitrogen

8.3.8 Chemicals, reagents and media

100x non-essential amino acids	Invitrogen
2x fast ligation buffer	Promega

Acetic acid anhydride	Merck
Acetic acid	Roth
Agarose	Invitrogen
Ammonium sulfate	Roth
Bacto-trypton	BD Biosciences
Bovine serum albumin BSA	NEB
Bromophenol blue	Sigma-Aldrich
Cresol red	Sigma-Aldrich
Dimethyl sulfoxide DMSO	Merck
Disodium hydrogen phosphate Na_2HPO_4 (dihydrate)	Roth
dNTPs (Deoxy-nucleotide triphosphate mix)	Invitek
Dulbecco's MEM with Glutamax-I	Invitrogen
Ethanol (absolute)	Roth
Ethidium bromide	Roth
Ethylenediaminetetraacetic acid EDTA	Roth
Ethyleneglycol tetraacetic acid (EGTA)	Roth
Fetal calf serum (FCS)	Sigma-Aldrich
Gelatine	Sigma-Aldrich
Glycerine	Roth
Glycerol	Roth
Hydroxide chloride HCl	Roth
Hyperladder I, II, or IV	Bioline
Isopropanol	Roth
L(+)-arabinose	Sigma-Aldrich
Lambda DNA/EcoRI+HindIII marker, 3 (125-21226)	Fermentas
Leukemia inhibitory factor LIF	A. Garratt, Lab of C. Birchmeier
Magnesium chloride $MgCl_2$	Roth
Monosodium phosphate NaH_2PO_4 (dihydrate)	Roth
N-lauroylsarcosine	Sigma-Aldrich
PBS (10x)	Invitrogen
Phenol	Roth
Phenol:chloroform:isoamylalcohol 25:24:1	Ambion
Potassium chloride	Merck
pUC19 DNA/MspI (HpaII) marker, 23 (26-501bp)	Fermentas
Salmon sperm DNA	Sigma-Aldrich
Sephadex G-50 (fine)	GE Healthcare
Sodium acetate	Merck
Sodium chloride NaCl	Roth
Sodium dodecyl sulfate SDS	Serva
Sodium hydroxide (NaOH)	Roth

Sucrose	Roth
Tris base	Roth
Tri-sodium-citrate	Roth
Trypsin-EDTA solution	PAN Biotech
Yeast extract	Roth
α-32P-dCTP with 1.85MBq (EasyTides, 25 µl with 250µCi (9.25 MBq)	PerkinElmer
β-mercaptoethanol (50mM)	Invitrogen

8.3.9 Kits

LigaFast Ligations-Kit	Promega
NucleoBond PC-500 Kit	Machery-Nagel
NucleoSpin-Extract II Kit	Machery-Nagel
Prime-It RmT Random Primer Labeling Kit	Stratagene
QuikChange® II XL Site Directed Mutagenesis Kit	Stratagene

8.3.10 Buffers and solutions

Buffers and solutions used are listed with the respective recipes in table B4. All solutions were diluted in ddH_2O. Many were autoclaved or sterile filtered.

Table B4

10x GS-PCR reaction buffer	1M Tris-HCL pH 8.8, 50ml cresol red solution, 1.5 ml H_2O, 3.5ml 1M $MgCl_2$, 1.454g $(NH_4)SO_4$, aliquoted, stored at -20℃
10x TAE buffer (Tris-acetate-EDTA)	48g Tris, 11ml glacial acetic acid, 20mM EDTA (0.5M, pH 7.5)
1x Denaturation solution	1M NaCl, 0.5M NaOH
20x SSC	3M NaCl, 0.3M tri-sodium-citrate, pH 7.0 with HCL
Block solution	100ml TE buffer, 500µg salmon sperm DNA
Cresol red solution	84.5mg cresol red in 100ml of T0.1E (10mM Tris, 0.1mM EDTA), aliquoted, stored at -20℃
Depurination solution	250mM HCl
Dilution buffer	100ml H_2O, 50ml of T0.1E (10mM Tris, 0.1mM EDTA), 0.8125ml cresol red solution, 50µl 4M NaOH, aliquoted, stored at -20℃
DNA extraction buffer	10mM Tris-HCl, ph 8.0, 100mM EDTA, pH 8.0, 0.5% (w/v) SDS
EDTA	0.5M EDTA, pH 8.0 with NaOH
ES cell lysis buffer	10mM Tris-HCl, pH 7.5, 10mM EDTA, pH 8.0, 10mM NaCl, 0.5% (w/v) N-lauroylsarcosine, 0.2mg/ml Proteinase K
ES cell medium	500ml Dulbescco's MEM with Glutamax-I, 90ml heat-inactivated FCS, 6ml 100x non-essential amino acids, 6ml Penicillin/Streptomycin solution, 1.2ml 50mM β-

	mercaptoethanol, 60µl LIF
Ethanol-sodiumacetate mix	0.15M sodiumacetate, pH 5.2 in ethanol
Fibroblast medium	500ml Dulbescco's MEM with Glutamax-I, 60ml heat-inactivated FCS, 5.7ml 100x non-essential amino acids, 5.7ml Penicillin/Streptomycin solution, 1.2ml 50mM β-mercaptoethanol
Freezing medium	20% (v/v) DMSO, 30% (v/v) FCS, 50% (v/v) ES cell medium
Hybridization solution (Church and Gilbert, 1984)	0.5M NaH_2PO_4, 1mM EDTA, pH 7.2 with NaOH, then at 68℃: 1% (w/v) BSA, 7% (w/v) SDS
LB medium	1% (w/v) bacto-trypton, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.5 with NaOH
Mitomycin C solution	0.2% (w/v) mitomycin C in PBS
Sodium acetate	3M sodium acetate, pH 5.2 with acetic acid
Sucrose solution	121.1g sucrose in 350ml H_2O, aliquoted, stored at -20℃
TE (Tris/EDTA) buffer	10mM Tris base, 1mM EDTA, pH 8.0 with HCL

8.3.11 Consumable materials

15ml & 50ml plastic tubes	VWR
Cell culture bottles	TPP
Cell culture dishes (several sizes)	TPP
Cell culture plates (6, 24, 48, 96 wells)	TPP
Cell scraper	TPP
Cryo tubes (1.5ml)	Nunc GmbH
Gene Pulser cuvettes, gap size: 0.1cm	BIO-RAD
Gene Pulser cuvettes, gap size: 0.4 cm	BIO-RAD
Hybond-XL nylon membrane	GE Healthcare
MicroSpin G-50 column	GE Healthcare
Nitrocellulose membrane (0.025 µM, White VSWP, 25 mm)	Millipore GmbH
PCR tubes and caps (0.2ml)	Applied Biosystems
Pipettes (1-25ml, one-way)	VWR
Reaction tubes 0.5, 1.5ml and 2ml	VWR
Syringes (1ml)	Braun
Tubes (1.5ml) with screw caps	NeoLab
X-ray film (Kodak BioMax MR Film), several sizes	Kodak

8.3.12 Technical equipment

Bacterial shaker Multitron	Infors HT
Beckman Avanti J.25 centrifuge	Beckmann Instruments GmbH
Bio vortex V1	Lab4you
Cellular Incubator 6000	Hereaus

Centrifuge 5417	Eppendorf
Centrifuge Varifuge 3.0R	Heraeus
CO_2 Incubator	Heraeus
Easy-cast Electrophoresis system	
Consort E333 Microcomputer 4 channel power supply	AGS
Electroporator (MicroPulser)	BIO-RAD
Eletroporations-Impulsgenerator	Dr. L. Fischer, Heidelberg
Gel electrophoresis chambers (several sizes)	AGS
Heraeus Varifuge 3.0	Heraeus
Homogenizer Ultra-Turrax T25	Ilka Labortechnik
Laminar airflow	BDK
Light microscope CKX31	Olympus
Microwave	Sharp
OV4 Compact Line Hybridization Oven	Biometra
PCR thermocycler	Biometra
pH-Meter 761 Calimatic	Knick
Photometer	AGS
Incubators	Heraeus
Spectrophotometer Ultrospec 2100 pro UV/visibe	GE Healthcare
Stirrer MR3002	Heidolph
Table top centrifuge 5424	Eppendorf
Thermocycler for PCR	Biometra
Thermomixer 5436	Eppendorf
Ultrapure Water Systems Milli-Q	Millipore
UV System	Intas
Vacusafe comfort pump	IBS
Video copy processor (PSI)	Mitsubishi
Vortexmixer (2TM Mixer 7-2020)	Neolab
Waterbath W6	Medingen

8.4 Methods

8.4.1 Molecular cloning

Standard methods were performed according to Sambrook and Russel (2001) and will not be described in detail (agarose gel electrophoresis, bacterial cultures, ethanol precipitation, restriction digestion, etc). DNA constructs were sequenced by Karin

Gottschling working in the laboratory of Prof. Dr. Carmen Birchmeier-Kohler or by the companies Invitek and AGOWA.

8.4.1.1 DNA-isolation and purification

The mini-preparation of BAC- and plasmid-DNA was done with a standard protocol of alkaline lysis. The DNA was finally diluted in 50µl TE and stored at 4℃. BAC- and plasmid-DNA isolation on a larger scale was conducted with the NucleoBond PC-500 Kit according to the manual. The DNA was usually diluted in 300µl TE and stored at -20℃. For the preparation of DNA fragments from agarose gels the NucleoSpin-Extract II-Kit was used according to the manual. The DNA was diluted in 10-25 µl TE and stored at -20℃.

In order to isolate genomic DNA from tissue for southern blot testing, livers from wild-type mice (CD1, C57BL/6J) were dissected. The livers were homogenized in PBS, centrifuged for 5 min at 500 g and resuspended in few ml PBS (ca. $5x10^7$ cells/ml). The suspension was added to DNA extraction buffer (2ml per 10^7 cells) containing 10µg/µl RNase A and incubated for 1 h at 37℃ with gentle agitation. 100µg/ml proteinase K was added and the suspension was digested for 3 h at 50℃. 1 volume phenol (Tris-Cl equilibrated, pH 8.0) was added and the solution was incubated overnight at 4℃. The next day, the reaction was centrifuged (10 min, 500 g, 10℃). The upper phase, containing the DNA, was transferred to a new tube. The phenol extraction was repeated and the upper phase was precipitated with 0.2 volumes 8.5M ammonium acetate and 2 volumes ethanol. With the help of a pasteur pipette, the DNA was ‘fished’ and washed for 5 min with cold 70% ethanol, airdried, diluted in TE for ca. 24 h and then stored at 4℃.

8.4.1.2 Isolation of DNA from embryonic stem cells (ESCs)

DNA was isolated from ESC cultured on 96 well or 6 well plates. Cells on 96 well plates were washed twice with 150µl PBS and digested overnight in 50µl ES lysis buffer at 60℃ in a humidity chamber. Subsequently, ESCs were incubated for 30 min at RT with 100µl ethanol-sodium acetate solution, washed three times with 70% ethanol, and finally air-dried.

For restriction digestion of ESC DNA, 20U of the appropriate restriction enzyme in a 35µl solution were added to the wells and incubated overnight at 37℃. The plates were

sealed with parafilm and agitated with 100 rpm in a bacterial shaker. The digested DNA was separated by means of gel electrophoresis and used for southern blot screening.
The 6 well plates were washed twice with PBS and 610µl of ES lysis buffer was added and the ESCs were digested overnight at 55℃. Then the cells were scraped off the plates and transferred into a tube. The DNA was extracted with 2x 25:24:1 phenol:chloroform:isomylalcohol (PCI) and precipitated with ammonium acetate and ethanol. Finally, the DNA was diluted in 100µl TE. After restriction digestion a re-screen with southern hybridization was conducted (5-10µg DNA).

8.4.1.3 Purification of plasmid- and BAC-DNA

In order to desalt plasmid- and BAC DNA solutions, ligation reactions and DNA fragments for electroporation, a nitrocellulose membrane for microdialysis was used. The liquid surface was swimming on ddH_2O in a cell culture dish. Up to four probes were carefully pipetted onto the membrane. The dialysis lasted 30 min up to 1 hour. Afterwards, the probes were used for electroporation.
The purification from lower molecular components (salts, phenol, ethanol, free nucleotides) of fragments from restriction digestions or PCI extractions was conducted by means of gel filtration with Sephadex G-50 columns. Sephadex G-50 was saturated with TE overnight at 4℃. The appropriate amount wa s then pipetted into a 1ml insulin syringe, which was previously sealed with a 0.45µm frit. The syringe was placed into a 0.5ml tube and excessive TE was removed by centrifugation at 1800 rpm for 3 min. The DNA solution was added on top of the prepared column and the syringe, after placing into a fresh tube, was again centrifuged at 1800 rpm for 3 min. The purified DNA was then diluted in 1x TE buffer.

8.4.1.4 Photometric determination of DNA concentration and quality

DNA concentration was measured either with concentration gels or photometrically. The DNA was diluted in TE and the absorption at 260 nm wavelength against a reference (TE) was measured. 1 OD = 50 µg/ml. The ratio of OD_{260}/OD_{280} was used to determine the purity of the DNA. The ratio lies between 1.75 and 2.0 for pure DNA.

8.4.1.5 PCR

PCR was used for amplification of DNA fragments for the cloning of the targeting vector. PCR fragments were PCI extracted or electrophoretically separated and isolated.

The following PCR mix was used:

7.2µl	sucrose solution
0.187µl	1:10 β-mercaptoethanol
1µl	dNTP mix (10mM)
2µl	10x GS-PCR reaction buffer
1µl	DNA (diluted)
3.5µl	dilution buffer
0.7µl	DMSO
2.3µl	H_2O
1µl	Primer 1 (10pM/µl)
1µl	Primer 2 (10pM/µl)
0.125µl	Taq or PfuUltra Polymerase

Step	**Temperature**	**Time**	**Cycle**
Denaturation	94℃	4 minutes	1x
Denaturation	94℃	30 seconds	25-30x
Annealing	55℃	30 seconds	
Elongation	72℃	1 minute	
Prolonged elongation	72℃	7 minutes	1x
Pause	4℃	∞	

8.4.2 Mutagenesis

For mutagenesis the ‘QuikChange® II XL Site Directed Mutagenesis Kit’ was used according to the manufacturer’s manual.

8.4.3 Bacteria- and stem cell culture

8.4.3.1 Transformation of bacteria

The transformation of bacteria with plasmid-DNA or ligation products was done by means of electroporation of electro-competent cells. Dialytically cleaned DNA

(membrane) was placed in a cooled electroporation-cuvette containing cool electro-competent cells (*E. coli* DH10B, DY380, or EL350) and mixed. With the help of an electroporator, the cells were transformed with 1.80kV, 25µF and 200Ω. 1ml LB medium was added and the suspension was incubated at 37℃ (*E. coli* DH10B) or 32℃ (*E. coli* DY380 or EL350) for 1 hour. Different amounts of the solution were distributed on LB Agar plates containing the appropriated selection antibiotic and incubated at the respective temperature overnight.

8.4.3.2 Homologous recombination in bacteria

The homologous recombination technique (Yu et al., 2000; Lee et al., 2001) was used to clone the targeting construct for the EF-hand mutagenesis into the TRPA1-locus. Electro-competent *E. coli* cells (DY380 and EL350) containing plasmid DNA were necessary. 5ml LB medium with the appropriate selection antibiotic was inoculated with bacteria from a glycerol stock and incubated overnight at 32℃. 1ml of this pre-culture was added to 100ml LB medium with the appropriate antibiotic and incubated at 32℃ until an OD_{600} of 0.6 was reached.

In order to transform DY380 and EL350 cells with plasmid DNA, a bacterial cell culture has to be made electro-competent. Therefore, the cultured suspension was kept in 50ml tubes and cooled for at least 10 min in ice water. Afterwards, it was centrifuged at 2500 g for 10 min at 4℃ and the supernatant was discarded. The pellet was resuspended in 10ml cooled ddH_2O and again centrifuged for 10 min at 4500 g at 4℃. After discarding the supernatant, 10ml of 10% glycerol were added to resuspend the pellet. The solution was centrifuged for 10 min at 4500 g at 4℃. 5ml of the supernatant were discarded. The pellet was resuspended in the residual 5ml and centrifuged for 10 min at 5000 g at 4℃. Finally, the cells were resuspended in a very small volume of residual 10% glycerol and aliquoted (26µl each). The cells can be used for electroporation immediately or stored at -70℃.

In order to induce homologous recombination in DY380 cells and to transform them afterwards, the cell culture was incubated for 15 min at 42℃. Afterwards, the transformation was prepared.

Cre-recombinase was induced in EL350 cells containing plasmid DNA after an overnight pre-culture at 32℃. They were grown until an OD_{600} of 0.6 was reached. L(+)-

arabinose (end-concentration: 0.1%) was added and the culture was incubated at 32℃ for one hour.

8.4.3.3 Culture of embryonic fibroblasts

An aliquot of fibroblasts was quickly thawed at 37°C and mixed with 9ml fibroblast medium at RT. After 3 min centrifugation at 1100 rpm, the cells were resuspended in 15ml fibroblast medium and distributed in 15cm cell culture plates. Subsequently, 15ml medium was added. The cells were incubated at 37℃ and 5% (v/v) CO_2. Medium was changed every two days until the fibroblasts were confluent. Afterwards, they were split, partially frozen, and inactivated to avoid further cell division.

In order to split the fibroblast, they were washed in PBS and incubated in trypsin-EDTA for 2-5 min at 37℃ to detach the cells from the pl ate. 5ml fibroblast medium was added and the suspension was centrifuged for 3 min at 1100 rpm at RT. Medium was added and the cells were distributed on 15cm cell culture dishes.

Before freezing, the cells were detached and pelleted and finally the cells were diluted in 0.5ml medium per cryo tube and cooled to 4℃. 0. 5ml cool freezing medium was added and the tubes were stored at -80℃ overnight and then transferred to liquid nitrogen.

In order to inactivate the division of the cells, the medium was aspirated except for 10ml, mitomycin C solution was added (1:100), and the cells were stored in the incubator for 2 h at 37℃. The medium was then disc arded and the cells were washed twice with PBS. New medium was added and the cells were then split.

8.4.4 Culture, transfection and selection of embryonic stem cells (ESCs)

8.4.4.1 Culture of ESCs

One aliquot for the TRPA1 targeting vector transfection was quickly thawed at 37℃ and mixed with 9ml ES medium at RT. After 3 min centrifugation at 1100 rpm, the cells were carefully resuspended in 5ml ES medium and added into a 6cm cell culture dish containing confluent and inactivated fibroblasts with ES medium. The cells were incubated at 37℃ and 5% (v/v) CO_2. Medium was changed every day until the ESC were densely grown but were not yet confluent (single colonies have to be still visible). Afterwards, they were split like the fibroblasts (see 8.4.3.3) using 1-3ml trypsin-EDTA, centrifuged at 900 rpm, resuspended in ES medium, and distributed on confluent and

inactivated fibroblasts in 10cm cell culture dishes containing ES medium. The medium was changed every day until they were almost confluent. Then, they were either used for transfection or they were frozen. The protocol for freezing is equivalent for the freezing of fibroblasts except that ES medium instead of fibroblast medium was used.
After screening for mutated clones with Southern hybridization, the positive stem cell clones were thawed and cultured in a fresh 96 well plate containing prepared inactivated fibroblasts and ES medium. The ESC culture was then successively expanded into 48, 24, and 6 well plates using the methods for splitting described above (see 8.4.3.3). Those plates always contained inactive fibroblasts and ES medium. Finally, the cells were divided into another 6 well plate and a gelatine-coated 6 well plate. The former plate was frozen and the latter was incubated for 7-8 hours. Afterwards, the DNA was isolated from the ES cells and screened again with Southern hybridization.

8.4.4.2 Transfection of ESCs

Densely grown ESCs (10cm culture dish) were used for transfection. The targeting construct was cut out of the targeting vector with the appropriate restriction enzymes. The reaction contained 80µg plasmid-DNA in 30µl and 100U of the enzyme, and was incubated overnight at 37℃. DNA was cleaned with PCI, precipitated with ethanol, diluted in sterile TE (1µg/µl), and stored at 4℃ until transfection. The ESCs were detached for transfection and carefully separated. The cell number was determined in a 'Neubauerkammer', the cells were centrifuged and diluted with PBS to get $1.2*10^7$cell/ml. 20µg of the targeting construct and 0.8ml of the cell suspension were pipetted into an electroporation cuvette (0.4cm). The cells were electroporated with a 2ms pulse at 300V and 1200µF. The transfected cells were shortly cooled on ice, transferred to ES medium and seeded on four 10cm cell culture dishes containing inactive fibroblast and ES medium. 10ml ES medium was added and the medium was changed every day until clone selection.

8.4.4.3 Selection of ESCs

For positive selection of clones containing the targeting construct, two days after electroporation ES medium containing G418 (400µg/ml) was added to the ESCS and changed every day. Clones were picked nine days after electroporation. They have to

be G418 resistant, round, not to large, and clearly bordered (not yet differentiated). In order to do so, the medium was aspirated, the cells were washed with PBS and the plates were refilled with 12ml PBS. One clone was soaked up in a volume of 23µl PBS and transferred to a 96 well plate. After picking clones for all 96 wells, 25µl trypsin-EDTA was added to each well and the plate was incubated 3-5 min at 37℃. 50µl ES medium was added, the cells were carefully resuspended and distributed to 96 well plates containing inactivated fibroblasts. The medium was changed every day. Three days later, fresh 96 well plates were covered with 150µl of 0.1% gelatine for 20 min and then airdried. The ESCs were washed with 100µl PBS, detached with 25µl trypsin-EDTA for 3-5 min at 37℃, and resuspended following adding of 200µl medium. Half of the suspension was transferred to the gelatine coated plates, and the other half was transferred to a fresh 96 well plate with inactive fibroblasts.

The gelatine treated plates were incubated for 7-8 days and the medium was changed every day. The cell were then used for DNA isolation and Southern blot screening to identify positive clones, which recombined the targeting construct and therefore contained the allele with the TRPA1 EF-hand point mutation.

The clones in the fibroblast containing dishes were cultured for two days, and then detached and cooled at 4℃. Afterwards, 25µl of cool freezing medium was added and the suspension was mixed. Then, the plates were packed into paper towels and stored at -70℃. Positive clones were later thawed and injected into blastocysts.

8.4.5 Southern hybridization

8.4.5.1 Radioactive labeling of DNA probes

The probes were amplified with PCR and appropriate primers and isolated by gel electrophoresis. The ‘NucleoSpin-Extract II-Kit’ was used for DNA extraction from agarose gels.

The ‘Prime-It RmT Random Primer Labeling-Kit’ was used according to the manual. 25ng of the cleaned PCR product was added to the reaction and after denaturation (10 min at 95℃) 5µl α-32P-dCTP with 1.85 MBq was added and incubated at 37℃ for 10 min. Afterwards, the labeled probe was added into a prepared MicroSpin G-50 column and centrifuged for 3 min at 2000 rpm and at RT. The probes were centrifuged through the columns into a tube containing 10µg block solution. 1ml of Southern hybridization

buffer was added and the reaction was incubated for 10 min at 95℃ for denaturation. Then, the probes were pre-hybridized at 65℃ for 30 min to 1 hour.

8.4.5.2 Southern-blotting

The agarose-gels with the separated DNA fragments were washed for 8 min in depurination buffer at RT and shortly washed in ddH_2O. Then, the gel was incubated twice in denaturation buffer for 20 min and afterwards washed in 20x SSC for 5 min. The gel was turned upside-down onto a glass-plate and wet with 20x SSC. A nylon membrane (Hybond XL) was cut to the size of the gel and wet in ddH_2O. It was put onto the gel and bubbles were removed. Three Whatman papers were humidified in 20x SSC and placed onto the membrane without bubbles. Many paper towels covered the Whatman papers and the whole construction is turned again to allow the transfer of the DNA to the membrane via gravity. The next day, the membrane was shortly washed in 2x SSC, airdried and either stored at 4℃ or direct ly used for Southern hybridization.

8.4.5.3 Southern hybridization

Southern hybridization was used to identify clones carrying the TRPA1 EF-hand point mutation after homologous recombination in the genome of the ESC.

The membrane from the Southern blot was shortly incubated in ddH_2O, un-coiled, and attached to the inner side of a glass tube with the help of a glass-pipette. The DNA side was facing to the interior of the tube. 5ml hybridization buffer was added and the membrane was pre-hybridized for 30 min to 1 hour in a hybridization oven at 65℃. Subsequently, the pre-hybridized probes were added to the tubes and incubated overnight at 68℃.

The next day, the hybridization reaction was discarded and exchanged by pre-warmed (68℃) solution I (1x SSC / 1% SDS) for 15 min at 6 8℃. The solution was then exchanged again with solution II (1x SSC / 0.1% SDS) for 30 min. Subsequently, the membranes were transferred to a plastic bowl and washed with solution III, IV and V (0.5x SSC / 0.1% SDS; 0.2x SSC / 0.1% SDS; and 0.1x SSC / 0.1% SDS) for 30 min each under gentle agitation. The radioactivity was measured. In case the radioactivity was only slightly higher than the background, the washing procedure was stopped. Afterwards, the membrane was packed in plastic foil and placed on an x-ray film in a

dark room. Exposure took place at -80℃ for 3 to 14 days and then the film was developed.

9 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

10 Publications and presentations

10.1 Publications

Zurborg S, Yurgionas B, Jira JA, Caspani O and Heppenstall PA (2007). Direct activation of the ion channel TRPA1 by intracellular Ca^{2+}. *Nature Neuroscience* 10(3), 277-9.

Steiner B, Zurborg S, Hörster H, Fabel K, Kempermann G (2008). Differential 24h responsiveness of Prox1-expressing precursor cells in adult hippocampal neurogenesis to physical activity, environmental enrichment, and kainic acid-induced seizures. *Neuroscience* 154, 521-529.

10.2 Presentations

'Defining a function for the ion channel TRPA1' at Max Delbrück Center (MDC), Berlin-Buch. Development and Function of Somatosensation and Pain.
May 15th 2008, Berlin, Germany

'Direct activation of the ion channel TRPA1 by calcium' at Freie Universität, Fachbereich Veterinärmedizin, Institut für Veterinär-Physiologie, Patch-Clamp-Colloquium; October 9th 2007, Berlin, Germany

'A Ca^{2+} activation domain in TRPA1'. Berlin Brain Days, Charité Universiätsmedizin, October 30th 2006, Berlin, Germany

10.3 Posters

Zurborg S, Caspani O, Heppenstall PA (2007). Regulation of TRPA1 expression levels in nerve injury and inflammatory pain. 37th Annual Meeting, Society for Neuroscience 2007, San Diego, CA, USA.

Zurborg S, Yurgionas B, Jira JA, Caspani O, Heppenstall PA (2006). Cold sensitivity of TRPA1 is dependent on Ca^{2+} binding at its EF-hand domain. 36th Annual Meeting, Society for Neuroscience 2006, Atlanta, GA, USA.

Zurborg S, Yurgionas B, Caspani O, Heppenstall PA (2006). Defining a function for TRPA1. Berlin Neuroscience Forum 2006, Liebenwalde, Germany.

Zurborg S, Tysiak E, Ritter P, Moosmann M, Wüstenberg T, Villringer A (2004). Automatic Classification of Vigilance Stages in Wakeful Subjects - A First Step towards Finding fMRI-Correlates of Vigilance. Berlin Neuroscience Forum 2004, Liebenwalde, Germany.

10.4 Honors

Attendance at the 57th Meeting of Nobel Laureate at Lindau, Germany
from July 1st to July 6th 2007

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12 Erklärung

„Ich, Sandra Zurborg, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Defining a function for the ion channel TRPA1“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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