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Distribution and function of TRP ion channels in primary sensory neurons

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Lund 2007

Cover page: Photos of a rat dorsal root ganglion (DRG) (*top*), a section of rat mesenteric artery with TRPV1 immunoreactive nerve fibres in the adventitia (*bottom left*) and a section of rat hindpaw skin with TRPV1 immunoreactive nerve fibres in the dermis/epidermis region (*bottom right*).

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E. Helena Axelsson

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Abstract

It is frequently argued that cannabinoids exert part of their analgesic and anti-inflammatory effects via activation of cannabinoid CB1 receptors on TRPV1-expressing primary sensory neurons (PSN) in peripheral tissues. However, we find no evidence of CB1 receptor immunoreactivity on nerve fibres (NF) in rat or mouse hindpaw skin and mesenteric artery. The CB1 receptor agonists anandamide and HU210 also fail to inhibit TRPV1-mediated neuronal CGRP release from rat hindpaw skin and mesenteric artery. Therefore, this study do not support the general view that the analgesic effect of CB1 receptor agonists is due to direct inhibition of TRPV1-expressing PSN in the periphery. Garlic contains a number of organosulfur compounds, including allicin and diallyl disulfide (DADS), some of which may contribute to its pungent and vasodilator properties. Our results show that raw garlic extract, allicin and DADS activate TRPA1 ion channels on PNS in culture and NF in the vascular system. These findings highlight TRPA1 as a novel drug target in the vascular system and provide unique pharmacological tools for investigating the role of this ion channel. Whether activation of TRPA1 in the vascular system explains the beneficial antihypertensive effect observed by garlic treatment remains to be shown. This study also expands our understanding of how TRPA1 is regulated at a molecular basis, which is of importance for development of novel drug therapies for pain, inflammation and vascular level. The skin is the body's largest sensory organ. The TRP ion channels TRPV2 and TRPM8 are expressed in PSN in animals and are therefore likely to be expressed in humans as well. Fluorescence immunohistochemistry was used to identify these channels and compare their distribution patterns with known neuronal markers of PSN in skin from healthy volunteers and from individuals with a mutation in the gene encoding NGFbeta that causes Norrbottnian congenital insensitivity to pain. This study shows for the first time the presence of TRPV2 and TRPM8 in PSN in the human skin. TRPV2 and TRPM8 as well as TRPV1 immunoreactive NF are present in unmyelinated NF in epidermis and papillary dermis, in nerve bundles, and around blood vessels and hair follicles. In contrast to TRPV1, TRPV2 and TRPM8 are found mainly in the papillary dermis and seem to be restricted to peptidergic NF, of which the majority contains the sensory neuropeptides CGRP or SP. There is a substantial loss of NF containing TRPV1, TRPV2 and TRPM8 in skin from individuals with Norrbottnian congenital insensitivity to pain. Insight into the role of TRPV2 and TRPM8 in human skin may open new avenues for treatment of neuropathic pain and inflammatory skin diseases.

Key words: allicin, calcitonin gene-related peptide, cannabinoids, capsaicin, congenital insensitivity to pain, garlic, immunohistochemistry, nociceptors, pain, skin, TRPA1, TRPM8, TRPV1, TRPV2

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Till Mamma, Pappa och Brorsan
Nu är jag fri som en fågel och kan ge mig ut på nya äventyr!

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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, referred to by their Roman numerals:

I. Helena E. Axelsson, Magnus Korsgren, Peter Ekström, Edward D. Högestätt, Peter M. Zygmunt. *Neuronal expression of the cannabinoid CB1 receptor and TRPV1 in rodent hindpaw skin and mesenteric artery.* (Submitted 2006)

II. Diana M. Bautista, Pouya Movahed, Andrew Hinman, Helena E. Axelsson, Olov Sterner, Edward D. Högestätt, David Julius, Sven-Eric Jordt, and Peter M. Zygmunt. *Pungent products from garlic activate the sensory ion channel TRPA1*. Proc Natl Acad Sci U S A, 102:12248-52, 2005.

III. Helena E. Axelsson, Jan K. Minde, Andreas Sonesson, Göran Toolanen, Edward D. Högestätt, Peter M. Zygmunt. *TRPM8, TRPV1 and TRPV2 immunoreactive nerve fibres in human skin and their disappearance in Norrbottnian congenital insensitivity to pain.* (Manuscript)

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ABBREVIATIONS

AITC	Allyl isothiocyanate
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
DAB	Diaminobenzidine
DADS	Diallyl disulfide
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
ED1	Ectodermal dysplasia
HEK	Human Embryonic Kidney
HRP	Horseradish peroxidase
5-HT	Serotonin
IB4	Isolectin-B4
NF200	Neurofilament 200 kD
NGFβ	Nerve growth factor beta
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PGP	Protein gene product 9.5
PNS	Peripheral nervous system
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rpHβ	Rat prolyl 4-hydroxylase
SP	Substance P
TG	Trigeminal ganglia
TH	Tyrosine hydroxylase
Δ^9-THC	Δ^9-tetrahydrocannabinol
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPC	A subgroup of the TRP ion channel family called “Canonical”
TRPM8	Transient receptor potential melastatin 8
TRPML	A subgroup of the TRP ion channel family called “Mucolipins”
TRPP	A subgroup of the TRP ion channel family called “Polycystin”
TRPV1	Transient receptor potential vanilloid 1
TRPV2	Transient receptor potential vanilloid 2
VIP	Vasoactive intestinal peptide

ABSTRACT

It is frequently argued that cannabinoids exert part of their analgesic and anti-inflammatory effects via activation of the cannabinoid CB1 receptor located on TRPV1-expressing primary sensory nerve fibres in peripheral tissues. However, we find no evidence of CB1 receptor immunoreactivity on nerve fibres in rat or mouse hindpaw skin and mesenteric artery. The CB1 receptor agonists anandamide and HU210 also fail to inhibit TRPV1-mediated calcitonin gene-related peptide (CGRP) release from primary sensory neurons in rat hindpaw skin and mesenteric artery. Therefore, this study do not support the general view that the analgesic and anti-inflammatory effects of CB1 receptor agonists are due to direct inhibition of TRPV1-expressing primary sensory nerve terminals in the periphery. Garlic contains a number of organosulphur compounds, including allicin and diallyl disulfide (DADS), some of which may contribute to its pungent and vasodilator properties. Our results show that raw garlic extract, allicin and DADS activate TRPA1 ion channels on primary sensory neurons in culture and nerve fibres in the vascular system. These findings highlight TRPA1 as a novel ion channel in the vascular system and provide novel pharmacological tools for investigating the role of this ion channel. Whether activation of TRPA1 in the vascular system explains the beneficial antihypertensive effect observed by garlic treatment remains to be shown. This study also expands our understanding of how TRPA1 is regulated on a molecular basis, which is of importance for development of novel drug therapies for pain, inflammation and vascular disease. The skin is a major sensory organ that contains a large number of nerves. The TRP ion channels TRPV2 and TRPM8 are expressed in the somatosensory nervous system in animals and are therefore likely to be expressed in humans as well. Fluorescence immunohistochemistry was used to identify these channels and compare their expression and distribution patterns with known neuronal markers of the sensory nervous system in skin from healthy volunteers and from individuals with a mutation in the gene encoding nerve growth factor beta (NGFβ) that causes Norrbottnian congenital insensitivity to pain. This study shows for the first time the presence of TRPV2 and TRPM8 in sensory nerves in the human skin. TRPV2 and TRPM8 as well as TRPV1 immunoreactive nerve fibres are present in unmyelinated nerve fibres in epidermis and papillary dermis, in nerve bundles, and around blood vessels and hair follicles. In contrast to TRPV1, TRPV2 and TRPM8 are found mainly in the papillary dermis and seem to be restricted to peptidergic nerve fibres, of which the majority contains the sensory neuropeptides CGRP or SP. There is a substantial loss of nerve fibres containing TRPV1, TRPV2 and TRPM8 in skin from individuals with Norrbottnian congenital insensitivity to pain. Insight into the role of TRPV2 and TRPM8 in human skin may open new avenues for treatment of neuropathic pain and inflammatory skin diseases.

INTRODUCTION

The bodily senses

The human body receives sensory information from its internal and external environment with the help of the visual, auditory, vestibular, somatosensory, gustatory and olfactory systems. A sensation is yielded upon a physical stimulus that is transformed (stimulus transduction) into nerve impulses. Although the senses differ in their modes of reception (light, sound, gravity, pressure, displacement, chemical, thermal or mechanical) they all convey four basic types of information when stimulated and hence have a similar organization. *Modality*, e.g. temperature, sound or pressure, defines a general class of stimulus, determined by the type of energy transmitted by the stimulus and the receptors specialized to sense that energy. The type of receptor activated by a stimulus plays the primary role in coding the stimulus modality. In each sensory system the initial contact with environment occurs through specialized neuronal structures called sensory receptors. The sensory receptor is the first cell in each sensory pathway that transforms stimulus energy to electrical energy. Each nerve fibre is primarily activated by a certain type of stimulus. Each class of sensory receptors makes connections with distinctive structures in the central nervous system which represent the six sensory systems. Humans have four classes of receptors, each of which is primarily sensitive to one form of physical energy; mechanical, chemical, thermal and electromagnetic. The mechanoreceptors of the somatosensory system mediate the sense of touch, proprioceptive sensations, and the sense of joint position, whereas the mechanoreceptors of the inner ear mediate hearing and the sense of balance. Chemoreceptors are involved in the senses of pain, itch, taste and smell. Thermoreceptors in the skin sense the body temperature and also the temperature of the ambient air and the objects that we touch. Humans possess only one type of receptor for electromagnetic energy; the photoreceptors in the retina. Each of the major modalities has several submodalities, e.g. taste can be sweet, sour, bitter or salty, because each sensory receptor class contains a variety of specialized receptors that respond to a limited range of stimulus energy. The *location* of the stimulus is represented by the set of sensory receptors within the sensory system that are active. The precision with which one stimulus can be located and differentiated from an adjacent one depends on the amount of convergence of neuronal input in the specific ascending pathway; the greater the convergence, the less the precision. Other factors affecting the precision are the size of the receptive field covered by a single neuron and the amount of overlap of nearby receptive fields.

The *intensity* of the stimulus is signalled by the response amplitude of each receptor, which reflects the total amount of stimulus energy delivered to the receptor. The electrical signal produced by the receptor is called the receptor potential and its amplitude and duration are related to the intensity and time course of stimulation. Hence, the intensity or amount of a sensation depends on the strength of the stimulus. The *timing* of stimulation is defined by when the response in the receptor starts and stops and is determined by how quickly the energy is received or lost by the receptor. The intensity and timing determines the firing pattern of an active sensory neuron. Taken together, all sensory systems respond to four elementary features of stimuli; modality, location, intensity and duration[1]. In this thesis, I have focused on the somatosensory system which will be further discussed.

Somatosensory system

Compared to the other sensory systems, the somatosensory system is much more complex and is least understood. The somatosensory system has four modalities; discriminative touch, proprioception, nociception and temperature sense. Discriminative touch and limb proprioception depend on encapsulated mechanoreceptors sensitive to physical deformation of the skin, stretch, or contraction of muscles, or the angle of individual joints. The sense of temperature is mediated by the bare endings of thinly myelinated or unmyelinated nerves sensitive to specific ranges of thermal energy. Separate classes of thermal receptors sense temperatures that are perceived as cold, cool, warm or hot. Painful sensations are mediated by free nerve endings, called nociceptors, which sense destructive mechanical stimuli, extreme temperatures and chemical substances released from cells as a result of tissue damage. The four modalities are conveyed in separate ascending pathways to the brain, at least in the early stages of information processing[1].

Primary sensory neurons

Primary sensory neurons are the sensory receptors of the somatosensory system. The cell bodies of these nerves are collected in sensory ganglia, such as the dorsal root ganglia (DRG) located along the spinal cord, and the nodose and trigeminal ganglia (TG) located in the neck and head region. The primary sensory neuron has as single process close to the cell body that divides into two branches forming a T-shape, with one branch extending to the periphery and the other to the spinal cord. This construction of a nerve is called pseudounipolar and is specific for the sensory neurons[2] (Fig. 1).

The primary afferent neuron have two functions; one is to convey sensory information from the periphery to the central nervous system (afferent function), and one is to release neurotransmitters from their peripheral ends (efferent function). These aspects will be further considered below.

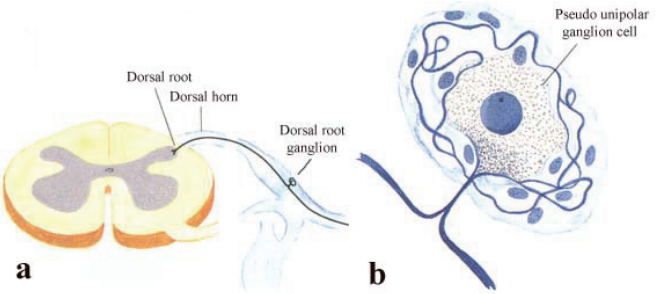

Fig. 1. (a) The dorsal horn with the dorsal root containing accumulations of sensory neurons. (b) The cell body, *soma*, of a primary sensory neuron, has two axons leaving to the peripheral and central parts of the body.

Pain is an afferent function of primary sensory neurons

The sensation of pain is a result of the activation of any of the three major classes of nociceptors called thermal, mechanical and polymodal nociceptors. The term nociceptor comes from the Latin word *noci*, meaning hurt[3] and was introduced by Sherrington[4], who defined nociceptors as those primary afferent neurones that can be activated by harmful or potentially harmful stimuli and then give rise to the sensation of pain[1,5]. Three classes of nociceptors can be distinguished on the basis of the type of stimulus upon which they are activated. Thermal nociceptors are thinly myelinated Aδ-fibres with a conduction velocity of ~ 20 m/s and are excited by extreme temperatures, ≥ 45 °C or ≤ 5 °C, as well as by strong mechanical stimuli. Mechanical nociceptors require strong tactile stimuli applied to the skin, which mediates sensations of sharp or pricking pain, and like the thermal nociceptors, their signals are conducted by thinly myelinated Aδ-fibres[1,6,7]. The last of the three nociceptors: the polymodals comprise the largest type and respond to a variety of destructive mechanical, thermal (both hot and cold) and chemical stimuli. These receptors are insensitive to gentle mechanical stimuli. Stimulation of these receptors in humans evokes sensations of slow, burning pain. Their signals are conducted by non-myelinated C-fibres of small diameter; the lack of myelin sheet reduces the conduction velocity to ~ 2 m/s.

All three classes of nociceptors are widely distributed in the skin and deep tissues where they often work together. They all have one common feature, they only respond to noxious stimuli[1,6-8].

Ascending pathways

Nociceptive afferent fibres of Aδ and C type terminate in the dorsal horn of the spinal cord. They are found particularly in the superficial dorsal horn, in the marginal layer (lamina I) and the substantia gelatinosa (lamina II), and to a minor extent, in the six other layers (lamina III-VIII). The major excitatory neurotransmitters, released by the central nerve endings of Aδ and C fibres, are the amino acid glutamate and neuropeptides. The release of glutamate gives fast synaptic potentials in dorsal horn neurons by acting on the AMPA (α-amino-3-hydroxy-5-methyl-isoxazole) type glutamate receptor, while neuropeptides elicit slow excitatory postsynaptic potentials[1,9,10]. These two types of neurotransmitters are released together from central terminals in spite of their distinct physiological actions on postsynaptic neurons[1]. The signals carrying information about noxious stimuli from the central neurons in the spinal cord are further sent to the brain through one of the five major ascending pathways (the spinothalamic, spinoreticular, spinomesencephalic, cervicothalamic and spinohypothalamic tracts). In the target area of the brain the nociceptive information is "translated" into an unpleasant warning signal[1].

Descending pathways

Except for the five ascending pathways there are also descending pathways with the purpose to inhibit neurotransmission and sensation of pain. In the spinal cord these descending pathways inhibit nociceptive neurons through direct connections or through inhibitory interneurons releasing enkephalins, endorphins and dynorphins, all of which are neuropeptides of the endogenous opioid system[1,11].

Efferent release of neurotransmitters from primary sensory neurons can result in sensitization and inflammation

Activation of the nociceptors by the different inflammatory mediators leads to a local release of substance P and calcitonin gene-related peptide (CGRP). They are synthesized in the cellbodies of such neurons and transported to the peripheral terminals, where they are stored and released upon depolarization of the terminals. Both substance P and CGRP act on mast cells in the vicinity of the injury and evoke degranulation with a following release of

histamine, which in turn directly acts on the nociceptors. The release of substance P and CGRP also causes vasodilation[1,12,13]. These events are followed by a local inflammation of the tissue. The three signs of inflammation are heat, redness and swelling. The two first symptoms are results of the dilation of peripheral blood vessels, whereas the last one is a result of plasma extravasation, a process where proteins and cells leak out of postcapillary venules[1,14]. The purpose of the complex cascade of chemical signalling that arises after tissue injury is not only to protect the injured area but also to promote healing and guard against infection[3].

Upon tissue injury and inflammation there are many inflammatory mediators that together enhance the sensation of pain. The phenomenon hyperalgesia is defined as an increased responsiveness to noxious stimulation in an area of damaged tissues. This is due to sensitization of nociceptors in the area of or nearby the damaged tissue[3,15]. The sensitization of nociceptors results from the release of a variety of chemicals by the damaged cells and tissues[1,15]. Some of the inflammatory mediators involved are bradykinin, serotonin (5-HT), histamine, potassium ions, acids, leukotrienes, prostaglandins, substance P and acetylcholine[15]. These pain-producing substances originate from different cells, but they all decrease the threshold for nociceptive activation. For examples bradykinin, can influence the activation in two ways, either by direct activation of the nociceptors or indirectly by stimulating the enzyme phospholipase A_2, giving rise to nociceptive prostaglandins, which in turn can activate the nociceptors directly. 5-HT and histamine have like bradykinin a direct activating effect on the nociceptors[1].

The molecular mechanism underlying nociception

The mechanism by which noxious stimuli depolarize free sensory nerve endings (nociceptors) and generate action potentials is only partly understood. The membrane of peripheral nociceptive neurons contains proteins such as ion channels that convert the thermal, mechanical or chemical energy of noxious stimuli into a depolarizing electric potential[1,16,17]. One group of ion channels that have received much attention in this regard is the transient receptor potential (TRP) ion channel family.

The TRP ion channel family

The transient receptor potential (TRP) ion channel family is composed of six mammalian members; TRPC, TRPV, TRPM, and TRPA, and the distantly related TRPP and TRPML[18-20]. It is a heterogeneous family of ion channels in the sense of activating stimuli, function and location within the body. However, they share some common features. All mammalian TRP channels are predicted to contain six transmembrane polypeptide sub-units that assemble as homo or hetero-tetramers to form nonselective cation-permeable channels. The pore of the channel is formed by the fifth and sixth transmembrane sub-units of the tetramer[21,22]. Functional properties common to many TRPs are their capacity to function as cellular sensors[19]. Most cells do express a number of different TRP ion channels and splice variants[19].

The discovery of a transient receptor potential (TRP) in response to light instead of a sustained response in a *Drosophila* mutant photoreceptor cell, made the foundation to explore a new family of ion channels[23,24]. The first mammalian TRP channel to be discovered was TRPC1[25] (canonical TRP). Of the mammalian TRP channels, members of the TRPC subfamily are the most closely related to *Drosophila* TRP. All seven members of the TRPC family share an invariant sequence in the C-terminal tail, called a TRP box, as well as 3-4 N-terminal ankyrin repeats[19,22]. In general, the TRPC channels are broadly expressed and are found as heteromultimers[21]. The TRPV family comprises six members of which TRPV1-V4 act as thermosensors[26-29]. TRPV5 and TRPV6 are highly expressed in the kidney and intestine, respectively, were they are essential for calcium ion reabsorbation[30,31]. All members of this family contain 3-5 N-terminal ankyrin repeats. TRPV1, the founding member of this group, was cloned a decade ago and have since then been extensively studied[26]. TRPM (melastatin) was initially identified as a transcript in metastatic melanoma cells[32] and contain eight members, none of which contains ankyrin repeats. One of the members, TRPM8 is over-expressed in organ-confined prostate cancer and therefore may have a role in the development from an organ-confined state to a metastatic state. This makes TRPM8 to a potential diagnostic tissue marker and prognostic indicator for the progress of prostate cancer[33]. The most recent member to take part in the TRP ion channel family is TRPA1 (ankyrin). It is distinguished by its 14 amino terminal ankyrin repeats and has been suggested as a target for noxious cold[34] and natural irritants[35] and to function as a mechanosensitive channel in the hair cells of the inner ear[36].

TRPP (polycystin) is comprised of five polycystic kidney disease 1 proteins; PKD1 (TRPP1), PKDREJ, PKD1L1, PKD1L2 and PKD1L3 and three polycystic kidney disease 2 proteins;

PKD2 (TRPP2), PKD2L1 (TRPP3) and PKD2L2 (TRPP5)[37]. TRPP1 and TRPP2 form complexes and then function as a non-selective calcium ion-permeable channel[38]. Mutations in these proteins cause the autosomal dominant polycystic kidney disease[39-41]. TRPML (mucolipin) is comprised of the mucolipins, MCOLN1 (TRPML1), MCOLN2 (TRPML2) and MCOLN3 (TRPML3), which are probably restricted to intracellular vesicles. Mutations in TRPML1 are associated with mucolipidosis type IV, a neurodegenerative lysosomal storage disorder[42]. TRPML3 is present in the cytoplasm of hair cells and the plasma membrane of stereocilia and a deletion of this ion channel in mouse results in deafness[43]. Several members of the TRP ion channel family have been shown to take part in the sensory systems, including the visual (Drosophila TRPs), olfactory (TRPC2), gustatory (TRPM5), auditory (TRPML3) and the somatosensory (TRPV1-V4, TRPM8 and TRPA1) systems[44]. In this thesis, four of these TRP ion channels have been studied and will be described in more detail below.

TRPV1

A decade ago, the first member of the TRPV family, TRPV1 (formerly called capsaicin or vanilloid receptor), was cloned by Caterina et al. (1997)[26]. TRPV1 responds to several stimuli and among them are noxious heat, low extracellular pH and chemical stimuli such as capsaicin, the active ingredient in hot peppers, and other related vanilloids[5]. TRPV1 is found mainly in primary nociceptive neurons and mediates the pain-producing actions of capsaicin, heat and protons, classifying TRPV1 as a polymodal nociceptor[1,14].

Localization of TRPV1

As mentioned above, TRPV1 is localized mainly on membranes of primary nociceptive neurons. It is through different studies including autoradiography and binding studies with resiniferatoxin that it has been possible to partly determine the anatomical localization and tissue specificity of TRPV1[5,45-47]. The first studies showed that TRPV1 are expressed along the entire length of primary nociceptive neurons, from the peripheral terminals to the central endings in the dorsal horn. Of the primary sensory neurons it is primarily the small diameter C-fibres and the thinly myelinated Aδ-fibres that express TRPV1[5,16]. Later it was shown that also vagal (nodose ganglion) neurons and certain brain areas, not associated with primary sensory neurons, express TRPV1[48,49]. Furthermore, capsaicin has always been regarded as “a remarkably selective tool for primary sensory neurons”, but it is now clear that not all actions of capsaicin can be referred to these neurons as TRPV1 seems to be localized in brain and non-neural tissues[50,51].

Activation of TRPV1

Among the many stimuli that activate TRPV1 on primary sensory neurons, low extracellular pH and noxious heat have been proposed as physiological activators of TRPV1[15,49,52]. Low extracellular pH or local tissue acidosis frequently occurs during inflammation and ischaemic conditions and is often also accompanied by pain. Normally a reduction in pH leads to a decrease in the capacity of the neurons to fire, but this is not the case for nociceptive neurons. Instead, they usually show ongoing spike activity when they are exposed to an acidic environment[16].

Chemical irritants are also activators of TRPV1. Capsaicin, the main ingredient in hot chilli peppers[53], played a key role in the cloning of TRPV1 and was first isolated in 1846[54]. The time passed to 1919 before the exact chemical structure of capsaicin was determined[55] and another decade had to pass before capsaicin for the first time was synthesized. Capsaicin has been used for a long time around the world for three main reasons: as a spice for enhancing the taste of food, to protect people against invaders by temporarily blinding them (pepper spray) and for treatment of pain (analgesic purposes)[50]. Other newly discovered vanilloids include the ultrapotent capsaicin analogue resiniferatoxin, which was first isolated from the cactus-like plant *Euphorbia resinifera*, the fungal terpenoid, isovelleral and scutigeral[52,56].

Effects due to activation of TRPV1

The activation of TRPV1 on primary nociceptive neurons by either extracellular pH, noxious heat or chemical stimuli leads to an increased intracellular concentration of, mainly calcium and sodium ions [50] (Fig. 2). The TRPV1 is designated as a non-selective cation channel with a slight selectivity for calcium ions[26,49]. The increased concentrations of calcium ions in primary nociceptive nerve endings result in the release of neurotransmitters, mostly CGRP and substance P, which in turn give rise to the various local effects, mentioned above[50].

Activation of TRPV1 can cause pain as well as analgesia, the latter possibly as a result of desensitization of the primary sensory neuron. Thus, both agonists and antagonists of TRPV1 may be useful as therapeutic agents for treatment pain and inflammation[50,57-59].

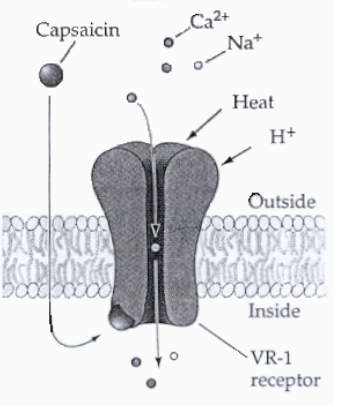

Fig. 2. TRPV1 has multiple activation alternatives, such as protons (H^+), heat and capsaicin. Activation leads to an increased intracellular concentration of ions, mainly calcium ions.

Drug-induced antinociception

The main purpose of the body's capacity to feel pain is to protect it from injury. If this helping system for some reason does not work, an innocent injury that normally should have been taken care of could lead to a life-threatening tissue damage. Alternatively the injury could lead to chronic pain. There are mainly two groups of drugs involved in the alleviation of pain. Aspirin-like drugs that block prostaglandin production by inhibiting cyclooxygenases and narcotic analgesics, for instance morphine that activates opioid receptors. These drugs give rise to a number of serious side effects[5]. None of the currently available drugs are known to block nociceptor-specific receptors or enzymes. Putative receptors of thermal, mechanical and chemical stimuli on nociceptors are promising targets for a new group of analgesics, possibly with fewer side effects. One mechanism by which an analgesic and anti-inflammatory drug could work is through inhibition of TRPV1 either directly by binding to TRPV1 as an antagonist, or indirectly by activating an inhibitory receptor signalling pathway on TRPV1 containing nerves. This latter mechanism is believed to contribute to the analgesic and anti-inflammatory effects of CB1 receptor agonists[60-63]. Hence, the cannabinoid system is of great interest in pain research[5].

The endocannabinoid system - CB1 and CB2 receptors

Cannabis is one of the oldest plant-derived recreational and therapeutic drugs used in human history. Extracts from the cannabis plant, *Cannabis sativa*, contain several related compounds named cannabinoids, most of which are extremely lipophilic. It was first in 1967 that the structure of Δ^9-tetrahydrocannabinol (Δ^9-THC) was determined[64]. Two cannabinoid receptors, CB1 and CB2 have so far been identified and cloned[65] (CB1 in 1990 and CB2 in 1993). Both receptors belong to the family of G-protein-coupled receptors, linked to inhibition of adenylate cyclase. They are also coupled to potassium channel activation and calcium channel inhibition, and thereby exert an inhibitory effect on the release of neurotransmitters, for instance CGRP and substanse P. These cellular effects resemble those of opioids on primary sensory neurons[65].

Localization of CB receptors

CB1 receptors are found in particularly high concentrations within the central nervous system (CNS) and to less extent in the peripheral nervous system (PNS) and in certain non-neuronal tissues. CB2 receptors occur mainly in immune cells where they mediate immunosuppressant effects. The identification of these cannabinoid receptors was followed by the discovery of endogenous cannabinoids, such as anandamide, which has been shown to function as both a neuromodulator and neurotransmitter[65,66].

The role of cannabinoids in antinociception

Much research on the antinociceptive properties of cannabinoids has been performed with the four main classes of cannabinoid receptor agonists. These are the "classical" cannabinoid Δ^9-THC, the "nonclassical" cannabinoid CP55940, the aminoalkylindole WIN55212 and the "eicosanoid" cannabinoid anandamide. Antinociceptive activity of cannabinoid receptor agonists has been tested in a wide range of animal pain models and there is no doubt that cannabinoids induce antinociception, at least in part through the activation of CB1 receptors[65]. It is mainly the CB1 receptors in the central nervous system (CNS) that seems to be important in antinociception, but it can not be excluded that a part of this effect is due to activation of peripheral CB1 receptors, although they are not as numerous as central CB1 receptors. In the periphery, CB1 receptors are localized in discrete regions, such as nerve terminals that form only a small part of the total tissue mass. Several studies have shown that CB1 receptors are found in a number of different regions within the peripheral nervous system. Among them are the dorsal roots, the dorsal root ganglia and the peripheral terminals of primary sensory

neurons[65]. Cannabinoid receptors and their endogenous ligands together constitute the endocannabinoid system, such as vanilloid receptors and their ligands constitute the vanilloid system (see below).

Anandamide - an endogenous cannabinoid and/or vanilloid?

When screening for endogenous cannabinoid receptor ligands in pig brain, Devane et al. (1992)[67] discovered anandamide – the first member of a group called endocannabinoids. Anandamide can cause inhibitory effects on sensory nerves by acting as an agonist at the CB1 receptor, which in turns activates adenylate cyclase and inhibits voltage-activated calcium channels. It is produced on demand from phospholipid precursors in various organs and cell types, including neurons, macrophages and endothelial cells. Inactivation of anandamide is brought about by cellular reuptake and enzymatic hydrolysis by fatty acid amidohydrolase (FAAH)[66]. Yet, there is no final proof of anandamide as a physiological important compound acting at cannabinoid receptors. In 1999, Zygmunt et al. discovered that anandamide acts on vanilloid receptors (TRPV1)[68-70]. In their study anandamide could activate TRPV1 on primary sensory neurons in arteries and in cell systems expressing the cloned TRPV1. Since this discovery, there has been a heated debate whether anandamide should be regarded as an endogenous ligand of the cannabinoid or the vanilloid receptor system[68-70].

Co-localization of TRPV1 and CB1 receptors

Both TRPV1 and CB1 receptors are localised on primary sensory neurons, but whether they are localized on the same neurons is controversial. TRPV1 is postulated to occur most commonly on small and medium size DRG neurons[26,48,71], while CB1 receptors are supposed to be present in large neurons[72]. Co-expression studies have shown conflicting results. For instance, one study showed that TRPV1 and CB1 receptors are co-expressed to a very high degree, while another study showed the opposite[72,73].

In favor of the presence of CB1 receptors at the peripheral terminals of primary sensory neurons is the observation that anandamide inhibits capsaicin-induced CGRP release in rat hindpaw skin[61]. However, whether more selective CB1 receptor agonists could mimic the effect of anandamide was not examined in this study. Furthermore, there is no immunohistochemical evidence that CB1 receptors are present on such nerves in the periphery.

TRPV2

The transient receptor potential vanilloid 2 (TRPV2) was discovered in 1999 by Caterina et al[27,74-81]. Although it is structurally related to TRPV1 (50 % sequence homology), it is not a target for capsaicin, protons or heat (43 ºC), but is instead activated by noxious heat with a threshold temperature of 52 ºC[16]. Currents mediated by TRPV2 show that it has the general properties of a non-selective cation channel. Numerous studies have shown that TRPV2 are found in rat and mouse sensory ganglia[27,74-81] and in the mammalian retina[82]. Within dorsal root and trigeminal ganglia, TRPV2 are expressed by medium to large neurons[27,74,83,84]. Studies have shown that TRPV2 is co-localized with CGRP[27,74,85] and NF200[27,83,86], and to a lesser degree with substance P (SP) and isolectin-B4 (IB4)[27], but not with TRPV1[27,77,83]. However, a few studies have reported a minor co-localization of TRPV2 and TRPV1 in sensory neurons[76,78]. TRPV2 is not restricted to peripheral sensory neurons but are also expressed in various other tissues including autonomic ganglia, larynx, lung, spleen, intestine, spinal cord and brain[27,83,86-92]. In these tissues TRPV2 may be activated by stimuli other than noxious heat, since 52 ºC is not a physiologically relevant temperature inside the body. Furthermore, the wide distribution of TRPV2 indicates that this ion channel may have other functions than to detect noxious heat. TRPV2 may function as a stretch sensor in vascular smooth muscles[93] and may have a role in sympathetically-mediated neuropathic pain and inflammatory hyperalgesia to noxious high temperature stimuli[94,95]. Co-localization of TRPV2 and calbindin D-28K, a protein associated with muscle spindles, may also indicate other functions than heat nociception. Understanding the distribution and function of TRPV2 in humans may result in novel drug therapies for treatment of pain, inflammation and disorders due to sensory nerve hyper reactivity.

TRPM8

In search for new prostate-specific tumor markers, a gene was discovered, termed trp-p8 (later called TRPM8), which resembles those for TRP ion channels[96]. mRNA transcripts of trp-p8 was detected in normal human prostate and testis, and in several cancer cell lines, but was not detected in tissues such as skeletal muscle, gastrointestinal tract, bladder, heart, lung, kidney or brain[96]. One year later, TRPM8 was cloned from mouse dorsal root ganglia[97] and rat trigeminal ganglia[98], and by the use of *in situ* hybridization TRPM8 was localized to small diameter neurons[97,98]. This is supported by studies showing TRPM8 in small DRG neurons of guinea pig and in TG neurons of rat[99,100]. Further analysis have demonstrated that TRPM8 is localized to neurons also expressing tyrosine kinase A (TrkA), but not NF200, TRPV1, CGRP or IB4, which indicates that TRPM8 is found on small nociceptive neurons distinct from the well defined heat nociceptor expressing TRPV1, CGRP and IB4[97,101]. However, other studies have reported co-localization of TRPM8 and TRPV1 on sensory neurons or a population of neurons sensitive for both menthol, cold and capsaicin[98,100,102-104]. These differences in expression between TRPM8 and TRPV1 could be explained by up-regulation of TRPV1 by the influence of NGF under culture conditions[34]. Furthermore, TRPM8 has also been detected on nerve fibres in rat tongue papillae[100] and in the urothelium and in nerves fibres of human urinary bladder[105]. TRPM8 is able to detect cold temperatures, with a threshold temperature of 23 ºC, and is activated by the cooling compounds menthol, icillin and eucalyptol[97,98,106]. Since this discovery of TRPM8, many have tried to disclose the mechanisms behind cold sensation in mammals, where menthol has been used as a tool for identification of cold-sensing neurons. Two main subpopulations have been detected, one menthol-sensitive and one menthol-insensitive, but further studies have shown that the pattern of cold-sensitive neurons in sensory ganglia is more complex and likely involves additional subgroups[103,107-110]. Recent studies indicate that TRPM8 can be a target for treating pain associated with tooth ache, overactive and painful urinary bladders as well as states of chronic pain[105,111,112].

TRPA1

TRPA1 (initially called p120 and ANKTM1) was first described when isolated from human cultured fibroblasts that had undergone oncogenic transformation, in a screen for down-regulated genes[113]. The topology of this novel TRP ion channel resembles that of other TRP ion channels, having six transmembrane units, but TRPA1 contains far more ankyrin repeats in the N-terminal region (14 altogether). Patch-clamp recordings indicate that TRPA1 is a non-selective cation channel, similar to many previously described TRP channels[34,35]. Story et al., (2003) showed that TRPA1 is activated by noxious cold with a temperature threshold of 17.5 ºC. Importantly, Jordt et al., (2004) identified TRPA1 as a sensor of chemical irritants in the somatosensory nervous system. TRPA1 is not only a target for natural irritants such as isothiocyanates, found in mustard oil and wasabi, and cinnamaldehyde but also by phospholipase C activation with e.g., bradykinin[35,114], an endogenous potent algogenic substance released at tissue injury and inflammation[115]. Furthermore, TRPA1 has also been suggested as a mechanosensitive channel in hearing, where the 14 N-terminal ankyrin repeats may form the gating spring of the hair cells[36,116]. However, the role of TRPA1 as a detector of noxious cold and as a mechanosensor is a matter of debate[35,116].

TRPA1 are expressed by sensory ganglia of mouse and rat as shown by *in situ* hybridization and immunohistochemistry[34,35,116]. TRPA1 is found exclusively in TRPV1 expressing neurons and many also contain the sensory neuropeptides CGRP or substance P[34,35], but are not found in NF200 or TRPM8 expressing neurons[34]. In addition to sensory ganglia, TRPA1 is found on nerve fibres in mouse urinary bladder and cornea[116]. Several studies have failed to detect mRNA transcripts of TRPA1 in tissues such as, heart, lung, skeletal muscle, kidney, liver and brain, indicating that this TRP ion channel is not as widely distributed as TRPV2 and TRPV1, making it an even more specific drug target than TRPV1 for treatment of pain, inflammation and disorders due to sensory nerve hyperreactivity.

AIMS OF THE THESIS

I. To challenge the general view that cannabinoids exert their analgesic and anti-inflammatory effects via the cannabinoid CB1 receptor located to TRPV1 expressing primary afferent nerves in peripheral tissues. No immunohistochemical evidence has been published in support of this. Therefore, immunohistochemistry in combination with functional experiments were performed to study the role of the CB1 receptor in sensory neurotransmission in the rat hindpaw skin and mesenteric artery. These tissues are frequently used to study pain and inflammation including sensory nerve-dependent vascular responses.

II. To understand the molecular basis of garlic's somatosensory actions, which contribute to its pungent and vasodilator properties. Using various imaging techniques, the pharmacology of garlic extract and its derivatives allicin and DADS were characterized in cultured rat trigeminal sensory neurons, rat mesenteric arteries and cells heterologously expressing TRPA1. The imunohistochemical localization of TRPA1 in rodent sensory ganglia and blood vessels was also examined.

III. To investigate the possibility that the TRP ion channels TRPV2 and TRPM8, which are expressed in the somatosensory nervous system in animals, exist in human skin. Fluorescence immunohistochemistry was used to identify these channels and compare their expression and distribution patterns with known neuronal markers of the sensory nervous system in forearm skin from healthy volunteers and from individuals with a mutation in the gene encoding nerve growth factor beta (NGFβ) that causes Norrbottnian congenital insensitivity to pain.

MATERIALS AND METHODS

Animals and tissues

The cerebellum (Fig. 3), hindpaw skin (Fig. 3) and mesenteric arteries (Fig. 3) were collected from female Wistar-Hannover rats (200-250 g; Scanbur BK, Sollentuna, Sweden) or female $CB1^{-/-}$ and $CB1^{+/+}$ mice[117] (Paper I). Dorsal root ganglia (Fig. 3) and mesenteric arteries were collected from female Wistar-Hannover rats (200-250 g; Scanbur BK, Sollentuna, Sweden), and trigeminal ganglia were dissected from newborn Sprague Dawley rats or from $TRPV1^{-/-}$ and $TRPV1^{+/+}$ mice. Cultured HEK293t cells and oocytes were used for calcium imaging and patch-clamp recordings (Paper II). Punch biopsies (3 mm in diameter) were collected under local anaesthesia from the lateral upper arm 10 cm below the lateral margin of the acromion of three healthy volunteers and of two homozygous and three heterozygous individuals, carrying the NGFβ gene mutation for Norrbottnian congenital insensitivity to pain (Paper III).

Fluorescence immunohistochemistry (Paper I-III)

Immunohistochemistry is a technique that makes it possible to visualize proteins in cells or tissues by using antibodies against a target protein (Fig. 4). Indirect fluorescence immunohistochemistry involves a primary antibody raised against a specific antigen and a secondary antibody coupled with a fluorochrome, that together form an antigen-antibody complex. When a cell or tissue containing such complexes is radiated with UV-light, the target protein can be detected by a fluorescence microscope as a coloured product. In this thesis, immunohistochemistry has been used as a tool to localize the cannabinoid CB1 receptor and TRP ion channels in various tissues.

The dorsal root ganglia, hindpaw skin and mesenteric artery from rat were fixed in a PBS solution containing paraformaldehyde (4 %) at 4 °C for 1 hour, 4 hours and 30 min, respectively. Biopsies from human skin were fixed in a Steffanini solution containing paraformaldehyde (2 %) and picric acid (0.2 %) in phosphate buffer solution (PBS), pH 7.2 at 4 °C for 24 hours. For cryoprotecting the tissues they were rinsed in a PBS solution containing sucrose (15 %) for two days with exchange to fresh solution twice a day. Fixed and cryoprotected tissues were mounted on cork plates with O.C.T. compound, frozen in iso-Pentane and stored at –70 °C. The frozen tissues were cut into sections by a cryostat (Leica CM 3050 S; Leica Microsystems, Wetzlar, Germany) at –20°C.

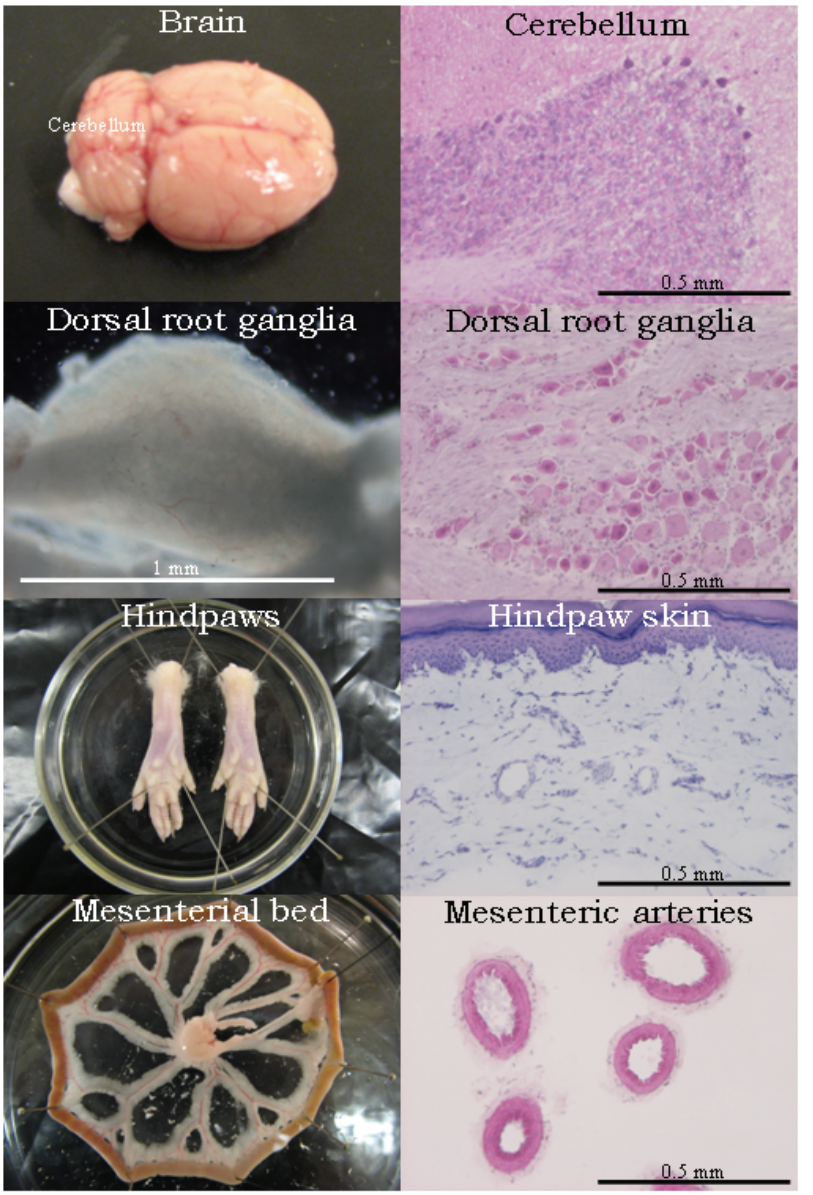

Fig. 3. Tissues used for fluorescence or enzymatic immunohistochemistry were dissected from rat (left panel) or mouse. The corresponding tissue sections stained with hematoxyline and eosine are shown in the right panel.

The sections were collected on chrome-alun coated (0.5 % gelatin and 0.05 % $KCr(SO_4)_2$) microscope slides and stored at –20°C.

Air dried tissue sections and whole mount preparations of rat mesenteric arteries, fixed in paraformaldehyde (4 %) at 4 °C for 30 min and washed with PBS for six times 10 min, were pre-incubated with a PBS solution containing Triton X-100 (0.2 %) and bovine serum albumin (0.1 %) for 2 hours, after which the tissues were incubated with the primary antibody, (Table 1) diluted in PBS containing Triton X-100 (0.2 %) and bovine serum albumin (0.1 %), over night at room temperature in a humid chamber. The tissues were washed from excess unbound primary antibodies with PBS for three times 10 min and then incubated with a fluorophore secondary antibody at room temperature, either Texas Red (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min, Fluorescein-isothiocyanate (Jackson ImmunoResearch Laboratories) for 1 hour or Alexa Fluor 488/555/568/594 (Molecular Probes, Eugene, OR) for 1 hour. The PBS wash was repeated before the tissues were dried and mounted with a PBS/glycerol solution. To reduce fading of FITC-conjugated sections, *p*-phenylenediamine was added to the mounting medium. When double immunohistochemistry was performed, the primary and secondary antibodies were incubated either separately or as a cocktail (Paper I). The primary antibodies were incubated as a cocktail over night and the secondary antibodies were incubated separately for 1 hour each with a PBS wash in between (Paper II and III).

The tissue sections were studied with a fluorescence microscope (Olympus Bx60F-3; Olympus America, Melville, NY) and images were acquired with a digital camera (Olympus DP50) attached to the microscope and analyzed with the software programs Studio Lite 1.0 and Viewfinder Lite 1.0 (Pixera Corporation, Los Gatos, CA) (Paper I and II). Confocal microscopy was performed on tissue sections and whole mount preparations of mesenteric artery with a confocal laser scanning microscope (Multiprobe 2001; Molecular Dynamics, Sunnyvale, CA). Auto focus and extended focus projections were made of the confocal section series with the software program ImageSpace 3.11 (Molecular Dynamics) (Paper I and II). Confocal microscopy was also performed with an Eclipse TE2000-S confocal laser scanning microscope (Nikon, Tokyo, Japan).

Table 1. Primary antibodies used in this thesis

Paper	Antibody	Host	Dilution	Source
I	CB1a	Rabbit	1:500	A gift from K. Mackie (Nyíri et al. 2005; last 15 AAs in C-terminus)
I	CB1b	Rabbit	1:250	A gift from K. Mackie (Hajos et al. 2000; entire C-terminus)
I	CB1c	Rabbit	1:250	A gift from K. Mackie (Twitchell et al. 1997; GST-CB1:1-77)
I	CB1d	Rabbit	1:250-1:500	Alexis, Lausen, Switzerland (210-197-1); Cayman Chemical, Ann Arbor, MI (101500)
I	CB1e	Rabbit	1:25	Biosource International, Camarillo, CA (44-310); Chemicon International, Hampshire, UK (AB9684)
I, II, III	CGRP	Guinea pig	1:8000, 1:180000	Euro-Diagnostica, Malmö, Sweden
I	ED1	Mouse	1:500	Serotec, Oxford, UK
I, III	NF200	Mouse	1:800, 1:8000	Sigma, St Louis, MO
I, III	PGP	Rabbit	1:2000	UltraClone, Isle of Wight, UK
I	rpHβ	Mouse	1:500	Medicorp, Montréal, Canada
III	SP	Guinea pig	1:32000	Euro-Diagnostica, Malmö, Sweden
III	TH	Mouse	1:1000	DiaSorin, Stillwater, MN
II	TRPA1	Rabbit	1:250, 1:1000	A gift from D. Julius
III	TRPM8	Rabbit	1:32000	Abcam, Camebridge, UK
I	TRPV1	Rabbit	1:100	EMD Biosciences, San Diego, CA
II	TRPV1	Goat	1:1000	Santa Cruz Biotechnology, Santa Cruz, CA
III	TRPV1	Rabbit	1:2000	Affinity BioReagents, Golden, CO
III	TRPV2	Rabbit	1:500	Chemicon International, Temecula, CA
III	VIP	Guinea pig	1:8000	Euro-Diagnostica, Malmö, Sweden

The primary antibodies ED1, NF200, rpHβ and TH are of monoclonal origin and the remaining of polyclonal origin. All antibodies are affinity purified. *Abbreviations are:* CB1; Cannabinoid CB1 receptor, CGRP; Calcitonin gene-related peptide, ED1; Ectodermal dysplasia, NF 200; Neurofilament 200 (kDa), PGP; Protein gene product 9.5, rpHβ; rat prolyl 4-hydroxylase, SP; Substance P, TH; Tyrosine hydroxylase, TRPA1; Transient receptor potential ankyrin 1, TRPM8; transient receptor potential melastatin 8, TRPV1; Transient receptor potential vanilloid 1, TRPV2; Transient receptor potential vanilloid 2, VIP; Vasoactive intestinal peptide.

Maximum projection images of the confocal section series were acquired with the software program EZ-C1 Gold version 3.0 (Nikon) (Paper III). Deconvolution microscopy was performed with a DMRA epifluorescence microscope (Leica Microsystems, Wetzlar, Germany), when immunofluorescence intensity was too weak to allow optimal confocal microscopy. Series of 10-bit grey-scale digital images were acquired with a C4742-95 CCD camera (Hamamatsu, Japan) attached to the microscope and the software program Openlab 3.5 (Improvision, Coventry, UK). The image series were deconvolved using a 3D adaptive blind deconvolution algorithm in AutoDeblur 9.0 (AutoQuant Imaging, Watervliet, NY). Then, the deconvolved image series were imported to ImageSpace 3.11 (Molecular Dynamics) and projections were obtained. For processing of all images, Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) was used (Paper I). When sections were analyzed for co-localization, each layer in the Z-stack was examined at both 2D and 3D levels (Paper I-III).

To check whether the secondary antibodies used caused any unspecific immunoreactivity, control experiments without the primary antibody was performed. The immunoreactivity found in tissue sections treated with the secondary antibody alone was considered as unspecific. Furthermore, to verify that the primary antibodies were bound to the synthetic peptide they were raised against, control experiments with blocking peptides were performed. The primary antibody was pre-incubated with or without its blocking peptide over night at 4°C, followed by immunohistochemistry. Tissue sections treated with the primary antibody alone or together with its blocking peptide were then studied and compared. The immunoreactivity found in tissue sections treated with the primary antibody together with its blocking peptide was considered as unspecific.

Enzymatic immunohistochemistry (Paper I-II)

In general, a weak signal was obtained with the CB1 receptor antibodies when fluorescence immunohistochemistry was performed. Therefore, a more sensitive enzymatic technique (Vectastain ABC kit; Vector laboratories, CA, USA) was mainly used to study CB1 receptor immunoreactivity (I). It was also used when dorsal root ganglion cells were tested for TRPA1 and TRPV1 immunoreactivity (II). The overall principle for enzymatic immuno-histochemistry is the same as for fluorescence immunohistochemistry (Fig. 4), but instead of a fluorochrome, biotin is coup led to the secondary antibody.

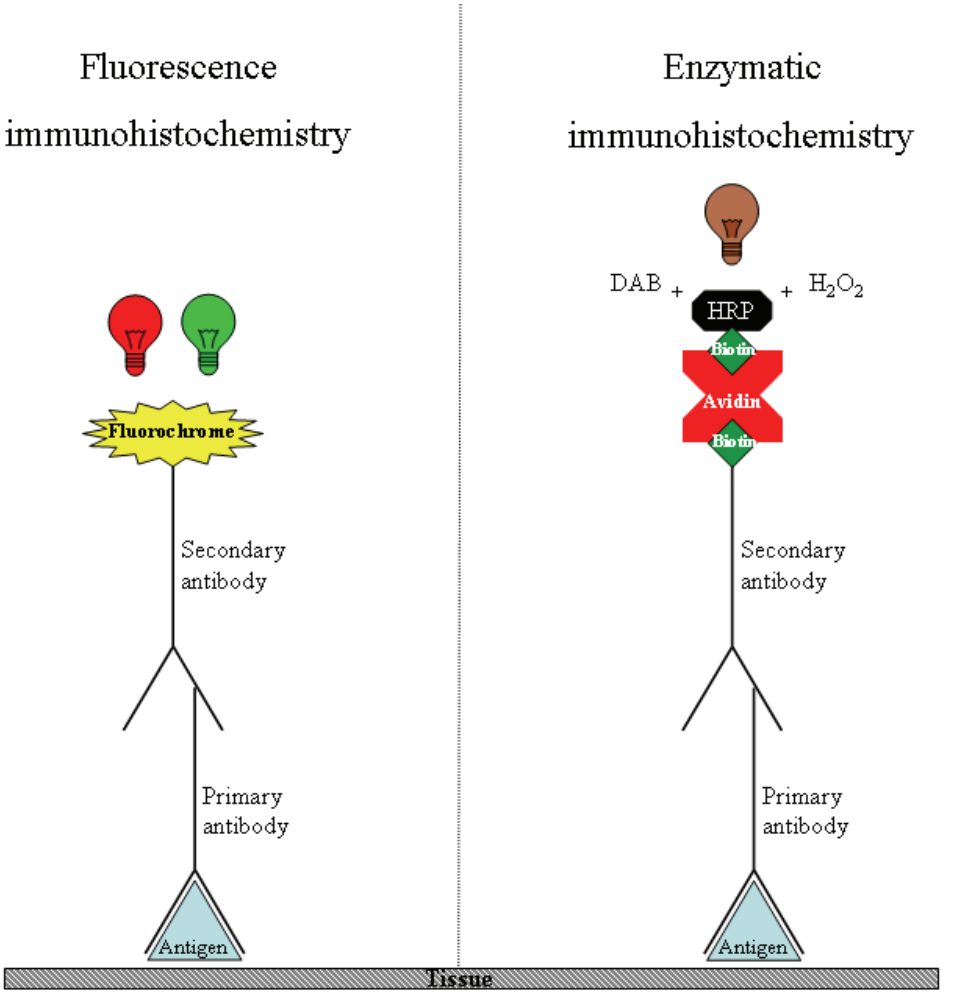

Fig. 4. Schematic drawing of the principles of fluorescence and enzymatic immunohistochemistry. Both techniques are based on an antigen-antibody reaction. The primary antibody (made in e.g. rabbit) binds to a short amino acid sequence found on the surface of the antigen. The secondary antibody (e.g. anti rabbit) then binds to the primary antibody. To be able to detect the antigen-antibody complex, the secondary antibody is coupled to a fluorochrome or a biotin/avidin complex in the fluorescence and enzymatic technique, respectively. When the flourochrome-coupled secondary antibody-antigen complex is radiated with UV-light, it appears as a coloured product, usually red or green, in the fluorescence microscope. The biotin/avidin-coupled secondary antibody-antigen complex is further treated with a biotin-coupled horse radish peroxidase (HRP), which together with diaminobenzidine (DAB) and H_2O_2, gives rise to a brown product that is observed with a light microscope.

The biotinylated secondary antibody binds to an ABC complex, containing avidin and biotinylated horseradish peroxidase (HRP), with very high affinity. Finally, when HRP are mixed with its substrate, diaminobenzidine (DAB) and H_2O_2, a brown precipitate is produced in the cells or tissues, which can be detected with a light microscope.

The cerebellum, dorsal root ganglia, hindpaw skin and mesenteric arteries were fixed, cryoprotected and sectioned as described for fluorescence immunohistochemistry. Air dried tissue sections (10 µm thick) were pre-incubated with a PBS solution containing Triton X-100 (0.3 %) for 5 min before H_2O_2 (1 %) was added to prevent endogenous peroxidase activity. After another 5 min the sections were washed with the PBS solution containing Triton X-100 (0.3 %). The sections were then incubated with normal goat serum, to prevent unspecific background staining, for 45 min at room temperature and washed once more before they were incubated with the primary antibody, diluted in PBS containing Triton X-100 (0.3 %), (Table 1) over night at room temperature in a humid chamber. The sections were washed from unbound primary antibodies and incubated with the biotinylated secondary anti rabbit antibody for 2 hours at room temperature. The wash was repeated and ABC-reagent was added to the sections for 1 hour at room temperature. The wash was repeated once more before the sections were pre-incubated in the dark with a PBS solution containing Triton X-100 (0.3 %) and diaminobenzidine tetrahydrochloride (DAB; 0.25 mg/ml), pH 7.6, for 10 min. H_2O_2 (0.03 %) was then added to the previous solution and the slides were removed after 100 s. Washed and dried sections were mounted with Keiser´s glycerol gelatin mounting medium. The tissue sections were studied with a light microscope (Olympus Bx60F-3; Olympus America, Melville, NY) and images were acquired with a digital camera (Olympus DP50) attached to the microscope and analyzed with the software programs Studio Lite 1.0 and Viewfinder Lite 1.0 (Pixera Corporation, Los Gatos, CA). Control experiments were performed as described for fluorescence immunohistochemistry.

Recording of tension (Paper I-II)

The organ bath technique was used to record vasorelaxation as a physiological readout of the efferent activity of primary sensory neurons.

Rat mesenteric arteries were cut into ring segments (1-2 mm long) and mounted on two parallel steel pins in tissue baths (Fig. 5), containing 2.5 or 5 ml physiological salt solution of the following composition (in mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2 and (+)-glucose 5.5.

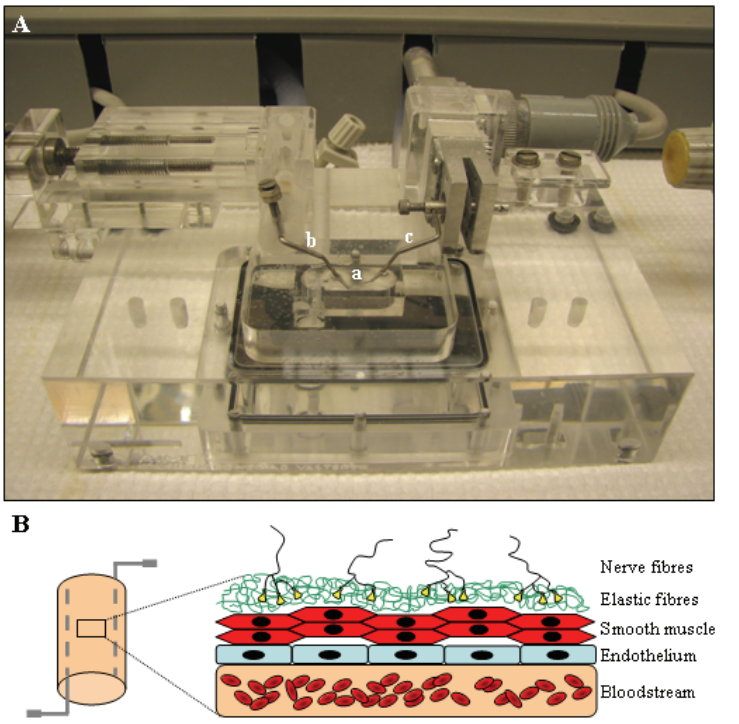

Fig. 5. Tissue bath used to record relaxation of blood vessels. **A**, A ring segment of a blood vessel is mounted on two parallel steel pins in a tissue bath that contains 5 ml of a physiological salt solution (a). One of the pins (b) is connected to a displacement device, which is adjusted until a proper resting tension of the vessel is obtained and the other (c) to a FT03C force-displacement transducer (Grass Instruments, USA). The latter is connected to a 7D polygraph (Grass Instruments), where the isometric tension is recorded. **B**, A schematic drawing of the blood vessel mounted in the tissue bath (a) and an enlargement of the vessel wall showing its different layers. Relaxation of the smooth muscle cells within the blood vessel, recorded by the polygraph, is due to the release of the neuropeptide CGRP from the nerve fibres in the outermost layer of the blood vessel.

The physiological salt solution was continuously bubbled with a mixture of 95 % O_2 and 5 % CO_2 at 37°C, resulting in a pH of 7.4. The arteries were allowed to equilibrate for about one hour and during this period they were repeatedly stretched until a stable resting tension of a certain value was obtained (for details, see Högestätt et. al 1983[118]). A force-displacement transducer (FT03C; Grass Instruments, USA) connected to a polygraph (Grass Instruments) was used to measure isometric tension. All experiments were performed in the presence of Nω-nitro-L-arginine (0.3 mM) and indomethacin (10 μM) to eliminate any contribution of nitric oxide and cyclooxygenase products, respectively. Relaxations were studied in arteries contracted with phenylephrine (3 μM).

When stable contractions were obtained, the agonists capsaicin, anandamide, allyl isothiocyanate (AITC), allicin or diallyl disulfide (DADS) were added cumulatively to determine concentration-response relationships. The effects of HU210 (0.1 μM), SR141716A (0.3 μM), ruthenium red (3 μM), capsazepine (3 μM), capsaicin (10 μM), 8-37 CGRP (3 μM) and vehicle (EtOH; 0.1 %) on vasorelaxation were recorded after pre-exposing arteries to these test substances for 30 min. Each vessel segment was exposed to only one treatment. Relaxation is presented as a percentage of the contraction caused by phenylephrine (3 μM).

Measurement of CGRP (Paper I)

The sensory neuropeptide calcitonin gene-related peptide (CGRP) is released from the peripheral terminals of primary sensory neurons, innervating tissues such as rat hindpaw skin and mesenteric artery, upon activation and can be detected using the radioimmunoassay (RIA) technique. This approach makes it possible to study pre-synaptic effects on sensory nerves and allowed us to examine the possible regulatory role of the cannabinoid CB1 receptor on TRPV1-mediated activation of such nerves.

Segments of rat hindpaw skin and mesenteric artery (1-2 cm) were equilibrated for 1 hour in aerated physiological salt solution (95 % O_2 and 5 % CO_2; 37 °C; pH 7.4) of the following composition (in mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2 and (+)-glucose 5.5, containing phenylmethylsulfonyl fluoride (0.1 mM) and thiorphan (10 μM) to prevent degradation of anandamide by fatty acid amide hydrolase and CGRP by endopeptidases, respectively.

The equilibrated skin segments were pre-treated with either capsazepine (10 μM), anandamide (0.01 μM or 10 μM), HU210 (0.1 μM), SR141716A (0.3 μM) or vehicle (EtOH; 0.1 %) in physiological salt solution for 20 min. The segments were then transferred to Eppendorff tubes containing a HEPES buffer solution (NaCl 119 mM, KCl 4.6 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 1.2 mM, HEPES 5 mM and (+)-glucose 6.0 mM), supplied with phenylmethylsulfonyl fluoride (0.1 mM), thiorphan (10 μM) and bovine serum albumin (0.05 %), and further treated with either capsaicin (0.01– 100 μM), anandamide (10 μM) or vehicle (EtOH; 0.1 %). After 15 min, the segments were removed and the solution in the test tubes subsequently evaporated using a SpeedVac AES 2000 (Savant Instruments, Farmingdale, NY). The amount of CGRP in the remaining pellet was determined using a rat ^{125}I-CGRP Radioimmunoassay kit (RIK-6006) from Peninsula Laboratories, San Carlos, CA. To determine the protein contents in the mesenteric arteries, a Coomassie Plus protein assay reagent kit from Pierce Biotechnology, Rockford, IL, was used.

Competitive radioimmunoassay

The kit is able to measure a CGRP concentration of 1-640 pg/ml and according to this interval a standard dilution series was made. The evaporated pellets were reconstituted with 500 μl RIA buffer. To 100 μl of standards and samples in duplicates, 100 μl of a primary antibody, raised in rabbit against rat α-CGRP, was added. The tubes were incubated over night at 4˚C. To compensate for efficiency differences between different gamma counters, the quantity of the tracer ^{125}I-CGRP was optimized to achieve a cpm-value between 10 000 and 15 000 cpm. When the desired cpm value was reached, 100 μl of the tracer was added to each tube and incubated over night at 4˚C. 100 μl of goat anti rabbit IgG serum and 100 μl normal rabbit serum was added to all tubes. After incubation for 90 min at room temperature, an additional volume of 500 μl RIA buffer was added and the tubes were centrifuged (Sigma 4K-1; Axel Johnson Instrument, Stockholm, Sweden) at 3000 rpm for 20 min at 4˚C. The supernatants were aspirated and the remaining pellets analysed of their CGRP content in a gamma counter (1277 Gammamaster; LKB Wallac, Turku, Finland). From a standard curve (prepared by the software program RiaCalc 2.50; LKB Wallac) the CGRP content in pg/ml was determined. The CGRP contents of the skin were then related to their weight and presented as fmol CGRP/g tissue, while the mesenteric arteries were related to their protein contents and presented as fmol CGRP/mg protein.

Protein assay

The mesenteric arteries used in CGRP release studies were homogenized by hand (Kontes glass CO, Duall 21), where after the homogenates were collected in glass tubes and centrifuged (Sigma 4K-1) at 3 000 rpm for 15 min at 10˚C. The supernatants were poured off and the pellets reconstituted with 150 µl 2 M NaOH and 150 µl 1 M HCl. A standard dilution series was made from an albumin standard (2 mg/ml). To 100 µl of standards and samples in duplicates, 400 µl of coomassie stain was added. 350 µl of the stained solutions were then added to a 96 well micro plate and read for protein content (Titertek Multiscan Plus spectrophotometer) at 595 nm.

^{3}H-noradrenaline release (Paper I)

This technique was used to study the effects of the cannabinoid agonists HU210 and anandamide on sympathetic nerves in rat mesenteric arteries. Briefly, rat mesenteric arteries pre-loaded with ^{3}H-noradrenaline were electrically stimulated to cause a depolarisation of the surrounding nerves, causing a release of neurotransmitters, among them ^{3}H-noradrenaline. The arteries were stimulated three times, but before the third stimulation the test substances were added. The released ^{3}H-noradrenaline was counted using a β-counter.

Segments of rat mesenteric arteries (1-2 cm) were equilibrated for 30 min in aerated physiological salt solution (95% O_2 and 5% CO_2; 37°C; pH 7.4) of the following composition (in mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2 and (+)-glucose 5.5, followed by another 30 min period in physiological salt solution, containing ^{3}H-noradrenaline (1 µM; specific activity 12 Ci/mmol). Preparations were then rinsed and transferred to temperature-controlled Perspex chambers perfused with physiological salt solution (95% O_2 and 5% CO_2; 37°C; pH 7.4) for 40 min before subjected to electrical stimulation. Electrical field stimulation of preparations was achieved by delivering square wave pulses with a duration of 0.3 ms at a frequency of 10 Hz in 1 s trains with 5 s interval over a period of 1 min. The preparations were stimulated 3 times (S_1-S_3) with 12 min intervals between stimulations. HU210 (0.1 µM) and anandamide (10 µM), vehicle or tetrodotoxin (1 µM) was introduced 8 min before the third stimulation period (S_3). The ^{3}H activity in collected samples of perfusate and in tissue was measured by a liquid scintillation spectrometer (RackBeta 1215, LKB, Sweden).

Calcium imaging and voltage-clamp recordings (Paper II)

The patch-clamp technique and calcium imaging were used in this thesis to study the ion channel TRPA1.

Calcium imaging

When the TRP ion channel, TRPA1, is activated it opens and extracellular calcium is allowed to pass into the cell. This raise in intracellular calcium can be measured by a calcium imaging technique, which is based on changes in light intensity that occurs when a calcium indicator, for example Fura-2, is bound to free intracellular calcium. In more detail, the cells are pre-loaded with the calcium indicator and exposed to UV-light, when a substance has been added to the cells. An absorption shift of Fura-2 occurs when it binds to calcium that can be observed by scanning the excitation spectrum between 300 and 400 nm, while monitoring the emission at 510 nm. The light intensity is measured over time, thus the more calcium that is passed into the cell and binds to Fura-2, the higher intensity.

Cultured trigeminal neurons[98] and Human Embryonic Kidney (HEK)293t cells expressing the human TRPA1[119] were loaded with the ratiometric calcium indicator Fura-2 acetoxymethyl ester (10 μM) diluted in Ringer's solution containing (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES 10, pH 7.4, with pluronic acid (0.02 %) for 60 min and in CIB buffer containing (in mM): NaCl 130, KCl 3, $CaCl_2$ 2.5, $MgCl_2$ 0.6, $NaHCO_3$ 1.2, glucose 10, HEPES 10, pH 7.45, with pluronic acid (0.02 %) for 30 min, respectively. The cells were rinsed twice before either garlic extract (diluted 1:10 000), allicin (40 μM) or diallyl disulfide (DADS; 200 μM) were added. Ratiometric calcium imaging was then performed, where dual images (340 and 380 nm excitation, 510 nm emission) were collected and pseudocolour ratiometric images monitored during the experiment. The cells were observed for 60-120 s.

Whole-cell patch-clamp

The cell membrane of a *Xenopus laevis* oocyte expressing human TRPA1 was patched by a glass-pipette electrode filled with a physiological salt solution, ND96 containing (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.6. When the electrode is in contact with this solution it conducts the electrical changes to a voltage clamp amplifier. The glass-pipette electrode is pressed against the cell membrane and a gentle suction is applied to the inside of the electrode to pull the cell's membrane inside the tip of the electrode.

The suction causes the cell to form a tight seal with the electrode, a so-called gigaohm seal. With the cell patched to the electrode a stronger suction was briefly applied which causes the patched cell membrane to break and the interior of the glass-pipette becomes continuous with the cytoplasm of the cell. This arrangement allows measurements of electrical currents from the entire cell and is therefore called the whole-cell recording method. The whole-cell configuration also allows diffusional exchange between the pipette and the cytoplasm, producing a convenient way to inject substances into the interior of a patched cell. Currents were recorded in the TRPA1 expressing oocyte in the absence and presence of allicin (3 µM), allyl isothiocyanate (AITC; 200 µM) or diallyl disulfide (DADS).

Quantitative real time PCR (qRT-PCR) (Paper III)

With quantitative real time PCR small amounts of mRNA encoding a specific protein in cells or tissues can be detected, since the measurement is made in real time. This technique was used to detect the mRNA of TRPA1, TRPM8, TRPV1, TRPV2 and TRPV3 in human skin biopsies of three healthy volunteers.

RNA isolation

Punch biopsies (3 mm in diameter) from the skin of the lateral upper arm 10 cm below the lateral margin of the acromion of three healthy volunteers (45 years, female; 52 years, female; 35 years, male) were collected under local anaesthesia. The biopsies were placed and homogenized in 800 µl Trizol (Invitrogen, Carlsbad, CA) reagent containing 10 µg glycogen (Invitrogen). The homogenized biopsies were incubated for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes. 160 µl chloroform (Sigma, St Louis, MO) was added to the homogenates and the tubes were shaked vigorously by hand for 15 s followed by a 3 min incubation at room temperature. The tubes were centrifuged at 10 600 rpm for 15 min at 4 °C. Following centrifugation, the mixture separates into, a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase containing RNA and glycogen was transferred to fresh tubes to which 400 µl isopropyl alcohol (Sigma) were added. The tubes were incubated for 10 min at room temperature and then centrifuged at 10 600 rpm for 10 min at 4 °C. The supernatants were poured off and the RNA pellets were washed twice with 800 µl 75 % ethanol. The samples were mixed by vortexing and centrifuged at 8400 rpm for 5 min at 4 °C. The supernatants were poured off and the RNA pellets were briefly air dried. Finally the RNA pellets were dissolved in DEPC

(Sigma)-treated water and incubated for 10 min at 60 °C. The concentration of total RNA was measured with a NanoDrop ND-1000 spectrophotometer (Saveen Werner, Malmö, Sweden).

cDNA synthesis

1 µl total RNA (250 ng) or 1 µl diethylpyrocarbonate (DEPC)-treated water was added to a PCR-tube containing $MgCl_2$ solution (25mM), RNase inhibitor (2000 Units), RT-buffer (10x), dNTP mix (with dTTP; 2.5 mM each), Random hexamers (50 µM), MultiScribe reverse transcriptase (5000 Units) and DEPC-treated water (to a final volume of 100 µl) from Taqman Reverse Transcriptase kit (Eppendorf, Hamburg, Germany). The PCR was run at 25 °C for 10 min, at 48 °C for 30 min and at 95 °C for 5 min with a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA).

RT-PCR

5 µl cDNA or water was added to a 96-well plate containing universal PCR master mix (2x; Applied Biosystems, Foster City, CA), Primer and Probe (20x; TaqMan Gene Expression Assays TRPA1, TRPM8, TRPV1, TRPV2, TRPV3 and β-actin (human); Applied Biosystems) and water (to a final volume of 25 µl). All samples were added as triplets. The RT-PCR reaction was run at 50 °C for 2 min, at 95 °C for 10 min, at 95 °C for 0.15 min (40 cycles) and at 60 °C for 1 min with an ABI Prism 7700 (Applied Biosystems). The samples were related to the house keeping β-actin (human). C_T-values below 35 are considered positive and low amounts are referred to as C_T 30-35.

RESULTS AND DISCUSSION

TRPV1 – a nociceptor modulated by the cannabinoid CB1 receptor? (Paper I)

Activation of TRPV1 on sensory nerves triggers pain and local vasodilatation[5,68,120]. One mechanism by which an analgesic drug could work is through inhibition of TRPV1 either directly by binding to TRPV1 as an antagonist, or indirectly by activating an inhibitory receptor signalling pathway on TRPV1-containing nerves in peripheral tissues. This latter mechanism is believed to contribute to the analgesic effect of CB1 receptor agonists[60-63]. However, there is no direct evidence of the presence of such neuronal CB1 receptors in peripheral tissues. We therefore used immunohistochemistry and assays of sensory neurotransmitter release to characterize the neuronal distribution and function of the CB1 receptor relative TRPV1 in the rat hindpaw skin and mesenteric artery, tissues frequently used in the study of sensory neurotransmission.

CB1 receptor immunoreactivity in hindpaw skin and mesenteric artery

The putative presence of peripheral neuronal cannabinoid CB1 receptors in rat and mouse hindpaw skin and mesenteric artery was examined by immunohistochemistry with five different CB1 receptor antibodies; two raised against the C-terminal and three against the N-terminal of the CB1 receptor. Immunoreactivity was observed with all five antibodies in perivascular nerve fibres (Fig. 3 in paper I), epidermal keratinocytes and vascular smooth muscle cells (tunica media) in hind paw skin (Fig. 1 in paper I). In addition, the C-terminal antibodies displayed immunoreactivity in skeletal muscle in the dermal layer. The N-terminal antibodies also recognized cells throughout the dermal layer. These cells were characterized as fibroblasts and macrophages/monocytes using antibodies against rpHβ and ED1, markers of fibroblasts and macrophages/monocytes, respectively (Figs. 1 and 4 in paper I). In the mesenteric artery, immunoreactivity was seen with all five antibodies in nerve fibres within the adventitia and in smooth muscle cells of the media (Fig. 5 in paper I). In addition, the N-terminal antibodies stained cells throughout the adventitia, which were characterized as fibroblasts and macrophages/monocytes (Fig. 6 in paper I). For each antibody, there was no difference in the staining pattern between rat and mouse and between wild-type and CB1 receptor-deficient mice. (Figs. 1, 3 and 5 in paper I). Taken together, these findings indicate that the staining obtained with all five CB1 receptor antibodies in skin and mesenteric artery of rat and mouse is unrelated to the CB1 receptor.

CB1 receptor immunoreactivity in cerebellum

CB1 receptors are widely distributed in the central nervous system, including the cerebellum, as shown by immunohistochemical studies[121]. We therefore used the rat and mouse cerebellum as positive controls of CB1 receptor immunoreactivity. The two C-terminal antibodies showed immunoreactivity in basket cells forming synapses with purkinje cells in rat and mouse cerebellum (Fig. 7A-B and D-E in paper I), consistent with findings in previous studies [121]. Such immunoreactivity was not present in CB1 receptor-deficient mice (Fig. 7C and F in paper I). Amongst the tested N-terminal antibodies, no immunoreactivity was detected with CB1c (Fig. 7G-I in paper I) and CB1e (Fig. 7M-O in paper I), whereas CB1d (Fig. 7J-L in paper I) displayed immunoreactivity in purkinje cells. However, this staining was also present in cerebellum of CB1 receptor-deficient mice. Taken together, only the two C-terminal CB1 receptor antibodies recognize the CB1 receptor in the cerebellum of rat and mouse. No immunoreactivity was observed with the N-terminal antibodies CB1c and CB1e, whereas the CB1d immunostaining turned out to be unspecific.

Immunohistochemical localization of TRPV1 in rat hindpaw skin and mesenteric artery

The normal distribution pattern of nerve fibres in the rat hindpaw skin and mesenteric artery is illustrated by the common nerve marker PGP. In skin, nerve fibres are found throughout the epidermal and dermal layers (Fig. 8A in paper I), while in artery, a dense network of nerve fibres is found in the adventitia (Fig. 9A in paper I). TRPV1 is often used as a marker for sensory nerves, which are known to innervate skin and blood vessels[122,123]. Thus, we find TRPV1 immunoreactive nerve fibres in the epidermal and upper dermal layers (Fig. 8B and E in paper I). These nerve fibres also show immunoreactivity for the sensory neuropeptide CGRP (Fig. 8C and D in paper I). In mesenteric artery, a dense network of TRPV1 immunoreactive nerve fibres are found in the outermost adventitia and these fibres are also immunoreactive for the sensory neuropeptide CGRP (Fig. 9C, E, and F in paper I). In dorsal root ganglia, TRPV1 is found on small and medium sized neurons, representing unmyelinated C-fibres and lightly myelinated Aδ-fibres, respectively[26]. To find out whether this is also true in the skin and mesenteric artery of rat the myelin marker NF200 was used to identify myelinated nerves. NF200 immunoreactive nerve fibres and nerve bundles (not shown) are found in the dermal layer of the skin (Fig. 8F in paper I), but these nerve fibres do not show immunoreactivity for TRPV1 (Fig. 8G in paper I). Likewise, the dense network of NF200 immunoreactive nerve fibres in the adventitia of mesenteric artery does not display immunoreactivity for TRPV1 (Fig. 9H and I in paper I).

The TRPV1 immunoreactivity in the rat hindpaw skin and mesenteric artery was validated in corresponding tissues from three TRPV1-deficient mice. Although the TRPV1 antibody used in these experiments was not the same as the TRPV1 antibody used in paper I, since the latter one was no longer available, the two antibodies displayed an identical staining pattern. Furthermore, there seems to be no difference in TRPV1 immunoreactivity in hindpaw skin and mesenteric artery between rat and mouse (Fig. 6).

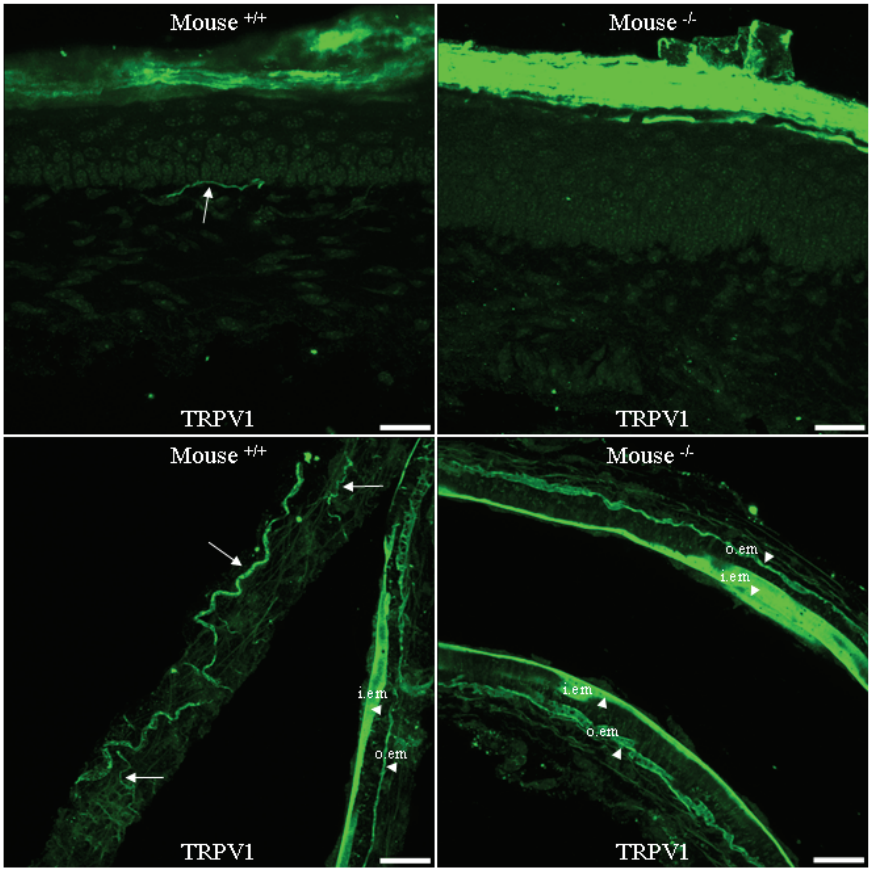

Fig. 6. Confocal images of TRPV1 immunoreactivity in hindpaw skin and mesenteric artery of TRPV1$^{+/+}$ and TRPV1$^{-/-}$ mice. TRPV1 immunoreactive nerve fibres (arrows) are found in the epidermis/dermis region of skin from normal mouse (upper row) and in the adventitia of mesenteric artery (lower row). No immunoreactivity in nerve fibres were observed in skin (upper row) or mesenteric artery (lower row) from TRPV1 deficient mouse. The inner elastic membrane (i.em, arrowheads) and the outer elastic membrane (o.em, arrowheads) were stained by the secondary antibody alone, thus considered as an unspecific immunoreactivity. Scale bars represent 25 µm in all images. The TRPV1 primary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the secondary antibody Alexa Fluor 488 from Molecular Probes (Eugene, OR).

Taken together, these findings indicate that the TRPV1 immunoreactive nerves in rat hindpaw skin and mesenteric artery are unmyelinated C-fibres, of which many contain the sensory neuropeptide CGRP.

Effects of HU210 and anandamide on TRPV1-mediated CGRP release in rat hindpaw skin and mesenteric artery

The effects of CB1 receptor activation on TRPV1-mediated CGRP release was studied in rat hindpaw skin and mesenteric artery. As expected from a previous study (Richardson et al. 1998), capsaicin evoked the release of CGRP in a concentration-dependent manner (Fig. 10A in paper I). The capsaicin-induced release of CGRP from rat hindpaw skin is inhibited by the TRPV1 antagonist capsazepine (Fig. 10A in paper I), indicating that the CGRP release is mediated through TRPV1. This is in agreement with our immunohistochemical findings. In addition to the endocannabinoid anandamide, the effect of the potent synthetic CB1 receptor agonist HU210 was also studied on capsaicin-induced CGRP release from rat hindpaw skin. Neither anandamide (0.01 μM) nor HU210 (0.1 μM) had any effect on CGRP release (Fig. 10B in paper I). A higher concentration of anandamide (10 μM) also has no effect on capsaicin-induced CGRP release (basal, 34.7 ± 5.4 fmol/g tissue; capsaicin, 528.7 ± 95.4 fmol/g tissue; capsaicin + anandamide, 387 ± 136 fmol/g tissue; n = 5-6, one-way ANOVA). Taken together, the CB1 receptor agonists HU210 and anandamide had no effect on the TRPV1-mediated CGRP release from rat hindpaw skin.

The rat isolated mesenteric artery is a useful model for studying vascular sensory nerve responses triggered by irritants such as capsaicin[68]. Via activation of TRPV1, capsaicin relaxes mesenteric arteries as a result of CGRP release from sensory nerves. In the present study, this relaxation was unaffected by the CB1 receptor agonist HU210 (0.1 μM; Fig. 11A in paper I).

TRPA1 - a nociceptor that recognizes pungent products from garlic (Paper II)

Garlic has long been used as a medical remedy for treating a wide range of ailments, including hypertension, high cholesterol and thrombosis[124], but it is also known to cause adverse effects such as cutaneous irritation, oedema and allergic contact dermatitis[125-127]. The mechanisms of action behind these effects are so far not clearly understood. Crushed fresh garlic is known to generate organosulphur compounds, such as allicin and diallyl disulfide (DADS)[128,129], which share certain chemical properties with allyl isothiocyanate (AITC), the pungent ingredient in wasabi and other mustard plants[130]. AITC and other plant-derived irritants, including capsaicin, are known to activate the TRP ion channels TRPA1 and TRPV1, respectively, on primary sensory neurons that can induce pain and vasodilatation[17,35,131]. In light of this, we studied the pharmacology of garlic extract and its derivatives allicin and DADS with reference to primary sensory neurons.

Garlic activates primary sensory neurons

Measurement of intracellular calcium levels in primary cell cultures of trigeminal ganglia (TG) and dorsal root ganglia (DRG) were used to study the effects of garlic on primary sensory neurons. When TG and DRG neurons were exposed to fresh garlic extract, purified allicin, or DADS, the intracellular calcium levels increased (Fig. 2A-C in paper II). This effect was completely lost when the sensory neurons were pre-treated with the non-selective TRP ion channel blocker, ruthenium red, which indicates that the calcium responses caused by garlic and its derivatives allicin and DADS are mediated by TRP ion channels. All TG and DRG neurons activated by AITC, which is a known TRPA1 agonist[35], also responded to garlic extract, purified allicin or DADS. This subtype of neuron is also sensitive to capsaicin[35], a TRPV1 receptor agonist, but represents only half of the capsaicin-sensitive population of neurons (Fig. 2D in paper II). This suggests that the TRP ion channel, TRPV1, is not the primary target for garlic. To further examine this possibility, calcium imaging was performed on TG neurons from wild-type and TRPV1-deficient mice exposed to garlic extract, purified allicin, DADS and AITC. The number of responding was the same in wild-type and TRPV1-deficient mice, which shows that TRPV1 is of minor importance in the action of garlic and mustard oil (Fig. 2E in paper II). Taken together, these results suggest that garlic and its derivatives allicin and DADS activate TRP ion channels different from TRPV1 on a mustard oil- and capsaicin-sensitive subtype of primary sensory neurons.

Garlic activates the TRP ion channel TRPA1

Since mustard oil- and garlic-sensitive neurons displayed a complete overlap, the effect of fresh garlic extract, purified allicin, or DADS intracellular calcium levels were studied in HEK293 cells expressing human or rat TRPA1. All compounds generated a robust increase in calcium (Fig. 3A-C in paper II). Thus, like AITC, garlic extract, allicin and DADS act as TRPA1 agonists. These results were supported by patch-clamp recordings of *Xenopus* oocytes expressing human TRPA1, which upon stimulation with allicin and DADS produced robust membrane currents (Fig. 3D and E in paper II).

Garlic produces vasodilatation of rat mesenteric artery

Peripheral nerve endings of primary sensory neurons (nociceptors) are known to release sensory neuropeptides such as CGRP and SP upon stimulation, which in mucous membranes may cause flare (vasodilatation) and oedema (vascular leakage), signs typical of inflammation[68,132,133]. TRPA1 and TRPV1 are expressed by such primary sensory neurons[34,35,49,116,134], the efferent function of which can be studied as vasodilatation in arterial segments of the rat mesenteric artery. This experimental model was used to understand the mechanism behind the vasodilatory effects of garlic compounds and mustard oil (Fig. 4A in paper II).

Fresh garlic extract, allicin, DADS and AITC all induced concentration-dependent and complete relaxations of mesenteric arterial segments (Fig. 4B-E in paper II). These relaxations were inhibited with the CGRP receptor antagonist, 8-37 CGRP, indicating that the vasodilatory responses to these compounds are due to a release of CGRP from primary sensory neurons. The non-selective TRP ion channel blocker ruthenium red, but not the TRPV1 antagonist capsazepine inhibited these vasodilatations, indicating that fresh garlic extract, allicin, DADS and AITC produce their effects by activating TRP ion channels different from TRPV1. The pharmacological profile of the garlic-derived compounds and mustard oil was consistent with an activation of TRPA1 on capsaicin-sensitive, CGRP-containing nerve fibres that innervate vascular smooth muscle.

TRPA1 immunoreactivity in primary sensory neurons

Immunohistochemistry was used to study the expression of native TRPA1 in rat DRG neurons. Approximately 22 % and 54 % of all DRG neurons showed TRPA1 and TRPV1 immunoreactivity, respectively (Fig. 5A-C in paper II). All TRPA1 immunoreactive neurons

also showed immunoreactivity for TRPV1 (Fig. 5D in paper II). Many of the TRPA1-containing neurons also contained CGRP (Fig. 5E in paper II). A similar pattern of TRPA1 immunoreactivity was seen in peripheral nerve fibres in mesenteric arteries (Fig. 5F in paper II). Taken together, these histological findings are in line with the physiological results indicating that TRPA1 activates a subtype of capsaicin-sensitive, CGRP-containing primary sensory neurons. Indeed, a recent study using TRPA1 knock-out mice shows that the effects of allicin and AITC are exclusively dependent on TRPA1[135].

Are TRPV2 and TRPM8 present on sensory nerve fibres in human skin? (Paper III)

Transient receptor potential vanilloid 1 (TRPV1), vanilloid 2 (TRPV2) and melastatin 8 (TRPM8) are thermosensitive cation channels expressed in primary sensory neurons[26,27,97,98]. In human skin, synthetic and endogenous TRPV1 agonists produce pain and vasodilatation, which are inhibited by the TRPV1 blocker capsazepine[136,137]. The TRPM8 agonist menthol produces a cooling sensation as well as thermal and mechanical hyperalgesia when topically applied on the skin and mucous membranes in man[138,139]. TRPV2 is activated by noxious heat (> 52ºC) and as such represents another potential molecular heat sensor[27]. Although such evidence suggests that TRPV2 and TRPM8 may have a role as molecular nociceptors in human skin, there is no immunohistochemical evidence of the existence of TRPV2 or TRPM8 in this tissue. In the present study, we used immunohistochemistry to study the expression of TRPV1, TRPV2 and TRPM8 in human skin from healthy individuals and individuals with a critical mutation in the gene encoding nerve growth factor beta (NGFβ) that causes Norrbottnian congenital insensitivity to pain[140].

Skin innervation in healthy volunteers

The skin is a complex organ with many structural and functional components. Many of which are richly supplied with nerve fibres, as demonstrated by the general nerve marker PGP (Fig. 2 and Table 2 in paper III). Nerve fibres immunoreactive for NF200, a marker of myelinated nerves, and the sensory neuropeptides calcitonin gene-related peptide (CGRP) and SP show a pattern of distribution similar to that of PGP immunoreactive nerve fibres (Table 2 in paper III). Noteworthy, only very few NF200 immunoreactive nerve fibres were found in epidermis, and although CGRP and SP displayed a similar pattern of immunoreactivity, substantially fewer fibres containing SP were found compared to CGRP. Tyrosine hydroxylase (TH) and vasoactive intestinal peptide (VIP), markers of nerve fibres belonging to the autonomic nervous system, are absent in the epidermis and papillary dermis, but otherwise have a similar pattern of distribution as PGP (Table 2 in paper III).

TRPV1, TRPV2 and TRPM8 immunoreactive nerve fibres are seen in the epidermis-papillary dermis region, and in nerve bundles and around blood vessels and hair follicles in the dermis (Table 2 in paper III). None of the TRP ion channels are present around eccrine sweat glands and arrector pili muscles (Table 2 in paper III). TRPV1 immunoreactive nerve fibres are most abundant in the epidermis and papillary dermis, while TRPV2 and TRPM8 immunoreactive nerve fibres are most abundant in the papillary dermis.

In general, there are fewer TRPV2 and TRPM8 than TRPV1 immunoreactive nerve fibres. To further characterize the TRPV1, TRPV2 and TRPM8 immunoreactive nerve fibres, co-localization experiments were performed (Table 3 in paper III). All three TRP ion channels co-localized with the sensory neuropeptides CGRP and to a lesser extent with SP, but not with NF200, VIP or TH (Figs. 3-5 in paper III). Taken together, these data indicates that TRPV1, TRPV2 and TRPM8 are expressed by unmyelinated C-fibres containing the sensory neuropeptides CGRP and/or SP in human skin.

Skin innervation in Norrbottnian congenital insensitivity to pain

In contrast to normal skin, which is densely innervated in the epidermis and dermis, skin from heterozygous and homozygous individuals, carrying the gene mutation for Norrbottnian congenital insensitivity to pain, shows only a sparse innervation, as demonstrated by the general nerve marker PGP (Fig. 6A, F and K in paper III). Some nerve fibres in normal skin are (i) myelinated, as shown by the nerve marker NF200 (Fig. 6B in paper III), (ii) CGRP or SP immunoreactive (Fig. 7 in paper III), or (iii) belong to the autonomic nervous system, as shown by the nerve markers VIP and TH (Fig. 6D and E in paper III). In heterozygous and homozygous individuals, only a few nerve fibres expressing NF200, CGRP or SP are present in the epidermis and papillary dermis (Fig. 6G and L, and Fig. 7 in paper III). A few VIP and TH immunoreactive nerve fibres are seen in between eccrine sweat glands in the deep part of dermis in heterozygous individuals, whereas no such nerves are found in homozygous individuals (Fig. 6I-J and N-O in paper III).

TRPV1, TRPV2 and TRPM8 are all found on nerve fibres in epidermis and papillary dermis of normal skin (Fig. 6C and Fig.7 in paper III), but only a few such nerve fibres are present in these regions in skin from heterozygous individuals (Fig. 6H and Fig. 7 in paper III). In skin from homozygous individuals, no TRPV1 immunoreactive fibres and only a few TRPV2 and TRPM8 immunoreactive nerve fibres are found (Fig. 6M and Fig. 7 in paper III). Taken together, a substantial reduction of various types of nerve fibre within both the sensory and autonomic nervous system is observed in skin from individuals with Norrbottnian congenital insensitivity to pain.

GENERAL DISCUSSION

Methodological aspects

Immunohistochemistry is a widely used technique in both cutting edge research and routine medical care to visualize proteins in biological tissues. In this thesis, immunohistochemistry is a key technique for studying the expression of proteins, such as TRP ion channels and CB1 receptors, within the somatosensory nervous system. The development of confocal microscopy has substantially advanced this methodology. In particular, confocal immunohistochemistry allows co-localization of two or more proteins in the same cell or tissue structure. I have personally experienced the advantage of this technique over traditional immunohistochemistry when co-localizing proteins in small tissue elements. As clearly demonstrated in paper I (Figs. 8E-G and 9G-I) and in paper III (Figs. 3-5), only confocal immunohistochemistry could adequately exclude co-localization of two proteins in small nerve fibres, because several layers throughout the entire tissue section could be analysed separately. The conclusions in many earlier studies of co-localization using traditional microscopy will probably need to be revised in the light of this technical advancement.

A main problem with immunohistochemistry, regardless of the microscopy technique used, is how to ensure that the staining obtained is indeed reflecting the expression of the target of interest. There are various procedures available to exclude off-target binding of the antibody. For example, monoclonal antibodies can be used instead of polyclonal antibodies to enhance the specificity of the staining. However, monoclonal antibodies are not easily available and are mainly produced in mouse. This is a clear disadvantage in double immunohistochemistry, which requires primary antibodies from different species. When performing fluorescence immunohistochemistry, whether using polyclonal or monoclonal primary antibodies, it is important to include appropriate control experiments. First, it is necessary to determine whether the tissue section under study produces auto fluorescence. This is a common problem with immunological cells and therefore other immunohistochemical techniques are often preferred. Second, it is important to make sure that a positive immunoreactivity is not due to the properties of the secondary antibody. The staining of the inner and outer elastic membranes of arteries in Fig. 6 (see Results and Discussion) and the outer epidermis in human skin (Fig. 1, paper III) are examples of such undesirable staining caused by the secondary antibody. Since this is a very common problem, control experiments should always be performed with each secondary antibody.

Third, the primary antibody should be incubated with the corresponding peptide used for immunization (blocking peptide) to exclude unspecific immunoreactivity. Any remaining staining in the tissue indicates that the primary antibody binds to structures different from the desired target. In paper III (Fig. 3), blocking of TRPV1 revealed that the intense small dots scattered throughout the epidermis were due to unspecific staining caused by the primary antibody. Fourth, the ultimate control experiment to verify antibody specificity is to compare immunoreactivity in normal tissue with tissue from knock-out animals, where the gene of the target protein has been immobilized. Such control experiments were performed with the TRPV1 (see Results and Discussion) and CB1 receptor (paper I) antibodies, revealing significant off-target binding of the latter antibodies. In some cases, immunohistochemistry may be combined with other techniques to support the presence of a particular protein. This was the case in paper III, in which TRPV1 gene transcripts were identified to support the existence of this ion channel in epidermis of human skin. In conclusion, immunohistochemistry is a powerful technique for localization of gene products in biological tissues, but it is important to recognize the pitfalls of the technique, some of which may be eliminated by adequate control experiments.

Cannabinoid CB1 receptors and primary afferents

In addition to directly affecting sensory neurotransmisson in the CNS, cannabinoids are believed to produce analgesia via an action in the periphery[141]. One such mechanism is activation of inhibitory CB1 receptors on TRPV1-containing nerve fibres[61]. This mechanism has received considerable attention, because it offers the possibility of designing novel cannabinoid analgesics devoid of psychotropic side effects. This thesis challenges this view. Using five different antibodies raised against the CB1 receptor, we could not find any immunohistochemical evidence of neuronal CB1 receptors in the rat and mouse hindpaw skin and mesenteric artery. The staining of nerve fibres and other cellular elements in wild-type animals was indistinguishable from that seen in CB1 receptor-deficient mice, clearly indicating off-target binding of the antibodies.

However, we cannot exclude the possibility that primary afferents projecting to other tissues are co-expressing CB1 receptors and TRPV1, or that neuronal CB1 receptors are expressed exclusively during disease. In fact, it has been claimed in a recent study that neuronal CB1 receptors are up-regulated during skin inflammation in rat[142]. However, they used the same N-

terminal CB1 receptor antibody as we did, the staining of which did not disappear in the hindpaw skin of CB1 receptor-deficient mice.

Our functional studies also do not support the view that the analgesic and anti-inflammatory effects of cannabinoids are caused by a direct inhibition of peripheral terminals of capsaicin-sensitive primary afferents. While we used HU210 in the different bioassays, most *in vivo* studies examining the possibility that cannabinoids activate peripheral CB1 receptors have used WIN55212, a compound that at high concentrations also interact with TRPV1 via CB1 receptor-independent mechanisms,[143-145]. Thus, our findings highlight the need for new CB1 receptor antibodies, the specificities of which should be confirmed in CB1 receptor-deficient animals, and calls for a re-evaluation of previous studies of CB1 receptor expression and cannabinoid activity in the periphery.

Role of TRPA1 in the somatosensory nervous system

The recent identification of TRPA1 as a detector of noxious stimulus such as cold[34] and chemical irritants[35,114] in primary sensory neurons has gained much interest. Whereas subsequent TRPA1 knock-out studies clearly confirm the role of TRPA1 as a chemical sensor, its role as a cold-receptor and mechanosensor is less obvious[135,146]. With regard to mechanosensation, knock-out studies failed to show a crucial role for TRPA1 in hearing, which does not exclude the possibility that this channel is indeed a mechanosensor in hair cells but may rather indicate that it is not the sole protein responsible for auditory signaling. Interestingly, a recent study shows that the peptide toxin GsMTX-4 from the spider *Grammostola spatulata*, which is used as a specific tool to study mechanosensitive ion channels, also interacts with TRPA1 possibly providing a link between TRPA1 and mechanosensation[147].

Our interest in TRPA1 has been mainly on its role in chemosensation and originates from early studies in our laboratory suggesting the existence of a novel ionotropic cannabinoid receptor belonging to the TRP ion channel family in the somatosensory nervous system[134]. These findings led to the identification of TRPA1 as the molecular target activated by the plant-derived cannabinoids Δ^9-THC and cannabinol[35]. Furthermore, it was shown that mustard oil, which is a commonly used tool to trigger pain and inflammation in animal studies, also activates TRPA1.

The different chemical properties of mustard oil and cannabinoids suggested that TRPA1 can be activated by different mechanisms. The present study extends these findings and show that other reactive molecules, such as the garlic-derived compounds allicin and DADS, also activate TRPA1. These findings may indicate a general role of TRPA1 in the detection of chemically reactive and potentially harmful compounds in both the external and internal environment. Two recent studies have suggested a covalent N-terminal modification of TRPA1 rather than a classical ligand-receptor interaction as the mechanism by which reactive chemicals, such as mustard oil, DADS and cinnamaldehyde, activate the channel[148,149]. Knowledge of the chemical properties of molecules interacting with TRPA1 may help identify endogenous substances and design drugs interacting with this channel.

CONCLUSIONS

I. We find no evidence of CB1 receptor immunoreactivity on nerve fibres in rat or mouse hindpaw skin and mesenteric artery. The CB1 receptor agonists anandamide and HU210 failed to inhibit TRPV1-mediated calcitonin gene-related peptide (CGRP) release from primary sensory neurons in rat hindpaw skin and mesenteric artery. Therefore, this study do not support the general view that the analgesic and anti-inflammatory effect of CB1 receptor agonists is due to direct inhibition of TRPV1 on peripheral terminals of capsaicin-sensitive primary afferent nerves. An important methodological aspect of this study is the antibody specificity. Whereas TRPV1 immunoreactivity disappeared in TRPV1 knock-out mice, experiments using CB1 receptor knock-out mice clearly show the lack of reliable antibodies to study CB1 receptor expression in the periphery.

II. This study shows that garlic extracts and some of its sulphur derivatives activate TRPA1 channels on sensory neurons present in the pain pathway and vascular system. It identifies TRPA1 as a novel ion channel in the vascular system and provides novel pharmacological tools for investigating the role of this ion channel. Whether activation of TRPA1 in the vascular system explains the beneficial antihypertensive effect observed by garlic treatment remains to be shown. This study also expands our understanding of how TRPA1 is regulated on a molecular basis, which is of importance for development of novel drug therapies for pain, inflammation and vascular disease.

III. This study shows for the first time the presence of the TRP ion channels TRPV2 and TRPM8 in sensory nerves in the human skin. TRPV2 and TRPM8 as well as TRPV1 immunoreactive nerve fibres are present in unmyelinated nerve fibres in epidermis and papillary dermis, in nerve bundles, and around blood vessels and hair follicles. In contrast to TRPV1, TRPV2 and TRPM8 are found mainly in the papillary dermis and seem to be restricted to peptidergic nerve fibres, of which the majority contain CGRP. There is a substantial loss of nerve fibres containing TRPV1, TRPV2 and TRPM8 in skin from individuals with Norrbottnian congenital insensitivity to pain. Insight into the role of TRPV2 and TRPM8 may open new avenues for treatment of inflammatory skin diseases.

SVENSK SAMMANFATTNING (SWEDISH SUMMARY)

Vad hade vi varit utan våra sinnen? Föreställ dig en värld utan att kunna se, höra, lukta, smaka och känna. Det är med hjälp av våra sinnen som kroppen kan känna vad som händer i vår omgivning. Ett av våra sinnen är smärtsinnet, som har till uppgift att varna oss för inre och yttre faror. Trots att smärta är den i särklass vanligaste orsaken till mänskligt lidande, så är kunskapen om de molekylära mekanismerna bakom dess uppkomst mycket bristfällig. Den vanligaste formen av smärta är nociceptiv och uppstår när olika typer av proteiner (jonkanaler) på smärtnerver (nociceptorer) ute i vävnaderna utsätts för kemisk, termisk eller mekanisk stimulering. En viktig sådan jonkanal är TRPV1 som förutom att aktiveras av pepparämnet kapsaicin också aktiveras av värme och sur miljö, tillstånd som bland annat kan ses vid inflammation. Andra potentiella angreppspunkter för läkemedelsbehandling är TRPA1 (senapsreceptorn), TRPM8 (mentolreceptorn) och TRPV2 (värmekänslig), som tillsammans med TRPV1 tillhör familjen TRP-kanaler. Dessa kanaler finns på primära sensoriska nerver (Aδ- och C-fibrer), som förutom att förmedla en signal till hjärnan om hotande vävnadsskada också deltar i regleringen av perifera organ, t.ex. genom att frisätta kärldilaterande substanser lokalt i vävnaden, och i olika reflexbågar av betydelse för funktionen i våra inre organ. Miktionsreflexen och hostreflexen är sådana viktiga reflexer.

Det har föreslagits att den smärtstillande och antiinflammatoriska effekten av Δ^9-tetrahydrocannabinol från växten *Cannabis sativa* och liknande substanser delvis kan förklaras av att dessa ämnen via sin receptor (CB1) hämmar smärtnerver och deras frisättning av sensoriska neuropeptider lokalt ute i vävnaden. Hittills har inga immunohistokemiska studier övertygande visat att CB1 receptorn finns på primärsensoriska nerver, som också uttrycker TRPV1. I det första arbetet har vi därför använt immunohistokemi och funktionella metoder för att studera en tänkbar interaktion mellan dessa två receptorsystem i perifera sensoriska nerver. En viktig slutsats är att de antikroppar som har använts för att studera CB1 receptorn inte är tillförlitliga för att studera denna receptor i periferin. Arbetet visar också vikten av att konfirmera specifik antikroppsinbindning genom användandet av möss som saknar CB1 receptorn. De immunohistokemiska och funktionella resultaten ifrågasätter den allmänna uppfattningen att CB1 receptoragonister utövar sin perifera smärtstillande effekt genom att hämma TRPV1 i de primärsensoriska nerverna.

I arbete två har vi visat att TRPA1 är den jonkanal som aktiveras av irriterande svavelhaltiga substanser från vitlök på de primärsensoriska nerverna. Dessa substanser utgör tillsammans med tidigare beskrivna TRPA1 agonister (isotiocyanater och kanelaldehyd) viktiga farmakologiska redskap för att förstå hur TRPA1 på molekylär nivå aktiveras av kemiskt irriterande substanser. Med immunohistokemisk analys av primärsensoriska nervcellskroppar i dorsalrotsgangliet finner vi att TRPA1 uttrycks i ungefär 22 % av alla nervceller. Vi visar också för första gången att TRPA1 finns uttryckt i kärlsystemet och att dess aktivering leder till frisättning av CGRP från primärsensoriska nerver i kärlväggen, vilket i sin tur leder till kärldilatation. Både immunohistokemi och funktionella studier på blodkärl tyder på att TRPA1 endast finns på en subpopulation av TRPV1-innehållande primärsensoriska nerver. Om denna grupp av nerver är inblandade i inflammatorisk smärta så kan TRPA1 vara en intressant angreppspunkt för nya smärtstillande läkemedel.

Huden är kroppens största sensoriska organ. I huden finns rikligt med primärsensoriska nerver, som kan registrera beröring och den omgivande temperaturen. Huden skyddar oss mot yttre våld och fungerar också som en barriär för skadliga främmande ämnen. I det tredje arbetet har vi studerat TRP-kanaler i hud från människa. Vi visar för första gången att TRPM8 och TRPV2 finns lokaliserat till primärsensoriska nerver runt blodkärl och hårfolliklar samt i det yttersta hudlagret (överhuden). I detta arbete undersöker vi också hudprover från individer med en medfödd okänslighet för smärta på grund av en mutation i en viktig tillväxtfaktor för nerver. Nerver som uttrycker TRPM8, TRPV2 och TRPV1 saknas i huden hos dessa individer. Arbetet väcker intressanta frågor angående vilken roll som de mindre väl karakteriserade TRP-kanalerna TRPV2 och TRPM8 spelar vid inflammatoriska tillstånd i huden.

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