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TRANSIENT RECEPTOR POTENTIAL VANILLOID 1, VANILLOID 2 AND MELASTATIN 8 IMMUNOREACTIVE NERVE FIBERS IN HUMAN SKIN FROM INDIVIDUALS WITH AND WITHOUT NORRBOTTNIAN CONGENITAL INSENSITIVITY TO PAIN

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Abstract—Transient receptor potential vanilloid 1 (TRPV1), vanilloid 2 (TRPV2) and melastatin 8 (TRPM8) are thermosensitive cation channels expressed on primary sensory neurons. In contrast to TRPV1, which is present on nociceptive primary afferents and keratinocytes in human skin, less is known about the distribution of TRPV2 and TRPM8 in this tissue. Immunohistochemistry of human forearm skin identified TRPV2 and TRPM8 immunoreactive nerve fibers in epidermis–papillary dermis and around blood vessels and hair follicles in dermis, although these nerve fibers were less abundant than TRPV1 immunoreactive nerve fibers throughout the skin. The TRPV2 and TRPM8 immunoreactive nerve fibers also showed immunoreactivity for calcitonin gene-related peptide (CGRP) and to a lesser extent substance P (SP). Neither of the TRP ion channels co-localized with neurofilament 200 kDa (NF200), vasoactive intestinal peptide (VIP) or tyrosine hydroxylase (TH). Nerve fibers immunoreactive for TRPV1, TRPV2, TRPM8, CGRP and SP were absent or substantially reduced in number in individuals with Norrbottnian congenital insensitivity to pain, an autosomal disease selectively affecting the development of C-fiber and Aδ-fiber primary afferents. Quantitative real time PCR detected mRNA transcripts encoding TRPV1 and TRPV2, but not TRPM8, in skin from healthy volunteers, suggesting that these ion channels are also expressed extraneuronally. In conclusion, nerve fibers in human skin express TRPV1, TRPV2 and TRPM8 that co-localize with the sensory neuropeptides CGRP and SP, but not with NF200, VIP or TH. A dramatic loss of such nerve fibers was seen in skin from individuals with Norrbottnian congenital insensitivity to pain, further suggesting that these ion channels are expressed primarily on nociceptive primary sensory neurons in human skin.

Key words: calcitonin-gene related peptide, immunohistochemistry, pain, sensory neuron, skin, transient receptor potential channels.

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Abbreviations: CGRP, calcitonin gene-related peptide; C_T, cycle threshold; F, female; HET, heterozygous; HO, homozygous; M, male; NF200, neurofilament 200 kDa; NGFβ, nerve growth factor beta; PBS, phosphate buffer saline; PGP, protein gene product 9.5; SP, substance P; TH, tyrosine hydroxylase; TRPM8, transient receptor potential melastatin 8; TRPV1, transient receptor potential vanilloid 1; TRPV2, transient receptor potential vanilloid 2; TRPV3, transient receptor potential vanilloid 3; VIP, vasoactive intestinal peptide.

Transient receptor potential vanilloid 1 (TRPV1), vanilloid 2 (TRPV2) and melastatin 8 (TRPM8) are thermosensitive cation channels expressed on primary sensory neurons (Caterina et al., 1997, 1999; McKemy et al., 2002; Peier et al., 2002). These proteins belong to a superfamily of ion channels, composed of six main subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) (Vennekens et al., 2008). The plant-derived irritant capsaicin produces pain via activation of TRPV1 on C-fiber and Aδ-fiber afferents (Caterina et al., 1997, 2000; Davis et al., 2000). Nociceptive primary sensory neurons may therefore be defined by their sensitivity to capsaicin (Szolcsanyi, 2004). Heat (>42 °C), acidosis and various membrane-derived lipids, such as N-acyl ethanolamines and lipoxygenase products, trigger the activation of TRPV1 (Caterina et al., 1997; Hwang et al., 2000; Movahed et al., 2005b; Zygmunt et al., 1999). As shown by immunohistochemistry, TRPV1 is present on nerve fibers and keratinocytes in both animal and human skin (Bodo et al., 2004; Stander et al., 2004). Exposure of human skin to synthetic and endogenous TRPV1 activators produces acute pain and vasodilatation, which are inhibited by the TRPV1 blocker capsazepine (Movahed et al., 2005a; Roosterman et al., 2006). Taken together, such evidence strengthens the role of TRPV1 as an important detector of painful stimuli in human skin.

In rodents, TRPV2 is expressed on mainly medium to large diameter neurons in dorsal root ganglia as well as on non-neuronal cells, such as larynx epithelial cells (Caterina et al., 1999; Hamamoto et al., 2008). TRPV2 was originally identified as a high-threshold (>52 °C) heat detector in rat primary afferents (Caterina et al., 1999). However, the human TRPV2 expressed in HEK293 cells is not activated by heat (Neeper et al., 2007). In an *ex vivo* characterization of cutaneous sensory neurons in mouse, TRPV2 was found in a majority of myelinated high-threshold mechanoreceptors, but only a small number of TRPV2 immunopositive cells

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responded to heat (Lawson et al., 2008). Furthermore, the wide distribution of TRPV2 in tissues, including the human urothelium (Caprodossi et al., 2008), where temperatures above 52 °C is unlikely to trigger its activation, indicates that this ion channel may have functions other than noxious heat detection (Vennekens et al., 2008). The lack of selective agonists and antagonists has made it difficult to study its role in nociception, and there are no reports on the presence of TRPV2 in human skin.

The cold-activated ion channel TRPM8 is expressed on a subpopulation of small diameter neurons in rodent dorsal root and trigeminal ganglia (McKemy et al., 2002; Peier et al., 2002; Story et al., 2003). Some of these neurons also express TRPV1 and respond to capsaicin, and thus display characteristics of nociceptive neurons (Babes et al., 2004; Okazawa et al., 2004; Abe et al., 2005; Xing et al., 2006). TRPM8 is upregulated in capsaicin-sensitive dorsal root ganglion neurons in rats subjected to chronic constrictive nerve injury, possibly explaining the occurrence of cold allodynia in this model of neuropathic pain (Colburn et al., 2007; Xing et al., 2007). TRPM8 is targeted by several plant-derived chemicals, including menthol, which produces a psychophysical sensation of cooling (McKemy et al., 2002; Peier et al., 2002). When topically applied on skin and mucous membranes, menthol produces spontaneous pain and cold hyperalgesia in man (Wasner et al., 2004; Namer et al., 2005). However, menthol is not as selective as capsaicin, and it may interact with TRP ion channels other than TRPM8 (Macpherson et al., 2006; Karashima et al., 2007). In contrast to the human urinary bladder, where TRPM8 is expressed on both nerve fibers and urothelial cells (Stein et al., 2004; Mukerji et al., 2006), there is no immunohistochemical evidence in support of the existence of TRPM8 in human skin.

In the present investigation, we have used immunohistochemistry and quantitative real time PCR to study the expression and distribution of TRPV2 and TRPM8 in human skin. To explore whether these ion channels are present on nociceptive nerve fibers, skin from normal subjects and individuals with Norrbottnian congenital insensitivity to pain was compared. The severe form of this disorder is inherited in an autosomal recessive manner and homozygous (HO) individuals are insensitive to pain at birth due to a selective loss of C-fiber and Aδ-fiber primary afferents (Einarsdottir et al., 2004; Minde, 2006).

EXPERIMENTAL PROCEDURES

Tissue specimens

Punch biopsies (3 mm in diameter) were collected under local anesthesia from the volar side of the forearm close to the cubital fossa of nine healthy volunteers (Normal 1, 45 years, female [F]; Normal 2, 52 years, F; Normal 3, 35 years, male [M]; Normal 4, 63 years, F; Normal 5, 63 years, F; Normal 6, 48 years, M; Normal 7, 37 years, F; Normal 8, 33 years, M; Normal 9, 36 years, F), and three heterozygous (HET1, 74 years, M; HET2, 79 years, M; HET3, 46 years, F) and three HO (HO1, 41 years, M; HO2, 23 years, F; HO3, 16 years, M; corresponding to patient 3, patient 2 and patient 1, respectively, in the study by Minde et al., 2004) individuals, carrying the nerve growth factor beta (NGFβ) gene mutation associated with Norrbottnian congenital insensitivity to pain. The study was performed with the approval from the local ethics committees at Lund and Umeå University.

Fluorescence immunohistochemistry

Skin biopsies were placed in Steffanini fixation, containing 2% paraformaldehyde and 0.2% picric acid in phosphate buffer saline (PBS, pH 7.2), for 24 h at 4 °C. The specimens were then cryoprotected in a PBS solution, containing 15% sucrose, for two days. The fixed biopsies were mounted in OCT compound (Tissue Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands), frozen in isopentane and stored at −70 °C. Sections of the skin biopsies (25 μm thick) were cut on a cryostat (Leica CM 3050 S; Leica Microsystems, Wetzlar, Germany) at −20 °C, collected on chromealun-coated microscope slides and stored at −20 °C until used. Air dried sections were pre-incubated with a PBS solution, containing 0.2% Triton X-100 and 0.1% bovine serum albumin, for 2 hours at room temperature and then incubated with the primary antibody (Table 1) overnight at room temperature in a humid chamber. The slides were washed from excess unbound primary antibodies with PBS and incubated with a secondary antibody (1:400) at room temperature for 1 h. The secondary antibodies (Alexa Fluor 488 goat antirabbit, Alexa Fluor 488 goat antimouse, Alexa Fluor 488 goat anti–guinea pig, Alexa Fluor 555 goat anti-mouse or Alexa Fluor 555 goat anti–guinea pig) were obtained from Molecular Probes (Eugene, OR, USA). The PBS wash was repeated before the slides were dried and mounted with a PBS/glycerol solution. When double immunohistochemistry was performed, the primary antibodies were incubated as a cocktail overnight and the secondary antibodies were incubated separately for 1 h each with a PBS wash in between.

To evaluate non-specific staining caused by the secondary antibodies, control experiments in the absence of primary antibody were performed. All secondary antibodies caused a profound staining of the stratum corneum and of collagen fibers in dermis (Fig. 1A). Likewise, a strong staining of secretory granules in eccrine sweat glands located deep in dermis was observed (Fig. 1B). This non-specific staining was seen in skin sections from both healthy volunteers and individuals with the NGFβ gene mutation.

To verify primary antibody specificity, blocking peptides against the TRPV1 (PA1-748, aa7-21: DLGAAADPLQKDT, Affinity BioReagents), TRPV2 (C-terminal: CKNSASEEDHLPLQVLQSP,

Table 1. Primary antibodies used for immunohistochemistry

Antibody	Host	Dilution	Source
CGRP	Guinea pig	1:180,000	B-GP 470-1; Euro-Diagnostica, Malmö, Sweden
NF200	Mouse	1:8000	AF5110-1; Sigma, St. Louis, MO, USA
PGP	Rabbit	1:2000	RA95101; UltraClone, Isle of Wight, UK
SP	Guinea pig	1:8000	B-GP 450-1; Euro-Diagnostica
TH	Mouse	1:1000	22941; DiaSorin, Stillwater, MN
TRPM8	Rabbit	1:32,000	ab3243; Abcam, Camebridge, UK
TRPV1	Rabbit	1:2000	PA1-748; Affinity BioReagents, Golden, CO, USA
TRPV2	Rabbit	1:500	AB5398P; Chemicon International, Temecula, CA, USA
VIP	Guinea pig	1:32,000	B-GP 340-1; Euro-Diagnostica

All antibodies were diluted in a PBS solution, containing 0.2% Triton X-100 and 0.1% bovine serum albumin. The primary antibodies for NF200 and TH are of monoclonal origin and the remaining ones are of polyclonal origin. All antibodies are affinity purified.