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Expression of substance P and vanilloid receptor (VR1) in trigeminal sensory neurons projecting to the mouse nasal mucosa ☆

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Abstract

Substance P and neurokinin A (NKA) have potent pro-inflammatory effects in the airways. The release of these neuropeptides from primary afferent (sensory) nerve endings to various stimuli is considered to be induced by activation of the capsaicin (vanilloid) receptor (VR1). In this study, retrograde neuronal tracing studies were combined with immunohistochemistry for VR1 and substance P to investigate the occurrence and distribution of substance P and VR1 receptor expression in mouse trigeminal neurons that were identified by retrograde labeling with Fast blue dye from the nasal mucosa. Fast blue signaling was observed in mucosa layers of the right nasal cavity and in sensory trigeminal neurons close to the division of the ophthalmic and maxillary nerve. Expression patterns of VR1 and substance P were found with different frequencies: 11.3 ± 1.2% (mean ± SEM) were immunoreactive for VR1, 4.9 ± 1.1% for VR1 and SP, and 6.4 ± 1.3% only for VR1 but not for SP. These VR1-positive neurons were partly binding to lectin I-B4, indicating VR1-expression in non-peptidergic upper airway C-fibers. In conclusion, based on the extent of SP and VR1 co-localization in nasal afferent neurons, the present study suggests that, following a peripheral activation of the VR1 receptor on SP afferents, there could be a triggering of SP-mediated phenomena, including those related to inflammation, such as plasma extravasation.

Keywords: Trigeminal ganglion; Substance P; Vanilloid receptor 1; Murine nasal mucosa; Mouse upper airway

1. Introduction

Airway response to a broad range of stimuli including capsaicin, bradykinin, hyperosmolar saline, tobacco smoke and cold dry air is mediated by C-fiber activation and following substance P (SP) release. The peptide SP is reported to be a key neuromodulator of the upper respiratory tract sensory airway innervation. SP is localized to afferent nerve terminals and sensory neuronal cell bodies of the trigeminal ganglion (TG) (Baraniuk and Kaliner, 1991; Baraniuk et al., 1991; Barnes, 1987; Barnes et al., 1991, 1998; Saria et al., 1988). In the periphery, released from the afferent nerve terminals, SP acts on NK1-receptors and mediates neurogenic inflammation.

Neurogenic inflammation can be described as a complex consisting of increased vascular permeability, plasma extravasation, glandular secretion and pro-inflammatory cell influx, which is mediated by SP. Recently, it has been proposed that the effects found in neurogenic inflammation may be partly mediated via activation of the capsaicin vanilloid receptor 1 (VR1) known as a polymodal receptor, which is stimulated by heat, protons or lipids (Caterina et al., 1997; Tominaga et al., 1998; Trevisani et al., 2002).

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As most of the co-localization studies for SP- and VR1-immunoreactivities (IR) were carried out only in the overall population of trigeminal primary sensory neurons of guinea pigs and rats (Guo et al., 1999; Hou et al., 2002), the present study aimed to examine the occurrence and distribution of SP-IR, VR1-IR and I-B4-lectin, a neurochemical marker for non-peptidergic C-fiber neurons in trigeminal sensory neurons that innervate the nasal mucosa of the murine upper respiratory tract. The mouse was used in the present study as in recent years, this species was commonly used to investigate immunological, genetic and neuro-immunological mechanisms underlying upper airway diseases such as allergic and non-allergic rhinitis (McCusker et al., 2002; Saito et al., 2001; van de Rijn et al., 1998). These chronic upper airway diseases are characterized by inflammation and nasal hyperreactivity and have been intensively characterized in the past years concerning both immune and neuroimmune mechanisms (Anggard, 1993; Baraniuk, 2001;Geppetti et al., 1988).

Using the mouse as species as it serves increasingly as species for animal models to study airway diseases due to the advantages of genetically altered mice with targeted disruption or over-expression of genes and VR1 as target, we here aimed to provide further details of the neurochemical nature of nasal mucosa afferents.

2. Material and methods

2.1. Animals

In total, 7 adult male and female BALB/c-mice weighing 15–20 g were used. The animals were kept under standard laboratory conditions. All protocols were performed in accordance with the Animal Welfare Act and the National Institutes of Health "Guidelines for the care and use of laboratory animals" (NIH publication 85-23, revised 1985) and approved by the state animal committee (0059/01).

2.2. Retrograde tracing and tissue preparation

Animals were anesthetized with intramuscular injections of ketamine hydrochloride (Ketanest, Parke-Davies, Freiburg, Germany, 10 mg/kg, body weight) and xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany, 10 mg/kg). The tip of a 10-μl syringe (Hamilton, Bonaduz, Switzerland) was inserted through the right nasal orifice into the nasal cavity and two 5-μl injections of Fast Blue tracing dye (2% aqueous solution containing 1% DMSO) were made into the anterior and posterior region of the right nasal cavity. The mice were then turned around their axis five times to ensure that the tracer reaches the entire nasal mucosa. All animals recovered undisturbed for a postoperative period of 7 days until they were killed by CO_2 asphyxiation. Directly after asphyxiation, the animals were perfusion-fixed retrogradely through the abdominal aorta with freshly prepared Zamboni's solution (2% paraformaldehyde, 15% picrinic acid, 0.1 M PBS, pH 7.4) for 5 min as described previously (Stefanini et al., 1967). This routine protocol was performed to ensure optimal conditions for immunohistochemistry and published previously for immunohistochemical analysis of ganglionic neuropeptide expression (Fischer et al., 1996; Fischer et al., 1996; Kummer et al., 1992). Dissection of the Zamboni-fixed trigeminal ganglia and the nasal mucosa samples from different locations of the nasal cavity was followed by rinses in 0.1 M phosphate buffer (pH 7.4) and cryoprotection with 18% sucrose in 0.1 M phosphate buffer overnight. Serial sections of the ganglia and nasal mucosa were cut on a cryostat (HM-500-O, Microm, Walldorf, Germany) to 8 μm and air dried for 30 min.

2.3. Immunohistochemistry for substance P, VR1 and I-B4-lectin binding

Ganglia and nasal sample sections were first incubated for 1 h with phosphate-buffered saline (PBS) containing 10% normal swine serum to block non-specific protein binding sites and 0.5% Tween 20 to enhance penetration of immunoreagents. Then, the sections were incubated overnight with a monoclonal antibody from rat raised against SP (Boehringer Ingelheim Heidelberg, Germany, dilution 1:400) in combination with a polyclonal antiserum to vanilloid receptor 1 from rabbit (Chemicon Inc. dilution 1:1000). After rinsing in phosphate buffer saline (PBS), a biotinylated sheep anti-rat-immunoglobulin (IgG) (Amersham, dilution 1:100) was used for 1 h. In separate studies, biotinylated I-B4-lectin (1:50; Sigma, Deisenhofen, Germany) was used to identify VR1-positive C-fiber neurons. After several washing steps, the tissues were incubated with the secondary reagents streptavidin-Texas-Red-conjugate (Amersham, dilution 1:200) and fluoresceinisothiocyanate (FITC)-conjugated goat anti-rabbit-IgG (Cappel, Ohio, USA, dilution 1:400) for 1 h. Finally, the slices were washed again in PBS and mounted in carbonate-buffered glycerol at pH 8.6. For immunohistochemical analysis, twenty randomly chosen sections were selected from the serial sections of every ganglia (14 ganglia of 7 animals) and all neurons with an apparent nucleus were analyzed by using an epifluorescence microscopy and appropriate filter combinations. Fast Blue was detected with the filter module U1 (excitation filter: band-pass 330–380 nm, barrier filter long-pass 418), Texas Red with the filter G1 module (band-pass 546/10 nm, long-pass 590 nm) and FITC with the B2 module (band-pass 455–490 nm, long-pass 515–560 nm).

To verify the immunoreactive specificity, pre-absorption studies of the polyclonal anti-vanilloid receptor

1 antiserum, as well as the monoclonal anti-SP antiserum with corresponding antigens was undertaken resulting in an absence of the labeling. Specific immunohistochemical signals also disappeared after replacement of the primary antisera by PBS or preimmune serum.

3. Results

3.1. Neuronal retrograde tracing

Fast blue dye was found in all layers of the nasal mucosa of the right but not of the contrary left nasal cavity. The dye appeared to diffuse through the basement membrane as it was also detected underneath the lamina propria of the nasal mucosa. Trigeminal neurons of all cell sizes were labeled with the tracer injected in the right nasal cavity, whereas labeled neurons in the left ganglion were very rare. Fast blue labeled neurons were predominantly located close to the division of the ophthalmic and maxillary nerves.

3.2. Immunohistochemistry for substance P, VR1 and I-B4-lectin reactivity in labeled sensory trigeminal sensory neurons and nasal mucosa

Within the ganglia of the right body side which project to the right nasal cavity, about 10–20 labeled neurons were found per section in every trigeminal ganglion examined. Some contra-lateral ganglia also displayed single traced neurons. SP-IR was present in 4.9 ± 1.1 (127/2605) of neuronal profiles, which were identified to innervate the right nasal mucosa by retrograde tracing. Within these retrogradely labeled perikarya, SP-IR was always co-localized with VR1-IR. VR1 was found to be more frequently expressed, and 6.4 ± 1.3 (167/2605) of the Fast Blue-labeled trigeminal sensory neurons and VR1-immunolabeled were negative for substance P-IR (Fig. 1). The vast majority of labeled trigeminal neurons was negative for both substance P-IR and VR1-IR. Most of them were found to be I-B4-lectin positive. Also, Fast blue negative neurons positive for I-B4-lectin-IR were abundantly present. Within the FB+/VR1+/SP+ neuronal population, no neurons were reactive to I-B4-lectin, whereas FB+/VR1+/SP− were partly positive for I-B4-lectin (Table 1, Figs. 1 and 2).

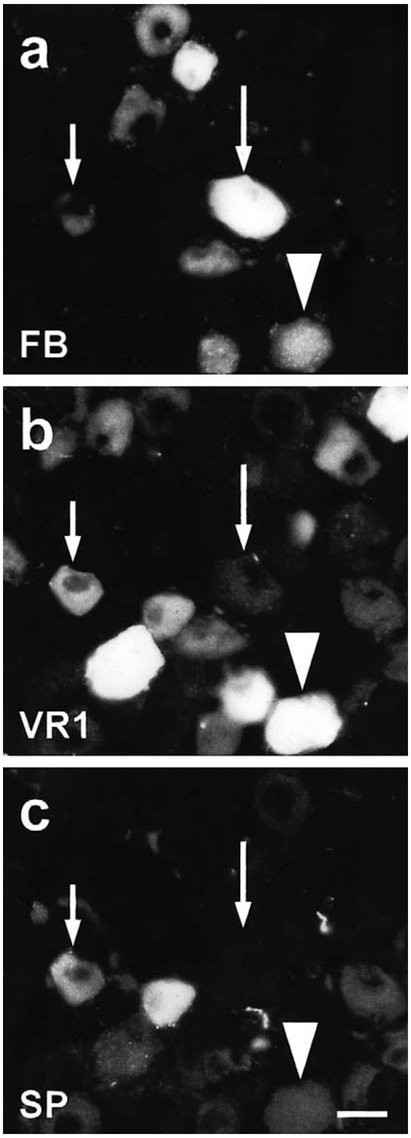

Fig. 1. Co-expression of SP- and VR1-immunoreactivities in retrogradely labeled neurons of trigeminal ganglion. Nasal mucosa-projecting murine neurons were identified by retrograde tracing (a) and examined for VR1 (b) and SP (c) by double-immunohistochemistry. Different combinations of SP- and VR1-expression were found: upper airway-projecting neurons simultaneously immunoreactive for SP (arrow in b) and VR1 (arrow in c), neurons positive for only VR1 and not for SP (arrowheads). A retrogradely labeled neuron which is negative for both SP- and VR1-immunoreactivities (large arrows). Scale bar represents 35 μm.

4. Discussion

In the present study, trigeminal sensory neurons innervating the nasal mucosa were identified by retrograde transport of Fast Blue dye from the nasal cavity. Using this technique of neuronal tracing, it is possible to investigate SP and VR1 receptor expression in upper airway-specific sensory neurons from the trigeminal ganglia that innervate the vessels and the epithelium of the nasal mucosa. Thus, these retrogradely identified sensory neurons are likely to be involved in mediating axon reflexes. The trigeminal sensory neurons innervating the nasal mucosa were predominantly located to a region close to the division of the ophthalmic and maxillary nerves as reported by a previous study (Hunter and Dey, 1998). This finding led to the as-

Table 1
Percentage of SP-and VR1-immunoreactivities in trigeminal sensory neurons projecting to the nasal mucosa

	Number of gangila	FB[a] (*n*)	FB/VR1[b] (%)	FB/SP/VR1[c] (%)	FB/SP[d] (%)
Trigeminal ganglion	7	2605	6.4 ± 1.3	4.9 ± 1.1	0

[a] Total number of retrogradely labeled neurons. Percentage of retrogradely labeled neurons which express.
[b] VR1 (vanilloid receptor 1).
[c] SP (substance P) and VR1.
[d] SP only. Data are expressed as means ± SEM. FB, Fast blue.

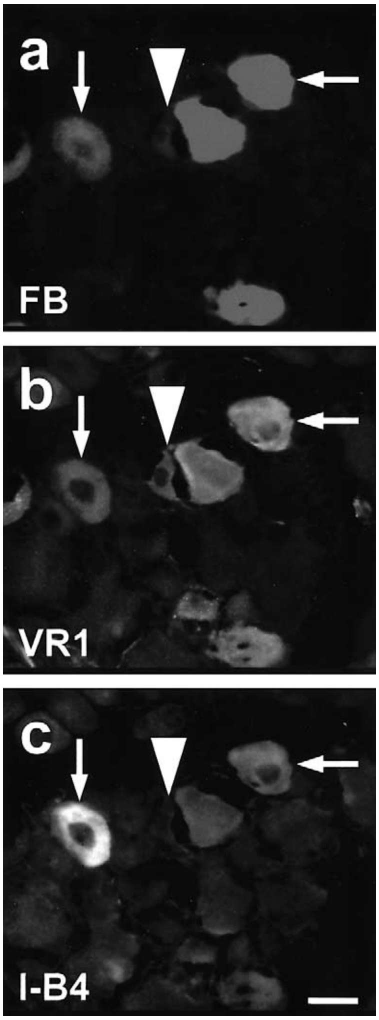

Fig. 2. Expression of VR1-immunoreactivity and I-B4-binding in retrogradely labeled trigeminal neurons. Nasal mucosa-projecting murine neurons were identified by retrograde tracing (a) and examined for VR1-expression (b) and binding to lectin I-B4 (c). Different combinations of lectin binding and VR1-expression were found: upper airway-projecting neurons simultaneously reactive for VR1 (arrow in b) and binding to I-B4 (arrow in c), or neurons positive for only VR1 but not binding to I-B4 (arrowheads). Scale bar represents 35 μm.

sumption that a somatotopic organization of these neurons within the trigeminal ganglion is very likely.

In the present study, primary afferent trigeminal neurons exhibiting SP immunosignaling displayed 4.9% of the total amount of retrogradely labeled trigeminal neurons. This percentage of SP-IR in neurons projecting to the nasal mucosa is relatively low if compared to the high percentage (81.6%) of trigeminal SP-positive neurons innervating the nasal epithelium of the species rat, as published previously (Hunter and Dey, 1998). Next to a species-specific difference which has been reported before for the neurochemical basis of the nervous system of different species such as the rat, guinea pig and mouse (Lazarov, 2002), this discrepancy may also arise from the different tracing substances used in the present and the previous studies. While the present experiments used Fast Blue which has the property to diffuse throughout the epithelium to the submucosa of the nasal mucosa, rhodamine-labeled latex microspheres as used previously for the rat study, is known to labeled only the epithelium (Hunter and Dey, 1998). In this respect, neurons specifically innervating the epithelial layer may express SP more frequently. Concerning species-specific differences, TG neurons displaying SP-IR have been previously reported to vary between different species including human and a percentage between 10% and 20% of the total neuronal population in the TG may be found (Lazarov, 2002). In the present study, substance P expression was examined in trigeminal sensory neurons of untreated, healthy, adult BALB/c-mice housed in a highly clean environment, since neuronal plasticity has been reported to be induced by a wide range of intrinsic and extrinsic factors such as airway inflammation, allergen sensitization and challenge or exogenously administered NGF (Fischer et al., 1996; Hunter et al., 2000; Undem et al., 1999). Concerning gender-based differences in SP or VR1 expression, the present study did not reveal significant differences.

Capsaicin-sensitive C-nerve fibers are likely to originate from the presently identified SP-containing trigeminal sensory neurons which represent the tachykinergic pathway to the nasal mucosa (Lundberg et al., 1984; Lundblad, 1984). Peripheral activation of these SP-expressing afferent nerve fibers can cause neurogenic inflammation which is characterized by increased vascular permeability, plasma extravasation, glandular secretion and chemotaxis of immune cells (Barnes, 1987; Barnes et al., 1991, 1998; Bertrand and Geppetti, 1996; Braunstein et al., 1994; Gawin et al., 1993; Lundberg et al., 1985; Nathan et al., 2001; Saria et al., 1988; Stjarne et al., 1989). In this respect, it was shown that SP levels were elevated in nasal lavages of allergic rhinitis patients as well as in the bronchial alveolar lavage fluid of animals with allergic airway inflammation (Fischer et al., 1996; Hunter et al., 2000; Nieber et al., 1992). Also, SP-positive nerve fibers were found to be increased in hyperreflectoric rhinitis suggesting a modulatory role of SP during nasal inflammation (Heppt et al., 2002).

The major aim of the present study was to investigate the distribution of VR1 in relation to SP in afferents of the nasal mucosa. Interestingly, all SP-positive sensory neurons also expressed VR1. This extent of presently examined total co-localization of SP and VR1 may indicate a functional role of VR1 in SP biosynthesis and release (Trevisani et al., 2002). In this context, using a VR1 antagonist termed capsazepin, it was shown that SP release from sensory nerves can be efficiently inhibited (McVey and Vigna, 2001; Nathan et al., 2001; Trevisani et al., 2002). The present neurochemical findings support the hypothesis, that the SP-pathway may be partly VR1-dependent (Trevisani et al., 2002). However, also VR1-positive, SP-negative neurons were found to innervate the nasal mucosa and these neurons were both reactive or unreactive to I-B4-lectin. Some of these neurons may therefore, belong to the non-peptidergic C-fiber population.

In conclusion, based on the extent of SP and VR1 co-localization in nasal sensory neurons, the present study suggests that, following a peripheral activation of the VR1 receptor on SP afferents, there may be a triggering of SP-mediated phenomena such as neurogenic inflammation.

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