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Characterization of Tachykinin Receptors in Human and Rat Skin

Masami Deguchi

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Receptors for the tachykinin family of peptides were investigated in human and rat skin areas using quantitative receptor autoradiography and emulsion autoradiography with \(^{125}\text{I}-\text{Bolton-Hunter substance P} (\^{125}\text{I}-\text{BH-substance P})\), a non-selective radioligand, and \(^{125}\text{I}-\text{Bolton-Hunter eledoisin} (\^{125}\text{I}-\text{BH-eledoisin})\), a selective radioligand for the tachykinin NK-2 receptor. There were no differences between localization of specific \(^{125}\text{I}-\text{BH-substance P}\) and \(^{125}\text{I}-\text{BH-eledoisin}\) binding sites in human and rat skin. The highest densities of the binding sites were in the dermal papillae of the human finger pad skin and the rat paw pad skin. In cold-ligand saturation experiments done in the presence of increasing concentrations of unlabeled substance P, \(^{125}\text{I}-\text{BH-substance P}\) binding to the rat and human dermal papillae was single and of a high affinity, \(^{125}\text{I}-\text{BH-eledoisin}\) bound to the dermal papillae with a much lower affinity than \(^{125}\text{I}-\text{BH-substance P}\) did. The rank order of potency of unlabeled tachykinin peptides to displace \(^{125}\text{I}-\text{BH-substance P}\) binding to the dermal papillae of the human finger pad skin and rat paw pad skin was substance P > eledoisin > neurokinin A >> neurokinin B. Thus, the methods we used revealed that the tachykinin NK-1 receptor is predominantly present in the skin dermal papilla.

Introduction

Primary sensory neurons with substance P, a member of the tachykinin family of peptides, originating from the dorsal root ganglia are widely distributed in skin. Skin organelles, such as sweat glands, hair follicles, and Meissner’s corpuscles are richly innervated by substance P-containing neurons. In addition to the organelles, skin blood vessels are possible target sites of the neurons, as evidence has revealed the pathophysiological significance of substance P in cutaneous inflammatory responses including vasodilation and plasma protein extravasation. Immunohistochemical studies have led to identification of free nerve endings of substance P neurons within dermal papilla, a finding which supports the role of substance P in pain transmission, as mediated by nociceptor in skin.

The mammalian tachykinin family of peptides, substance P, neurokinin A, neurokinin B, neuropeptide K and neuropeptide \(\gamma\), which have an amino acid sequence, Phe-X-Gly-Leu-Met at the C terminus in common, exert physiological functions, by interacting with at least three subtypes of receptors, the NK-1, the NK-2 and the NK-3 receptors. In contrast to morphological and functional findings, far less is known of properties of tachykinin receptors in areas of the skin. Using the quantitative receptor autoradiographic method with two radioligands, \(^{125}\text{I}-\text{Bolton-Hunter substance P} (\^{125}\text{I}-\text{BH-substance P})\), a radioligand for the tachykinin receptors, and \(^{125}\text{I}-\text{Bolton-Hunter eledoisin} (\^{125}\text{I}-\text{BH-eledoisin})\), a radioligand for the NK-2 receptor, we investigated tachykinin receptors in rat and human skin.

Materials and Methods

Materials

\(^{125}\text{I}-\text{BH-substance P}\) and \(^{125}\text{I}-\text{BH-eledoisin}\) were purchased from the New England Nuclear, U.S.A., and peptides and drugs used were from the Peninsula lab., U.S.A., the Sigma Chemical Co., U.S.A., and the Peptide Institute, Japan.

Male Wistar rats weighting 250g to 300g were given standards chow (F-2, Funabashi Farm Co., Japan) and water ad libitum and were housed at 24°C, with lights on from 07:00 hr to 19:00 hr at the laboratory Animal Center for the Biomedical Research, Nagasaki University School of Medicine, Japan. The rats were decapitated between 10:00 hr and 12:00 hr, and skins from the dorsum, abdomen and paw pad were immediately removed. Human dorsal and pad skins of a finger and toe were obtained from 7-, 12- and 18-month-old boys and girls with polydactyly. Human scalp skins were surgically dissected from three patients undergoing treatment for alopecia. Rat and human skins were immersed at -30°C, and stored at -80°C. Frozen sections, 16-\(\mu\text{m}\)-thick, were cut on a cryostat at -16°C, thaw-mounted on gelatin-coated glass slides and stored overnight under vaccum at 4°C.

Quantitative Receptor Autoradiography

Related tissue sections were labeled in vitro with \(^{125}\text{I}-\text{BH-substance P}\) (specific activity, -81.4 TBq/mmol) or \(^{125}\text{I}-\text{BH-eledoisin}\) (specific activity, -81.4 TBq/mmol) in 2.0 ml of incubation box, under the conditions described. After preincubation in the incubation buffer at room temperature...
(23°C) for 15 min, related tissue sections were incubated with the radioligands at 23°C for 90 min in 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 mg/ml bovine serum albumin, 4 μg/ml bacitracin, 4 μg/ml leupeptin, 50 μg/ml chymostatin, and 5 mM MnCl2. Non-specific binding was determined in adjacent sections, incubated with the same amounts of the radioligands in the presence of 1.0 μM unlabeled substance P, eledoisin, neurokinin A or neurokinin B. To characterize the binding sites, we incubated consecutive, related sections with a fixed amount of the radioligands in the absence or presence of increasing concentrations of unlabeled peptides, ranging from 1.0 pM to 10 μM. After incubation, the slides were washed four times (30 sec each) at 4°C in 50 mM Tris-HCl buffer (pH 7.4) and rinsed quickly in ice-cold distilled water. Sections were dried under a stream of cold air and exposed to [3H]-Ultralight film (LKB, U. S. A.) with calibrated [3H]-standards ([3H] micro-scale, Amersham). The films were developed at 4°C for 7 min, using a D19 developer (Eastman Kodak, U.S.A.). The optical densities, measured by computerized microdensitometry (UHG-101, Unique Medical Co., Japan), were related to the concentration of radioactivity, as based on a comparison with standard curves generated from processing sets of standards with each autoradiogram.

**Emulsion autoradiography**

To observe cellular localization of [3H]-BH-substance P and [3H]-BH-eledoisin binding sites, we performed emulsion autoradiography. After exposure to [3H]-Ultralight film, related tissue sections were washed three times (15 sec each) in cold (4°C) distilled water. Sections were defatted in xylene and alcohol rinses, and then dipped into Kodak NTB-3 nuclear emulsion (Eastman Kodak, U.S.A.). Coated slides were stored in dark at 4°C for 5 days in the case of [3H]-BH-substance P binding and for 10 days for [3H]-BH-eledoisin binding. After these exposure, the slides were developed at 16°C for 5 min, using a D19 developer and counterstained with eosin, and coverslips were added.

**Data analysis**

The data obtained by quantitative receptor autoradiographic studies were analyzed using the LIGAND computer program. Results were expressed as means ± S.E.M. Differences in the data were assessed by one-way analysis of variance (ANOVA) using the F test.

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**Results**

**Localization of [3H]-BH-substance P and [3H]-BH-eledoisin binding sites in human and rat skin**

Fig. 1 shows receptor autoradiographic localization of [3H]-BH-substance P binding sites in the finger pad skin obtained from a thumb excised from a 12-month-old boy with polydactyly (A), and from a rat paw pad skin (B). High densities of [3H]-BH-substance P binding sites were present in the dermal papilla and sweat gland. [3H]-BH-eledoisin also richly labeled the dermal papilla and sweat gland. There were no differences between the receptor autoradiographic localization of [3H]-BH-substance P and [3H]-BH-eledoisin binding sites in human and rat skin. Non-specific binding sites were detected in adjacent sections when the incubations were carried out in the presence of 1.0 μM unlabeled substance P and eledoisin, respectively (Figs. 1 and 2 #2 panel).

Light microscopic examination of emulsion autoradiography revealed [3H]-BH-substance P binding in the dermal papilla of the human finger pan skin to be over the vascular endothelial cells (arrow A in Fig. 3) and areas devoid of vascular components (arrow B). We compared binding sites of [3H]-BH-substance P with those of [3H]-BH-eledoisin. As shown in Fig. 4, cellular localization of [3H]-BH-eledoisin binding sites was comparable to that of [3H]-BH-substance P.
Fig. 2. Typical receptor autoradiograms of \(^{125}\text{I}-\text{Bolton-Hunter eledoisin}\) \((^{125}\text{I}-\text{BH-eledoisin})\) binding sites in the finger pad skin obtained from a rat paw pad skin (A) and from a thumb excised from a 7-month-old with polydactylia (B). Consecutive, 16-\(\mu\text{m}\)-thick tissue sections were incubated with 350 pM \(^{125}\text{I}-\text{BH-eledoisin}\), without (total binding, 2) or with 10 \(\mu\text{M}\) unlabeled eledoisin (non-specific binding, 3). Dried sections were exposed to \(^{3}\text{H-Ultrofilm}\) for 10 days. Adjacent sections were stained with hematoxylin-eosin to observe the anatomy (1). DP, dermal papilla; SG, sweat gland. Bar = 1.0 mm

Fig. 4. An emulsion autoradiogram of \(^{125}\text{I}-\text{Bolton-Hunter eledoisin}\) \((^{125}\text{I}-\text{BH-eledoisin})\) binding to dermal papilla of the finger pad skin obtained from a thumb excised from a 7-month-old boy with polydactylia (upper panel). Dots indicated by arrows A and B represent \(^{125}\text{I}-\text{BH-eledoisin}\) binding to the capillary endothelium and areas which lack vascular components, respectively. A dark-field photomicrograph of the autoradiogram are shown in the lower panel of the figure. DP, dermal papilla; E, epidermis. Bar = 40 \(\mu\text{m}\).

Concentrations of specific \(^{125}\text{I}-\text{BH-substance P}\) and \(^{125}\text{I}-\text{BH-eledoisin}\) binding sites in human and rat skin

Apparent specific \(^{125}\text{I}-\text{BH-substance P}\) and \(^{125}\text{I}-\text{BH-eledoisin}\) binding concentrations in human and rat skin were calculated by subtracting non-specific binding concentrations obtained at incubation with 100 pM of the radioligands in the presence of 1.0 \(\mu\text{M}\) unlabeled substance P or 10 \(\mu\text{M}\) eledoisin from total binding concentrations (Table 1). Among the human skin tissues examined, the highest \(^{125}\text{I}-\text{BH-substance P}\) and \(^{125}\text{I}-\text{BH-eledoisin}\) binding densities were noted in the dermal papilla of the finger pad skin. In case of rat skin, the paw pad skin had the highest densities of both radioligands in the dermal papilla and the sweat gland. Therefore, we focused the following binding experiments on the dermal papillae of the rat paw pad skin and...
Table 1. Quantitative determination of $^{125}$I-Bolton-Hunter substance P ($^{125}$I-BH-SP) and $^{125}$I-Bolton-Hunter eledoisin ($^{125}$I-BH-EL) binding sites in human and rat skin. Sections were incubated with 100 pM of the radiolabeled ligands. Optical densities were converted into fmo/mg. Non-specific binding was subtracted from all readings, individually. Results are means ± S.E.M. of findings in 3-6 human and rat skin samples.

<table>
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<tr>
<th></th>
<th>Dermal papilla</th>
<th>Sweat gland</th>
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<tbody>
<tr>
<td></td>
<td>$^{125}$I-BH-SP</td>
<td>$^{125}$I-BH-EL</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scalp skin (3)</td>
<td>1.39 ± 0.43</td>
<td>0.40 ± 0.16</td>
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<tr>
<td>Finger dorsal skin (6)</td>
<td>2.52 ± 0.52</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>Finger pad skin (6)</td>
<td>2.76 ± 0.14</td>
<td>0.54 ± 0.15</td>
</tr>
<tr>
<td>Toe tip skin (6)</td>
<td>2.33 ± 0.62</td>
<td>0.36 ± 0.11</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Back skin (6)</td>
<td>1.04 ± 0.24</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td>Abdominal skin (6)</td>
<td>0.58 ± 0.13</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Paw pad skin (6)</td>
<td>2.10 ± 0.34</td>
<td>0.36 ± 0.09</td>
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*Data on binding sites in the hair follicle.

Characteristics of $^{125}$I-BH-substance P and $^{125}$I-BH-eledoisin binding to dermal papillae of rat paw pad and human finger pad skin

Specific $^{125}$I-BH-substance P and $^{125}$I-BH-eledoisin binding characteristics in the dermal papillae of the rat paw pad skin and the human finger pad skin were examined in a cold-ligand saturation study, in the presence of fixed amounts of the radioligands, 85 pM or 94 pM $^{125}$I-BH-substance P, and 350 pM pM $^{125}$I-BH-eledoisin, respectively, and increasing concentrations of unlabeled substance P or eledoisin, ranging from 10 pM to 10 μM. $^{125}$I-BH-substance P binding to the dermal papillae of the rat paw pad skin and the human finger pad skin was displaced by unlabeled substance P, with a high affinity (Fig. 5, inset). The program LIGAND fitted the data to a one-site model significantly better than it did to a two-site model, as evidenced by the straight line of the Scatchard plot (Fig. 5). $^{125}$I-BH-substance P thus presumably bound to a single population of sites in the dermal papillae of the rat paw pad skin and the human finger pad skin, with a dissociation constant (Kd) of 297 pM and 744 pM, respectively.

$^{125}$I-BH-eledoisin binding to the dermal papillae of the rat paw pad skin and the human finger pad skin was also saturable and single (Fig. 6 and Fig. 7). However, in contrast to $^{125}$I-BH-substance P binding, the affinities of $^{125}$I-BH-eledoisin by unlabeled eledoisin (inset in the figure), analyzed using the computer program LIGAND.
Fig. 7. A typical cold-ligand saturation experiment of specific $^{125}$I-Bolton-Hunter eledoisin ($^{125}$I-BH-eledoisin) binding to the dermal papilla of the rat paw pad skin (radioligand concentration, 350 pM). A Scatchard plot was obtained by displacing the binding of $^{125}$I-BH-eledoisin by unlabeled eledoisin (inset in the figure), analyzed using the computer program LIGAND. The binding characteristics, dissociation constant ($K_d$) and maximum binding capacity ($B_{max}$) were calculated to be 16.5 nM and 9.2 fmol/mg. BH-eledoisin binding calculated were much lower. Furthermore, the radioligand for the tachykinin NK-2 receptor bound to the dermal papilla of the rat paw pad skin with a higher affinity than it did to the human finger pad skin. The binding parameters, $K_d$ and maximum binding capacity ($B_{max}$) of specific $^{125}$I-BH-substance P and $^{125}$I-BH-eledoisin binding to the dermal papillae of the rat paw pad skin and the human finger pad skin are listed in Table 2.

Table 2. Binding parameters, dissociation constant ($K_d$) and maximum binding capacity ($B_{max}$) of specific $^{125}$I-BH-substance P and $^{125}$I-BH-eledoisin binding to dermal papillae of rat paw pad skin and human finger pad skin. Results are means ± S.E.M. of findings in 3 human and 6 rat skin.

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<tr>
<th>Peptide</th>
<th>Rat</th>
<th>Human</th>
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<tr>
<td>$^{125}$I-BH-substance P</td>
<td>$K_d$ (nM)</td>
<td>0.30 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>$B_{max}$ (fmol/mg)</td>
<td>11.3 ± 3.5</td>
</tr>
<tr>
<td>$^{125}$I-BH-eledoisin</td>
<td>$K_d$ (nM)</td>
<td>13.3 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>$B_{max}$ (fmol/mg)</td>
<td>7.5 ± 2.6</td>
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$P < 0.01 (a < c, b < d)$. We then compared the potencies of the tachykinin family of peptides, substance P, eledoisin, neurokinin A and neurokinin B, at displacing $^{125}$I-BH-substance P binding to the dermal papillae of the rat paw pad skin and the human finger pad skin (Fig. 8 and Table 3). $^{125}$I-BH-substance P binding was potently inhibited by substance P. The potency of eledoisin for displacing binding was lower than that of substance P. Neurokinin B was the weakest inhibitor, however, interestingly, the peptide with a high affinity for the NK-3 receptors displaced $^{125}$I-BH-substance P binding to the rat dermal papilla more potently (Fig. 8 right side panel) than to the human dermal papilla (left side panel). Also, we noted the less potency of neurokinin A, a tachykinin for the NK-2 receptor, at displacing $^{125}$I-BH-substance P binding to the human dermal papilla, as compared with that in the case of binding to the rat dermal papilla. Thus, the rank order potency to displace $^{125}$I-BH-substance P binding to the rat and human dermal papillae was substance P > eledoisin > neurokinin A > neurokinin B, and substance P >> eledoisin >> neurokinin A >> neurokinin B, respectively.

Table 3. Affinities (IC$_{50}$, nM) of related tachykinins for specific $^{125}$I-Bolton-Hunter substance P ($^{125}$I-BH-substance P) binding to dermal papillae of rat paw pad skin and human finger pad skin. $IC_{50}$ is concentration of peptide displacing 50% of specifically bound $^{125}$I-BH-substance P. Results are means ± S.E.M. of 3 determinations.

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<tr>
<th>Peptide</th>
<th>Rat IC$_{50}$ (nM)</th>
<th>Human IC$_{50}$ (nM)</th>
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<tr>
<td>substance P</td>
<td>0.99 ± 0.56</td>
<td>0.72 ± 0.33</td>
</tr>
<tr>
<td>eledoisin</td>
<td>9.32 ± 2.56</td>
<td>9.88 ± 5.43</td>
</tr>
<tr>
<td>neurokinin A</td>
<td>92.7 ± 15.3</td>
<td>960 ± 249</td>
</tr>
<tr>
<td>neurokinin B</td>
<td>730 ± 195 x 10</td>
<td>&gt; 10$^4$</td>
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$P < 0.01 (a < c, b < d)$. We then compared the potencies of the tachykinin family of peptides, substance P, eledoisin, neurokinin A and neurokinin B, at displacing $^{125}$I-BH-substance P binding to the dermal papillae of the rat paw pad skin and the human finger pad skin (Fig. 8 and Table 3). $^{125}$I-BH-substance P binding was potently inhibited by substance P. The potency of eledoisin for displacing binding was lower than that of substance P. Neurokinin B was the weakest inhibitor, however, interestingly, the peptide with a high affinity for the NK-3 receptors displaced $^{125}$I-BH-substance P binding to the rat dermal papilla more potently (Fig. 8 right side panel) than to the human dermal papilla (left side panel). Also, we noted the less potency of neurokinin A, a tachykinin for the NK-2 receptor, at displacing $^{125}$I-BH-substance P binding to the human dermal papilla, as compared with that in the case of binding to the rat dermal papilla. Thus, the rank order potency to displace $^{125}$I-BH-substance P binding to the rat and human dermal papillae was substance P > eledoisin > neurokinin A > neurokinin B, and substance P >> eledoisin >> neurokinin A >> neurokinin B, respectively.

**Discussion**

The mammalian tachykinin family of peptides, substance P, neurokinin A, neurokinin B, neuropeptide K and neuropeptide γ, participate in physiological functions as neuro transmitters in the central and peripheral nervous system, and chemical mediators related to inflammatory
responses.22 These peptides increase cytosolic free Ca\(^{2+}\) concentration in target cells, presumably by interacting with at least three types of specific receptors, functionally linked to the activation of phospholipase C and to the mobilization of intracellular Ca\(^{2+}\). Three distinct receptors, classified recently as three subtypes\(^{13,14,15}\), the NK-1 receptor, preferentially recognized by substance P with a much higher affinity than neurokinin A and neurokinin B, the NK-2 receptor, which is mainly activated by neuropeptide γ and is bound by eleidoisin with a high affinity, and the third type of the NK-3 receptor.

These receptors have been extensively studied in the central nervous system and the gastrointestinal tract system.\(^{15,25,26,27,28}\) However, there is little information on the receptor, as related to skin, although it has been established that sensory nerves are involved in cutaneous inflammatory responses including vasodilation and plasma protein extravasation.\(^{5,9}\) We found specific \(^{125}\)I-BH-substance P and \(^{125}\)I-BH-eledoisin binding sites to be discretely localized in rat and human skin areas correspondingly anatomically to the dermal papilla. As presented, \(^{125}\)I-BH-substance P specifically bound to the dermal papilla with a K\(_d\) value in a subnanomolar range, and the K\(_d\) of the binding sites for \(^{125}\)I-BH-eledoisin, a radioligand for the NK-2 receptor,\(^{14}\) was calculated to have a supernanomolar value. Our emulsion autoradiography revealed that there were no differences in cellular localization of \(^{125}\)I-BH-substance P and \(^{125}\)I-BH-eledoisin binding sites. Taken together with the observation that neurokinin A, an endogenous ligand for the tachykinin NK-2 receptor,\(^{20}\) and neurokinin B, a selective ligand for the NK-3 receptor,\(^{26}\) were weak displacers for \(^{125}\)I-BH-substance P binding, it may be true that specific \(^{125}\)I-BH-substance P binding sites present in the dermal papilla is characteristically the NK-1 receptor.

We also noted the difference between the affinity of neurokinin B at displacing \(^{125}\)I-BH-substance P binding to the rat and human dermal papillae. This may be evidence suggesting the existence of a further heterogeneity of the NK-1 receptor. Moreover, \(^{125}\)I-BH-eledoisin bound to the rat dermal papilla with a much higher affinity than it did to the human dermal papilla. The possibility of the existence of a tiny amount of the NK-2 receptor in the rat dermal papilla is not ruled out. Neurokinin A-containing sensory neurons coexisting with substance P-containing neurons are present in the rat skin.\(^{19}\)

Density and localization of the tachykinin NK-1 receptor correspond to the distribution of substance P-containing neurons in the skin dermal papilla.\(^{4,29,30}\) This differed from evidence on the receptor-transmitter mismatch in the substance P system in the central nervous system.\(^{30}\)

Of particular interest was the present observation that the highest density of the tachykinin receptor was in the dermal papilla, since the area contains substantial numbers of substance P-containing free nerve endings.\(^{4,14}\) The free nerve endings are thought to be a nociceceptor exerting pain

transmission.\(^{9,30}\) Taking note of the present observation that the tachykinin receptors are in an area of the dermal papilla which lacks the vascular component such as the capillary endothelium, the possibility that the tachykinin receptors are located on presynaptic sites of the free nerve endings would have to be considered. Furthermore, the number of the receptor in the dermal papilla was the highest in the finger pad skin, an area most richly innervated by primary sensory neurons, as compared with abdominal, dorsal, and scalp skin.\(^4,30\) Therefore, substance P released from free nerve endings may relate to pain transmission, by interacting with the tachykinin NK-1 receptor.

Substance P released by antidromic stimulation to axon collaterals of the primary sensory neurons seems to act on the capillary epithelium, and results in an increase in plasma extravasation.\(^{32,33}\) If such is indeed the case, then this would explain mechanisms related to neurogenic inflammation. There is the view that substance P participates in this phenomenon by stimulating receptors present in mast cells to release histamine.\(^{30}\) Our evidence for the existence of the tachykinin NK-1 receptor over the capillary epithelium indicates that substance P may be involved in neurogenic inflammation.

In summary, we have obtained evidence to support the idea that tachykinin functions within the skin dermal papilla as a neurotransmitter and/or neuromodulator. Although the NK-1 receptor seems to be predominant, the existence of the other subtype of the tachykinin receptors cannot be excluded. The precise heterogeneity of the receptors can be elucidated by in situ hybridization technique with the respective receptor gene probe.

Acknowledgements

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