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Characterization of Tachykinin Receptors in Human and Rat Skin

Masami Deguchi

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Materials and Methods

Materials

$^{125}$I-BH-substance P and $^{125}$I-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 polydactylia. 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-μm-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 $^{125}$I-BH-substance P (specific activity, -81.4 TBq/mmol) or $^{125}$I-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-Ultrofilm (LKB, U. S. A.) with calibrated ²⁵I-standards ([²⁵I 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 processing sets of standards with each autoradiogram.¹⁷

Emulsion autoradiography
To observe cellular localization of ²⁵I-BH-substance P and ²⁵I-BH-eledoisin binding sites, we performed emulsion autoradiography.¹⁸ After exposure to ³H-Ultrofilm, 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 ²⁵I-BH-substance P binding and for 10 days for ²⁵I-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.¹⁷ 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.

Results

Localization of ²⁵I-BH-substance P and ²⁵I-BH-eledoisin binding sites in human and rat skin
Fig. 1 shows receptor autoradiographic localization of ²⁵I-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 ²⁵I-BH-substance P binding sites were present in the dermal papilla and sweat gland. ²⁵I-BH-eledoisin also richly labeled the dermal papilla and sweat gland. There were no differences between the receptor autoradiographic localization of ²⁵I-BH-substance P and ²⁵I-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 ²⁵I-BH-substance P with those of ²⁵I-BH-eledoisin. As shown in Fig. 4, cellular localization of ²⁵I-BH-eledoisin binding sites was comparable to that of ²⁵I-BH-substance P.

Fig. 1. Typical receptor autoradiograms of ²⁵I-Bolton-Hunter substance P (²⁵I-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). Consecutive, 16-μm-thick tissue sections were incubated with 100 pM ²⁵I-BH-substance P, without (total binding, 2) or with 1.0 μM unlabeled substance P (non-specific binding, 3). Dried sections were exposed to ³H-Hyperfilm for 4 days. Adjacent sections were stained with hematoxylin-eosin to observe the anatomy (1). DP, dermal papilla; SG, sweat gland. Bar = 1.0 mm.
Fig. 2. Typical receptor autoradiograms of $^{125}$I-Bolton-Hunter eledoisin ($^{125}$I-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 polydactyia (B). Consecutive, 16-μm-thick tissue sections were incubated with 350 pM $^{125}$I-BH-eledoisin, without (total binding, 2) or with 10 μM unlabeled eledoisin (non-specific binding, 3). Dried sections were exposed to $^{3}$H-Ul trofilm 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. 3. An emulsion autoradiogram of $^{125}$I-Bolton-Hunter substance P ($^{125}$I-BH-substance P) binding to dermal papilla of the finger pad skin obtained from a thumb excised from a 12-month-old boy with polydactyia. Dots indicated by arrows A and B represent $^{125}$I-BH-substance P binding to the capillary endothelium and areas which lack vascular components, respectively. N, nucleus of capillary endothelial cells; DP, dermal papilla; E, epidermis. Bar = 40 μm.

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

Apparent specific $^{125}$I-BH-substance P and $^{125}$I-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 μM unlabeled substance P or 10 μM eledoisin from total binding concentrations (Table 1). Among the human skin tissues examined, the highest $^{125}$I-BH-substance P and $^{125}$I-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 fmol/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>
<thead>
<tr>
<th></th>
<th>Dermal papilla</th>
<th>Dermal papilla</th>
<th>Sweat gland</th>
<th>Sweat gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{125}$I-BH-SP</td>
<td>$^{125}$I-BH-EL</td>
<td>$^{125}$I-BH-SP</td>
<td>$^{125}$I-BH-EL</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scalp skin (3)</td>
<td>1.39 ± 0.43</td>
<td>0.40 ± 0.16</td>
<td>(0.08 ± 0.45*)</td>
<td>(0.09 ± 0.05*)</td>
</tr>
<tr>
<td>Finger dorsal skin (6)</td>
<td>2.52 ± 0.52</td>
<td>0.28 ± 0.09</td>
<td>2.08 ± 0.31</td>
<td>0.26 ± 0.11</td>
</tr>
<tr>
<td>Finger pad skin (6)</td>
<td>2.76 ± 0.14</td>
<td>0.54 ± 0.15</td>
<td>2.41 ± 0.32</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>Toe tip skin (6)</td>
<td>2.33 ± 0.62</td>
<td>0.36 ± 0.11</td>
<td>1.63 ± 0.70</td>
<td>0.21 ± 0.10</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Back skin (6)</td>
<td>1.04 ± 0.24</td>
<td>0.13 ± 0.06</td>
<td>0.47 ± 0.45</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Abdominal skin (6)</td>
<td>0.58 ± 0.13</td>
<td>0.07 ± 0.03</td>
<td>0.30 ± 0.18</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Paw pad skin (6)</td>
<td>2.10 ± 0.34</td>
<td>0.36 ± 0.09</td>
<td>2.04 ± 0.61</td>
<td>0.23 ± 0.15</td>
</tr>
</tbody>
</table>

*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 $^{125}$I-BH-eledoisin, respectively, and increasing concentrations of unlabeled substance P or eledoisin, ranging from 10 pM to 10 uM. $^{125}$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 ($K_d$) 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 affinites of $^{125}$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 ($K_d$) of 297 pM and 744 pM, respectively.

Fig. 5. A typical cold-ligand saturation experiment of specific $^{125}$I-Bolton-Hunter substance P ($^{125}$I-BH-substance P) binding to the dermal papillae of the finger pad skin obtained from a thumb excised from a 12-month-old boy with polydactyly (0) (94 pM) and the rat paw pad skin (●) (85 pM). Scatchard plots were obtained by displacing the binding of $^{125}$I-BH-substance P by unlabeled substance P (inset in the figure), analyzed using the computer program LIGAND.

Fig. 6. A typical cold-ligand saturation specific experiment of $^{125}$I-Bolton-Hunter eledoisin ($^{125}$I-BH-eledoisin) binding to the dermal papillae of the finger pad skin obtained from a thumb excised from a 7-month-old boy with polydactyly (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.
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 were calculated 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.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rat ($K_d$)</th>
<th>Human ($K_d$)</th>
<th>Rat ($B_{max}$)</th>
<th>Human ($B_{max}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}$I-BH-substance P</td>
<td>0.30 ± 10</td>
<td>0.74 ± 0.70</td>
<td>13.3 ± 4.3^a</td>
<td>224 ± 160^a</td>
</tr>
<tr>
<td>$^{125}$I-BH-eledoisin</td>
<td>1.3 ± 3.5</td>
<td>8.2 ± 2.0</td>
<td>7.5 ± 2.6^a</td>
<td>225 ± 123^a</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. Results are means ± S.E.M. of 3 determinations.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rat ($IC_{50}$)</th>
<th>Human ($IC_{50}$)</th>
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<tbody>
<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).

Discussion

The mammalian tachykinin family of peptides, substance P, neurokinin A, neurokinin B, neuropeptide K and neuropeptide γ, participate in physiological functions as neurotransmitters in the central and peripheral nervous system, and chemical mediators related to inflammatory
responses. 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\(^{2+}\); 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 \(\gamma\) and is bound by eledoisin 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.\(^{15,17,18,25,27}\) 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-eledoisin 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, 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, and neurokinin B, a selective ligand for the NK-3 receptor, 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.\(^{4,20,30}\) This differed from evidence on the receptor-transmitter mismatch in the substance P system in the central nervous system.\(^{31}\)

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.\(^{4,20}\) The free nerve endings are thought to be a nociceptor exerting pain transmission.\(^{9,20}\) 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.\(^{4,20}\) 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.\(^{34}\) 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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