Use of NK\textsubscript{1} knockout mice to analyze substance P-induced edema formation

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Cao, Thong, Norma P. Gerard, and Susan D. Brain. Use of NK\textsubscript{1} knockout mice to analyze substance P-induced edema formation. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R476–R481, 1999.—The mechanisms involved in tachykinin-induced neurokinin-1 (NK\textsubscript{1}) receptor-mediated edema formation have been studied in anesthetized wild-type and NK\textsubscript{1} knockout mice. Intradermally injected substance P (30-300 pmol), NK\textsubscript{1} agonists septide (3–30 pmol) and GR-73632 (3–30 pmol), and the mast cell-degranulating agent, compound 48/80 induced dose-dependent edema in wild-type skin, measured by the accumulation of intravenously injected \textsuperscript{125}I-labeled albumin. Septide was 3–10× more potent than substance P. The tachykinins were inactive in knockout mice, but compound 48/80 induced a significantly greater edema (P < 0.05) than that observed in paired wild-type mice. Capsaicin (which releases endogenous neuropeptides) and exogenous tachykinins induced edema formation, which was reduced by the mast cell amine histamine H\textsubscript{1} antagonist mepyramine (P < 0.05). These findings confirm that tachykinins mediate edema formation via the NK\textsubscript{1} receptor and provide direct evidence that the septide-sensitive binding site is on the NK\textsubscript{1} receptor. Furthermore, results suggest that edema induced by the tachykinins, although totally dependent on NK\textsubscript{1} receptor-mediated mechanism, contains a mast cell-dependent component. The evidence is in keeping with an NK\textsubscript{1} receptor on mast cells.

neurokinin-1 receptor; substance P; neurokinin-1 knockout mouse; edema formation; rat skin; tachykinins; neurokinins

The tachykinin substance P is contained in and released from sensory nerves (mainly C fibers) in various tissues, including skin. The release of neuropeptides from nerves such as the saphenous nerve in rat skin leads to an acute inflammatory response that is observed as edema formation (24). This is a consequence of increased microvascular permeability, which, in combination with an adequate blood flow, leads to substantial cutaneous edema formation (6, 15). Tachykinins (e.g., substance P), and in addition the potent vasodilator neuropeptide caldocrin gene-related peptide (CGRP), play major roles in these responses (6, 12, 14, 23).

Substance P can act via two mechanisms to mediate increased microvascular permeability. The neurokinin-1 (NK\textsubscript{1}) receptor mechanism is the most common, and it is generally considered that the receptor is situated on postcapillary venule endothelial cells. However, there is evidence for the presence of NK\textsubscript{1} receptors on mast cells, for example in the guinea pig lung (25) and in a cloned mast cell line (22). By comparison, it is considered that the most usual interaction by which substance P can mediate mast cell-dependent edema formation is via a sialic acid residue on the mast cell membrane that interacts with the basic N-terminal of substance P (13, 17). This is a receptor-independent mechanism by which substance P, together with other peptides and the pharmacological tool compound 48/80, can activate mast cells and stimulate vasoactive responses that include the triple response in human skin (19). On the other hand, on the basis of studies of human skin slices and rat skin, it is suggested that endogenous substance P cannot activate mast cells via these mechanisms (21, 29).

The neurokinin receptors are members of the seven transmembrane receptor superfamily, and mammalian neurokinins all show some degree of affinity for each of the receptors, with the traditionally established order of potency for the NK\textsubscript{1} receptor being substance P > neurokinin A > neurokinin B (for review see Ref. 26). There is evidence for a binding site for the NK\textsubscript{1} agonist septide, which is separate from that of substance P, and a distinct receptor subtype has been suggested (26–28). In more recent studies results have indicated that the NK\textsubscript{1} receptor is able to express two different conformations, as opposed to there being two different NK\textsubscript{1} receptors (11). These findings are relevant to skin in that septide has been shown to be more potent than substance P at inducing edema formation in rat skin (2).

The development of transgenic animals has allowed the involvement of the NK\textsubscript{1} receptor in pathophysiological processes to be examined. NK\textsubscript{1} receptor (Sv129 + C57BL/6) knockout mice (NK\textsubscript{1} –/–) were developed and used by Bozic and co-workers (5). They demonstrated that the neurogenic response to capsaicin applied topically to the ear was reduced in NK\textsubscript{1} receptor knockout mice, as was inflammation observed in a model of immune complex-mediated lung injury. Since then, a number of reports using NK\textsubscript{1} receptor knockout mice have implicated the NK\textsubscript{1} receptor in pain responses (8, 16), Clostridium difficile-induced enteritis (9), hypoalgesia (30), and IL-1-stimulated neutrophil migration (1).

In this study we have investigated the involvement of the NK\textsubscript{1} receptor-mediated edema responses in the mouse dorsal skin cutaneous microvasculature by comparing responses between wild-type and NK\textsubscript{1} receptor knockout mice to synthetic NK\textsubscript{1} agonists in addition to the natural agonist substance P. We have also investi-
gated the possibility that an NK<sub>1</sub> receptor is normally on cutaneous mast cells in wild-type mice.

**METHODS**

Animal preparation. Male and female Sv129 + C57BL/6 mice (30–35 g) were obtained from a breeding stock that was generated at the Perlmutter Laboratory (Children's Hospital, Boston, MA). Mice are either genetically unaltered wild-type (+/+-) or knockout mice with the gene for the NK<sub>1</sub> receptor removed (-/-) (see Ref. 5). Animals were raised in a climatically controlled environment and allowed food and water ad libitum. Both strains of mice displayed normal growth and behavioral characteristics. All studies were carried out on animals anesthetized with urethane (70 µg/10 g body wt ip). A heating blanket was used to sustain animals at a constant (37°C) temperature.

Measurement of dorsal skin edema formation. After urethane administration, the dorsal skin was shaved and a maximum of four injection sites were selected. Edema formation was measured by the extravascular accumulation of intravenously injected [125]labeled BSA, [125]labeled albumin (1.25 µCi) with Evans blue (1.25% wt/vol in saline) was injected (0.1 ml iv into tail vein) and flushed through with 0.1 ml heparinized saline (10 U/ml). After 5 min, test agents (50 µl in Tyrode solution) were injected intradermally into the dorsal skin. Animals were left for 30 min to allow plasma extravasation to occur, after which a blood sample (0.5–1.0 ml) was taken by cardiac puncture. The blood samples were centrifuged at 10,300 g for 4 min, after which 100 µl plasma was taken for measurement of plasma radioactivity. Animals were then killed by cervical dislocation. Dorsal skin was removed and 8-mm-diameter circles of skin at injection sites were punched out and counted with plasma samples for radioactivity. Edema formation in dorsal skin was calculated as microliters of plasma per site.

The following protocols were carried out in wild-type mice with substances obtained from Sigma (Dorset, UK) unless stated. All agents were stored as aliquots at −20°C until required and then prepared for injection in modified Tyrode solution. Dose-related responses to substance P (30–300 pmol), the NK<sub>1</sub> agonists GR-73632 (6-Aminovaleryl [Pro<sup>9</sup>, N-Me-Leu<sup>16</sup>]-substance P (7-11), 3–30 pmol; Peninsula Laboratories, Merseyside, UK) and septide ([p-Glu<sup>6</sup>,Pro<sup>9</sup>]-substance P(6-11), 3–30 pmol) all produced dose-related responses in wild-type mice. Comparative studies were made between wild-type and knockout mice. In a separate series of experiments, the ability of histamine and 5-hydroxytryptamine (5-HT) to induce edema formation was determined, and the ability of the H<sub>1</sub> histamine antagonist mepyramine (3 mg/kg ip 15 min before treatment) on capsaicin-induced ear edema was investigated in paired experiments.

Statistical analysis. Statistical analysis was carried out by ANOVA followed by Bonferroni’s multiple comparisons test for dose-response data obtained in wild-type mice. Comparative results between wild-type and knockout mice, and also between mepyramine pretreatment and saline pretreatment, were analyzed by unpaired t-test. Results are expressed as means ± SE.

**RESULTS**

Substance P (30–300 pmol), GR-73632 (3–30 pmol), and septide (3–30 pmol) all produced dose-related responses in wild-type mice. Data show that 30 pmol GR-73632 and septide both produce larger edema formation than 300 pmol substance P (see Fig. 1), and results indicate that GR-73632 and septide are 3–10× more potent than substance P. Figure 2 shows a comparison of septide and compound 48/80-induced edema formation in wild-type mice. Compound 48/80 was chosen because it is a nonneuropeptide mediator of microvascular permeability that acts through a mast cell-mediated mechanism, binding to the mast cell membrane and stimulating release of edema-inducing histamine and 5-HT.

CGRP has been demonstrated to potentiate edema formation induced by substance P in the rat (6). The response is a consequence of the ability of CGRP to increase microvascular blood flow (7) and thus potentiate edema formation induced by mediators of increased microvascular permeability. Figure 3 shows that mouse dorsal skin edema induced by substance P and septide are significantly potentiated by 30 pmol CGRP. CGRP has no effect on edema formation when injected alone as expected (6).

Capsaicin acts as a potent stimulator of neurogenic inflammation by causing release of endogenous neuropeptides. In agreement with previously published data showing that capsaicin produces ear edema formation...
in wild-type but not NK₁ receptor knockout mice (5), edema induced by wild-type mice was $104.0 \pm 20.8$ µl/g ear tissue, and that induced by knockout mice was $3.1 \pm 1.7$ µl/g ear tissue (means ± SE, $n = 4$; $P < 0.001$). This experiment established the lack of NK₁ receptor in knockout mice. Furthermore, the application of capsaicin led to a significant increase in ear weight loss from capsaicin-treated ears compared with control-treated ears when wet – dry weight difference for each was compared with data as follows: $16.05 \pm 0.06$ vs. $12.1 \pm 0.6$ mg weight loss, capsaicin-treated ear compared with control ear (means ± SE, $n = 6$; $P < 0.01$).

The effects of substance P, the NK₁ agonist septide, and compound 48/80 on edema formation in wild-type and knockout mice are shown in Fig. 4A. Doses were chosen that gave equivalent edema responses in wild-type mice. Substance P and septide did not produce equivalent responses in knockout mice. Compound

Fig. 2. Effect of mast cell-degranulating agent compound 48/80 (C48/80) on edema formation in wild-type mouse dorsal skin. Response to intradermally injected C48/80 and septide (for comparison) is shown as plasma extravasation (µl/site). Results are expressed as means ± SE, $n = 7$. *$P < 0.05$, ***$P < 0.001$ compared with vehicle control (modified Tyrode).

Fig. 3. Potentiation of septide and substance P (SP)-induced edema formation by calcitonin gene-related peptide (CGRP) in wild-type mouse dorsal skin. Edema formation induced by septide (30 pmol, open columns) and SP (300 pmol, solid columns) in presence and absence of CGRP (30 pmol) is shown. Responses to Tyrode and CGRP alone are also shown. Results are shown as plasma extravasation (µl/site) and expressed as means ± SE, $n = 11$–12. *$P < 0.05$ compared with SP alone; **$P < 0.01$ compared with septide alone.

Fig. 4. Comparison of edema formation in wild-type and NK₁ receptor knockout mice dorsal skin in response to C48/80, SP, and septide (A), and effect of potentiating dose of CGRP on tachykinin-induced edema formation in wild-type and knockout mice (B). Edema response to intradermally injected C48/80 (500 ng), SP (300 pmol), and septide (30 pmol) (A) and response to substance P (300 pmol), GR-73632 (30 pmol), and septide (30 pmol) in presence of CGRP (30 pmol) (B) is shown for wild-type and knockout mice. Results are expressed as plasma extravasation (µl/site) with control values (Tyrode and CGRP alone) substracted to reveal any residual responses. Results are expressed as means ± SE, $n = 6$–10. *$P < 0.5$, **$P < 0.01$, ***$P < 0.001$ compared with results obtained in wild-type mice.
48/80, however, produced an edema response that was significantly larger than that in the wild-type mice. As can be seen, there is a minimal response in the knockout mice to substance P and septide. It is possible that there may be some receptor-mediated increase in vascular permeability that is not observed as significant edema formation as a result of insufficient dilation of vascular beds. Thus an attempt was made to potentiate this response in the knockout mice by use of the vasodilator CGRP, as shown in Fig. 3. The results in Fig. 4B show that there is no significant potentiation of any minor residual response that was possibly present. It is concluded that no responses of any significance or nature were observed in knockout mice when treated with NK1 agonists.

It is unclear from the results described above whether the NK1 receptor involved in mediating increased microvascular permeability is present on microvascular endothelial cells alone or also on mast cells. This was investigated by carrying out experiments to determine the mast cell amine component in the NK1 receptor-induced edema formation in wild-type mice. Compound 48/80 was shown to induce edema in mouse dorsal skin (Fig. 2), thus implicating the involvement of mast cell amines in edema formation. A histamine and 5-HT dose response was first established because these are the two amines considered to be involved in rodent mast cell microvascular changes (18). The results demonstrate that histamine but not 5-HT was functional in producing an edema response in wild-type mice (see Fig. 5). 5-HT however, did not produce any edema, even at doses that were 10-fold higher than those of histamine. A similar lack of edema formation to 5-HT was also observed in NK1 knockout mice (results not shown).

To elucidate the receptor-mediated action of histamine, mepyramine (an H1 receptor antagonist) was administered before histamine treatment. Mepyramine (3 mg/kg ip 15 min before treatment) abolished histamine-induced edema formation at the two lower doses and substantially inhibited it at the higher dose, as shown in Fig. 5. Having demonstrated that mepyramine pretreatment reduces histamine edema response, we proceeded to give mepyramine pretreatment before inducing an edema dose response with septide and GR-73632. This was to determine if there was an NK1 mast cell-dependent component in the edema response to NK1 agonists. The results shown in Fig. 6 demonstrate that mepyramine significantly inhibited edema formation induced by septide and GR-73632. This is evidence to suggest that an NK1 receptor antagonist is present on mast cells and supported by the finding that capsaicin-induced edema formation is inhibited by mepyramine. Capsaicin (100 µg/ear)-induced edema in intraperitoneally saline-pretreated wild-type mice was 207.6 ± 39.6 µl plasma/g tissue compared with 101.0 ± 42.6 µl plasma/g tissue in mepyramine (3 mg/kg ip)-pretreated mice (means ± SE, n = 5; P < 0.05).

**DISCUSSION**

The lack of the NK1 receptor in the mice used in this study was first demonstrated by Bozic and co-workers (5). Since this study, other groups have produced their own NK1 receptor knockout mice or mice with the PPT-A gene removed to remove physiological NK1 receptor agonists (8). All the workers have confirmed the results of the successful removal of NK1-mediated effects by using the capsaicin ear edema model. We too
have used capsaicin, which binds to a vanilloid receptor to stimulate an acute release of neuropeptides via a calcium-dependent mechanism (10), to confirm a functional NK1 receptor deletion in these mice. Recently, a receptor for capsaicin has been demonstrated on mast cells, but this has been shown to be a low-affinity C-type receptor that does not degranulate mast cells (4). The results in studies with NK1 knockout mice support the concept that capsaicin cannot directly activate mast cells to release edema-inducing agents.

We have carried out a study with substance P and the NK1 receptor agonists GR-73632 and septide on mouse dorsal skin edema formation. All the agonists stimulated plasma extravasation, observed as edema formation, in a dose-related manner. In addition, our results show that septide and GR-73632 are 3-10× more potent than substance P (for review see Ref. 27) in mediating edema formation in the mouse cutaneous microvasculature. Ahluwalia and co-workers (2) have shown septide to be 10-fold more potent than substance P in the rat dorsal skin edema model. In knockout mice it is clear that there is no significant skin edema response to substance P or septide. This directly demonstrates the involvement and necessity of NK1 receptors in septide as well as substance P-induced skin edema formation in mouse dorsal skin.

The edema response to substance P and septide in the wild-type mouse was significantly potentiated by the vasodilator CGRP, as would be expected from studies carried out in a range of species (6). Potentiation of edema formation by CGRP was not seen in the knockout mice. This is important, because our results (see Fig. 4A) could be interpreted to suggest that, although substance P does not produce a significant edema formation in knockout mice, it does produce a nonsignificant residual edema response that is slightly higher than that of Tyrode (drug vehicle) and septide. A possible mechanism for this could be that, although substance P is acting to increase microvascular permeability, it is not acting to promote edema formation, because there is insufficient microvascular vasodilatation (12, 15). However, the inability of the potent vasodilator CGRP to potentiate this minor residue would suggest that this is not the case and is further evidence that substance P acts solely through the NK1 receptor to mediate edema formation in these mice.

The NK1 receptor on postcapillary venule endothelial cells is considered to act primarily to mediate plasma extravasation, although NK1 receptors may be present on mast cells, and furthermore substance P may activate mast cells via an NK1-independent mechanism (see introduction). A neurogenic mast cell-dependent response to substance P has been shown in rat and guinea pig airways and in human skin (3, 19). This is in comparison with studies in rat and human skin wherein evidence suggests that endogenous neuropeptides cannot contribute to neurogenic mast cell-dependent responses, possibly because of released tachykinins not reaching sufficient concentrations at the mast cell surface (20, 21, 29). Thus the situation is complex, and we therefore decided to investigate for mast cell-dependent mechanisms in these mice.

Edema formation responses induced by tachykinins were compared with those induced by compound 48/80. Compound 48/80 stimulated edema in wild-type and knockout mouse skin. Furthermore, C48/80 was shown to be active in NK1 knockout mice and to produce a larger edema than that of the wild-type mice. This could suggest that the knockout mice may have developed a compensatory mechanism to account for the loss of NK1 receptor activity. Also, if NK1 receptors are present on mast cells, our results show that their absence does not affect mast cell integrity or function. To pursue the possibility of a mast cell NK1 receptor involvement in mouse dorsal skin edema formation, we carried out a series of experiments involving specific mast cell amines and mast cell amine inhibitors. The results show that histamine-induced edema in a potent and dose-dependent manner, but 5-HT did not. It was surprising, in light of results from Inoue and colleagues (18), that 5-HT is involved in capsaicin-induced ear edema. It is possible that the functional involvement of 5-HT in edema formation is strain dependent. This does not mean that 5-HT is lacking in the dorsal skin mast cells of these mice, but it does indicate that 5-HT does not act to induce microvascular permeability. Histamine acts via the expected H1 receptors because mepyramine, an H1 receptor antagonist, inhibited edema formation. Furthermore, the use of mepyramine significantly reduced NK1 receptor agonist and capsaicin-induced edema. These results indicate that exogenous and endogenous tachykinin NK1 agonists can mediate mast cell-dependent edema formation. This suggests that there are NK1 receptors present on mouse dorsal skin mast cells.

In conclusion, the results in this study demonstrate that substance P and neurokinin agonists do not mediate edema formation in mice in which the NK1 receptor has been deleted. This confirms results of studies using NK1 receptor antagonists in a variety of animal models wherein the NK1 receptor has been shown to play a major role in mediating neurogenic edema formation. The results demonstrate that septide and GR-7362 are more potent than substance P in inducing edema responses in the mouse, in keeping with the concept of a peptide-sensitive binding site or receptor subtype (see Ref. 26). These responses are lost in the NK1 knockout mouse. This provides good evidence that septide-like agonists do in fact bind to the NK1 receptor and that subtypes of this receptor are unlikely to exist. Evidence is also provided to indicate the presence of functional NK1 receptors on cutaneous mast cells in the mouse.

Perspectives

The development of transgenic mice has allowed the involvement of the NK1 receptor in pathophysiological processes to be examined. In this study, the mechanisms involved in tachykinin-induced NK1 receptor-mediated edema formation have been studied in anesthetized wild-type and NK1 knockout mice. Substance P is known to stimulate inflammatory edema formation
by two mechanisms. The first is via NK<sub>1</sub> receptors, usually considered to be sited on endothelial cells, although there is some evidence that they can also be found on mast cells. The second is by an NK<sub>1</sub> receptor mechanism by which the N-terminal of substance P can activate mast cells. There is evidence for a binding site for the NK<sub>1</sub> agonist septide, which is distinct from the substance P binding site at the NK<sub>1</sub> receptor. Originally it was thought that there may be separate NK<sub>1</sub> receptor subtypes, but more recent evidence indicates that the NK<sub>1</sub> receptor is able to express two different conformations. The major finding from this study is that neither substance P nor septide can induce edema formation in NK<sub>1</sub> knockout mice. This implies first that substance P cannot activate mast cells independently of NK<sub>1</sub> receptors in mouse skin, at least in the skin of the strain of mice used in this study. Second, the study implies that the NK<sub>1</sub> receptor does not exist in subtype forms.

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