Intradermal Cholinergic Agonists Induce Itch-Associated Response via M₃ Muscarinic Acetylcholine Receptors in Mice

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ABSTRACT—In the present study, we examined whether cholinergic agonists would elicit an itch-associated response in mice. When mice were given an intradermal injection of carbachol (1 – 10 nmol) or bethanechol (0.3 – 100 nmol) into the rostral back, they showed the dose-dependent increase of scratching. Nicotine (1 – 10 nmol) showed no effect. Pretreatment with naloxone, but not with terfenadine, significantly suppressed the carbachol-induced scratching. When intradermally co-injected with carbachol, atropine and 4-DAMP but neither methoctramine nor pancuronium significantly inhibited the carbachol-induced scratching. Muscarinic agonists are suggested to produce itch through activation of M₃ muscarinic receptors in the skin.

Keywords: M₃ acetylcholine receptor, Scratching behavior, Itch

Pruritus is a common symptom in various skin diseases. Severe pruritus of skin diseases such as atopic dermatitis is often resistant to the histamine H₁ receptor antagonists, which are usually prescribed as antipruritics. At present, the precise mechanisms and endogenous mediators of pruritus are unclear. Previously, we have demonstrated that itch-associated responses are induced by an intradermal injection of not only histamine but also other endogenous substances, such as serotonin, substance P and leukotriene B₄, in mice (1 – 4). Thus, several endogenous substances may be involved in itch.

Acetylcholine (ACh) is an endogenous substance released from cholinergic nerves in the skin. ACh is generally considered to be an algesiogenic agent because it has been demonstrated that intradermal injection of ACh elicits burning pain in human skin (5) and nociceptive responses in rat isolated skin (6). On the other hand, intradermal injection of ACh elicits the itch sensation in patients with atopic eczema (5). Thus, it has been suggested that ACh acts as endogenous pruritogen in some skin diseases such as cholinergic urticaria and aquagenic pruritus (7). Thus, ACh may be an important itch mediator. In the present study, we examined whether intradermal injections of various cholinergic agonists would produce itch-associated responses in mice.

Male ICR mice (Japan SLC, Shizuoka) were used at 6 weeks of age. They were housed in a room under controlled temperature (23 ± 1°C), humidity (55 ± 10%) and light (light on 07:00 – 19:00 h). Food and water were freely available. Procedures for animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. The hair of mice was clipped over the rostral part of the back on the day before experiment. For the observation of scratching, four mice were put into an acrylic cage (26 × 18 × 30 cm) composed of four cells for at least 1 h acclimation. Immediately after intradermal injection of agents, they were put back into the same cell and videotaped with any experimenters kept out from the observation room. Scratching of the injected site by the hind limbs was counted as an index of itch-associated responses (8).

Carbachol chloride, histamine dihydrochloride and atropine sulfate were purchased from Wako Pure Chemical Industries Ltd. (Osaka). Bethanechol chloride, pirenzepine dihydrochloride, methoctramine tetrahydrochloride, pancuronium bromide and terfenadine were from Sigma Chemicals Co. (St. Louis, MO, USA). 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) and naloxone hydrochloride were from Biomol Lab. Inc. (Plymouth Meeting, PA, USA) and from Aldrich Chemicals Co. (Milwaukee, WI, USA), respectively. Carbachol and bethanechol were dissolved in physiological saline and

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intradermally injected in a volume of 0.05 ml per animal. Atropine, pancuronium, pirenzepine, methoctramine or 4-DAMP was injected as a mixture solution with carbachol. Naloxone was dissolved in physiological saline and subcutaneously injected 15 min before carbachol injection. Terfenadine was suspended in 1% carboxymethylcellulose and administered perorally 30 min before carbachol injection. Values are represented as the mean and S.E.M. Statistical significance was determined with analysis of variance followed by Dunnett’s multiple comparisons. For all tests, differences were considered as significant at $P<0.05$.

We first examined the pruritogenic effect of the ACh receptor agonist carbachol in mice; we did not use ACh as an agonist because the metabolic rate of ACh is too fast to observe the effect for 1 h. When injected intradermally into the rostral back, carbachol (1–10 nmol/site) produced dose-dependent increase of scratching (Fig. 1). The highest dose (100 nmol) did not elicit scratching. This may be due to suppression of the itch sensation by pain, because intradermal injection of ACh was reported to elicit burning pain (5). Bethanechol, a muscarinic receptor agonist, also induced the scratching dose-dependently at intradermal doses of 0.3–3 nmol/site in mice (Fig. 1). It induced scratch responses even at a higher dose of 100 nmol/site. On the other hand, intradermal injection of nicotine, a nicotinic ACh-receptor agonist, did not induce scratching at doses of 1 and 10 nmol/site (Fig. 1). Although an injection of algogenic agent, such as capsaicin and dilute formalin, into the hind paw elicits pain-related behavior such as licking, its injection into the rostral back does not elicit apparent behavioral responses including scratching in mice (8). Stimulation of nicotinic receptors in the skin is thought to elicit pain (6, 9). Thus, nicotine at doses tested and carbachol at the high dose (100 nmol) might elicit pain rather than itch in mice. This may be a reason why they did not elicit any apparent behaviors. Taking account of these findings, the present results suggest that the itch-associated responses were induced by activation of muscarinic ACh receptors, but not nicotinic ones, in the skin.

Pretreatment with the opioid antagonist naloxone (1 mg/kg) significantly suppressed the carbachol (10 nmol)-induced scratching, as compared with the saline control (Fig. 2A). Opioid antagonists suppress itching of patients with pruritic diseases (10) and pruritogen-induced scratching in animals (2, 4). Thus, the result suggests that carbachol-induced scratching is an itch-associated response.

**Fig. 1.** Dose-dependent increase of scratching after intradermal injection of cholinergic agonists in mice. Carbachol, bethanechol, nicotine and saline (open columns) were injected into the rostral part of the back skin. The scratching to the injected site by hind limbs was counted for 1 h following injection. Values represent the means ± S.E.M. of 8 mice. *$P<0.05$ compared with the saline control (Dunnett’s multiple comparisons).

**Fig. 2.** Suppression by naloxone but not by terfenadine of carbachol-induced scratching in mice. The scratching of the carbachol (10 nmol)-treated site by hind limbs was counted for 1 h following injection. A: Naloxone was injected subcutaneously at a dose of 1 mg/kg 15 min before carbachol injection. The control group was given saline. B: Terfenadine was administered perorally at a dose of 30 mg/kg 30 min before carbachol injection. The control group was given 1% carboxymethylcellulose as vehicle. Values represent the means ± S.E.M. of 8 mice. *$P<0.05$ vs control group (Dunnett’s multiple comparisons).
Pretreatment with the histamine H\textsubscript{1} receptor antagonist terfenadine (30 mg/kg) did not affect the carbachol (10 nmol)-induced scratching (Fig. 2B). Terfenadine at the same dose significantly suppressed histamine-induced scratching (1). Therefore, the present result suggest that histamine is not involved in carbachol-elicited scratching and that the mechanisms of carbachol-elicited scratching are not similar to those of cholinergic urticaria, in which excessive sudoriferous stimulation induces the degranulation of cutaneous mast cells to release histamine.

Co-injection of atropine, but not pancuronium, at a dose of 1 nmol/site, with carbachol (10 nmol) significantly decreased the scratching, as compared with carbachol alone (Fig. 3A). Since atropine inhibited the carbachol-induced scratching, we examined the effect of selective antagonists against the subtypes of muscarinic ACh receptor on the carbachol-induced scratching. Co-injection of pirenzepine, a selective M\textsubscript{1} receptor antagonist, with carbachol slightly but not significantly suppressed the scratching, as compared with carbachol alone (Fig. 3B). Co-injection of methoctramine, a selective M\textsubscript{2} receptor antagonist, with carbachol did not suppress but rather significantly increased the scratching (Fig. 3B). An injection of methoctramine alone induced a slight but not significant increase of scratching (data not shown). Co-injection of 4-DAMP, a selective M\textsubscript{3} receptor antagonist, with carbachol significantly suppressed the scratching in a dose-dependent manner (Fig. 3B). These results suggest that carbachol induced the scratching via M\textsubscript{3} muscarinic ACh receptor.

Muscarinic receptors are present in the skin and afferent C-fibers (9, 11). M\textsubscript{3} receptors are localized on epidermal keratinocytes in rat and human skin (9, 11). Epidermal keratinocytes not only have muscarinic receptors but also release ACh itself (12). These findings taken together suggest that keratinocytes is one of the effector cells for intradermally injected carbachol. Muscarinic M\textsubscript{2}, M\textsubscript{3} and M\textsubscript{4} receptors are localized on the small- or middle-sized cells in the dorsal root ganglia (13). Although it is unclear which function M\textsubscript{3} receptors on afferent C-fibers are involved in, the stimulation of M\textsubscript{3} receptors on C-fibers evokes the release of neuropeptide such as calcitonin gene-related peptide in tracheal tissue (14). Therefore, direct stimulation of cutaneous C-fibers by carbachol may also be involved in eliciting scratching responses.

The stimulation of muscarinic receptors on the nociceptive afferent nerves produces an antinociceptive effect, which may be caused by desensitization of the neurons via activation of M\textsubscript{2} muscarinic receptors (9). Additionally, the stimulation of cutaneous M\textsubscript{2} receptor inhibits the release of neuropeptides from cutaneous nerve induced by noxious stimuli in vitro (15). With these findings taken into account, the potentiating effect of methoctramine on carbachol-induced scratching may be associated with the disinhibition of nociceptive afferent nerves through the inhibition of M\textsubscript{3} receptors.

In conclusion, ACh may act as an itch mediator acting on M\textsubscript{3} muscarinic receptors in the skin.

REFERENCES


