Muscarinic M2 Receptors on Peripheral Nerve Endings: A Molecular Target of Antinociception

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We recently described a novel endogenous mechanism of peripheral antinociception, possibly involving activation of muscarinic M2 acetylcholine receptors that are expressed on nociceptive nerve endings and decrease their sensitivity. In the present study, this mechanism was scrutinized in skin taken from mice with targeted deletions of the muscarinic M2 receptor gene and, for control purposes, of the M4 receptor gene. Two different approaches were taken. Electrophysiologically the effects of muscarine on nociceptive afferents were investigated using the mouse skin–saphenous nerve preparation, *in vitro*. Muscarine did not excite nociceptors in the wild-type littermates (WT) and M4 knock-out (M4 KO) mice, but almost all fibers exhibited marked desensitization to mechanical and heat stimuli. Surprisingly, in the M2 KO mice, muscarine was able to excite C-nociceptors and to induce a mild sensitization to heat but caused no alteration in mechanical responsiveness tested with von Frey hairs. In the second, neurochemical approach, the heat-induced cutaneous release of calcitonin gene-related peptide (CGRP) was investigated to gain comparative data on the neurosecretory (vasodilatory) functions of the primary afferent neurons. The substantial increase of CGRP release evoked by noxious heat (47°C) was diminished under muscarine by >50% in the WT and M4 KO animals but remained unaltered in the M2 KO mice. Together, these data provide direct evidence that M2 receptors on cutaneous nerve endings mediate effective depression of nociceptive responsiveness. This observation should be of interest for the development of novel classes of analgesic agents.

Key words: primary afferents; cholinergic; desensitization; noxious heat; mechanosensitivity; M2 knock-out; M4 knock-out; pain; analgesia

Muscarinic acetylcholine receptors are known to be involved in the control of many peripheral as well as central cholinergic responses (Caulfield, 1993). Five different muscarinic receptor subtypes (M1–M5) have been identified and characterized (Bonner et al., 1988, 1989), all of which are members of the G-protein-coupled receptor superfamily. The M1, M3, and M5 receptors act preferentially through stimulation of the phosphoinositol cascade (via G_q/G_{11}), whereas the M2 and M4 receptors mainly mediate the inhibition of adenylyl cyclase (via G_i/G_0) (Caulfield and Birdsall, 1998). Many studies reported the presence of muscarinic receptors on peripheral nociceptors (Steen and Reeh, 1993; Wanke et al., 1994; Bernardini et al., 1999, 2001a,b; Haberberger et al., 1999; Tata et al., 2000), although their involvement in pain control mechanisms was not proven until recently. In an electrophysiological study, we demonstrated previously that muscarine treatment of polymodal nociceptors left almost all fibers with a marked and sustained desensitization to mechanical and heat stimulation (Bernardini et al. 2001b). This effect could be prevented by the non-subtype-selective muscarinic antagonist scopolamine and by the M2-preferring antagonist gallamine; moreover, the M2-preferring agonist arecaidine mimicked the desensitizing effect of muscarine. In a further study, we demonstrated that muscarine and arecaidine dose-dependently decreased the basal and heat-induced CGRP release from isolated rat skin (Bernardini et al., 2001a). Together, these data suggest an involvement of the M2 receptor in muscarinic nociceptor desensitization and in control of "neurogenic inflammation." However, the lack of muscarinic ligands with a high degree of receptor subtype selectivity and the fact that primary sensory neurons co-express more than one muscarinic receptor subtype (Bernardini et al., 1999; Tata et al., 2000) make it difficult to conclusively assign specific functional responses to individual receptor subtypes.

Thus, the availability of mutant mice that lack either M2 or M4 receptors (Gomeza et al., 1999a,b) has provided the opportunity to scrutinize the physiological role of muscarinic receptors in peripheral nociception in a more direct manner. Recent studies with M2 and M4 receptor knock-out (KO) mice had already

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revealed a crucial role of the muscarinic M2 receptor subtype in mediating the central analgesic effects of oxotremorine, as assessed in the hot plate and the tail-flick tests (Gomeza et al., 1999a). The present electrophysiological and neurochemical studies focused on comparing the effects of muscarine on peripheral nociceptors in skin prepared from M2 and M4 receptor KO mice and their WT littermates.

MATERIALS AND METHODS

Animals. The generation of M2 and M4 muscarinic receptor knock-out mice has been described previously (Gomeza et al. 1999a,b). The M2 KO and the corresponding WT mice are 129"J1"/CF-1 hybrids, whereas the M4 KO and the corresponding WT mice are 129SvEvTac/CF-1 hybrids. For this study we used 4- to 8-week-old male mice from the F3 generation.

Electrophysiology. This study was performed using the *in vitro* skin–saphenous nerve preparation that has been described previously in detail (Reeh, 1986, 1988).

The preparations were obtained from M2 KO and WT (three each) and M4 KO and WT mice (four each), killed in a pure CO_2 atmosphere. The saphenous nerve in continuity with the dorsal hindpaw skin was subcutaneously dissected and excised. The skin was pinned out, corium side up, in a Perspex chamber and kept under laminar superfusion (16 ml/min). The saphenous nerve was pulled through a hole into a second chamber where the aqueous solution was overlaid with paraffin oil; here, the nerve was subdivided into smaller and smaller filaments until single-fiber unitary activity could be recorded via gold wire electrodes.

The skin was superfused with "synthetic interstitial fluid" (SIF) containing (in mM): 108 NaCl, 3.48 KCl, 3.5 $MgSO_4$, 26 $NaHCO_3$, 1.7 NaH_2PO_4, 1.5 $CaCl_2$, 9.6 sodium gluconate, 5.55 glucose, 7.6 sucrose (Bretag, 1969) at 32°C.

Receptive fields of C-fibers were searched for by probing the corium side of the skin with a blunt glass rod. The nerve endings were electrically stimulated in their receptive fields via Teflon-insulated steel microelectrodes (impedance 6–10 MΩ) to measure conduction velocity and establish the identity of mechanically and electrically evoked impulses using the "marking phenomenon" (Weidner et al., 1999). The thresholds to mechanical stimulation were tested with a set of 17 von Frey hairs calibrated from 1 to 256 mN in a geometric series ($x_i = x_{i-1} * \sqrt{2}$). Heat responsiveness was examined by focusing a halogen lamp through the translucent bottom of the skin chamber onto the epidermal side of the isolated receptive field. At the opposite corium side, the linearly increasing temperature (from 32 to 46°C in 20 sec, which corresponds to a peak temperature of 52°C at the epidermal surface) (Reeh, 1986) was feedback controlled by a thermocouple. To isolate the receptive field, a metal ring was placed over the respective corium area, and the SIF content was evacuated. The temperature corresponding to the second spike of the heat response of a fiber was considered as heat threshold. Heat and mechanical testing were always performed before and after drug administration (i.e., at a 6 min interval).

The metal rings to isolate receptive fields were also used for chemical stimulations. Muscarine 10^{-4} M in SIF was applied to the receptive fields for 5 min, and then the mechanical and heat thresholds were redetermined.

The single nerve fiber activity was recorded with a low-noise AC-coupled amplifier and monitored on a loudspeaker and an oscilloscope. The recordings were digitized and processed in an AT-type computer using a DAP 1200 interface card (Microstar, Redmond, WA). The data were analyzed off-line using the Spike/Spidi software package that provides a template-matching procedure for automatic spike discrimination (Forster and Handwerker, 1990).

The magnitude of a chemical or heat response was assessed as the total number of spikes counted during the 5 min or 20 sec of stimulation, respectively.

CGRP release. We used M2 KO and WT (nine each) and M4 KO and WT mice (eight each). Animals were euthanized by exposure to a pure CO_2 atmosphere, and the hairy skin from both hindpaws was subcutaneously dissected from below the knee. The skin flaps obtained ($n = 68$) had an average weight of ~100 mg (range 80–120 mg); they were wrapped around acrylic glass rods (Ø = 6 mm) with the corium side exposed and fixed with surgical silk. The preparations were washed for 30 min in SIF. A series of four or five consecutive glass tubes were filled with 1 ml SIF, which had been previously bubbled with carbogen (95% O_2, 5% CO_2), and positioned in a shaking bath (32°C). The skin flaps were incubated for 5 min in the first test tube and then forwarded to the next tube for 5 min and so forth; the third tube always contained the stimulating solution on the basis of SIF at 47°C. Muscarine chloride 10^{-4} M (Sigma) was added to the heated solution. Both skin flaps of one animal were always examined in parallel; one side was control, and the other was treated with muscarine.

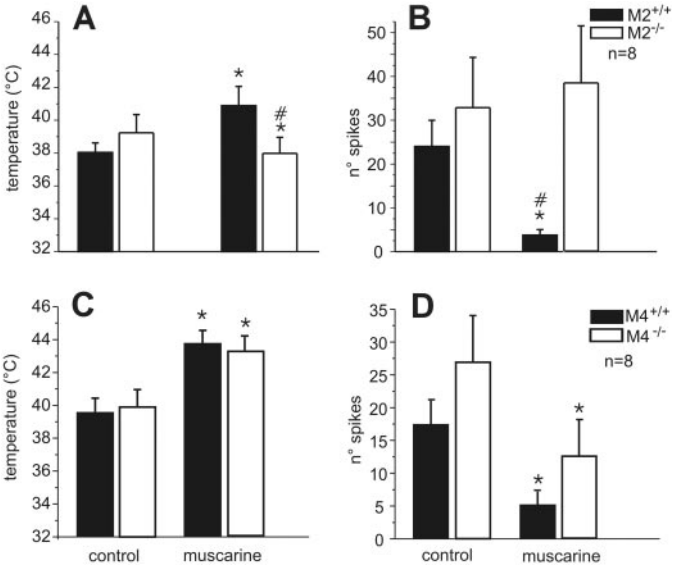

Figure 1. Heat responsiveness. Mean heat-induced discharge (*B*, *D*) and heat threshold (*A*, *C*) of primary afferent C-MH fibers before (*control*) and after administration of muscarine 10^{-4} M for 5 min to the receptive fields in the isolated skin–nerve preparation. *n* refers to the number of units tested. *Asterisks* indicate the intragroup significance (*Wilcoxon matched pairs test comparing to the control heat response), and the *# symbol* indicates significance between KO (−/−) mice and the corresponding WT (+/+) littermates (# Mann–Whitney *U* test).

The CGRP content of the incubation fluid was measured using commercial enzyme immunoassays (EIAs) (Cayman Chemical, Ann Arbor, MI; distributed through SPIbi, Massy, France) immediately after the experiment as described in detail (Averbeck and Reeh, 2001). All EIA plates were determined photometrically using a microplate reader (Dynatech Alexandria, VA).

The values of CGRP were calculated referring to 1 gm fresh weight of skin. Average results are given as mean ± SEM. In the figures, *n* refers to the number of different animals used. For better comparison of effect magnitudes, we used normalized values in the figures. For this, the actual value was divided by the value of the second baseline sample, which was the last one collected before stimulation. Nonparametric statistical comparisons within groups of identical experiments were made using the Wilcoxon matched pairs test, and the Mann–Whitney *U* test was used to compare among different groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Single fiber recordings

Altogether, we examined 32 mechano-heat-sensitive C-fibers (C-MH), eight units from each of the four groups of experimental animals. Conduction velocities ranged from 0.3 m/sec to 0.5 m/sec; no fiber showed spontaneous activity. All receptive fields were tested with muscarine at 10^{-4} M concentration, which is a supramaximal concentration with respect to nociceptor desensitization (Bernardini et al., 2001b).

M2 WT

All units revealed a marked and significant desensitization against heat stimulation after muscarine administration (Fig. 1*A,B*). The vast majority of the C-MH fibers showed a gradual

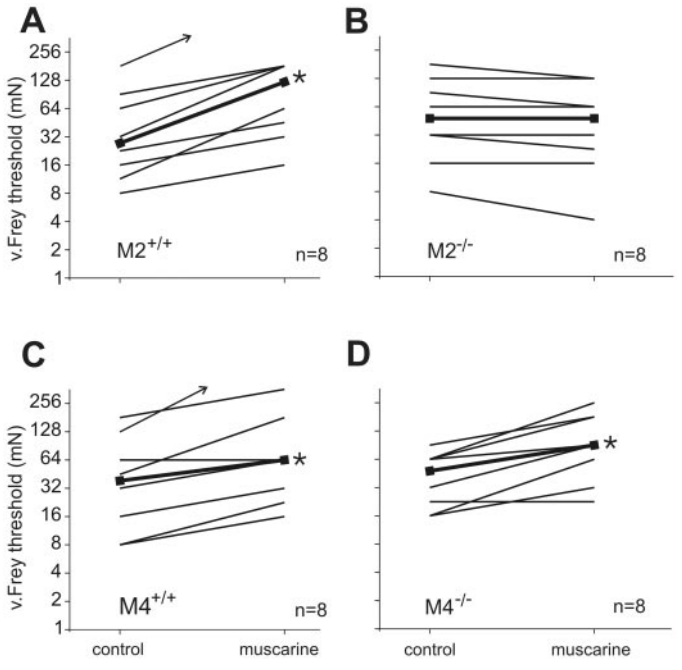

Figure 2. Von Frey thresholds. Mechanical thresholds tested with von Frey hairs before (*control*) and after administration of muscarine (10^{-4} M) to the receptive fields of the C-MH fibers. *Arrowheads* in *A* and *C* indicate nociceptive nerve fibers responding only to glass rod pressure (~1000 mN). The *bold lines* indicate the median von Frey thresholds. *Asterisks* indicate the intragroup significance (*Wilcoxon matched pairs test).

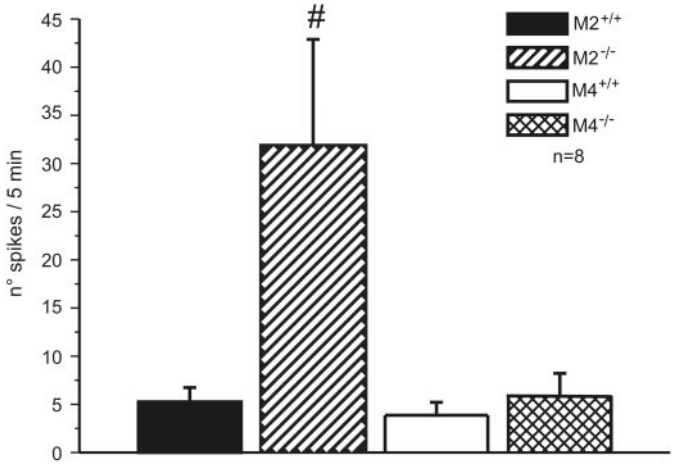

Figure 3. Muscarine-induced excitation. Cutaneous C-MH fiber discharge during 5 min of muscarine (10^{-4} M) treatment. *n* refers to the number of fibers examined. The *# symbol* indicates a significant difference between the M2 KO mice (−/−) and the other groups (# Mann–Whitney *U* test).

increase of the heat threshold; one was no longer excited by heat stimulation up to 46°C, but its heat responsiveness recovered after a 20 min washout period (data not shown). The increase of the heat threshold was always accompanied by a decrease in the total number of spikes during heat stimulation. The fibers tested were also significantly desensitized by muscarine to mechanical stimulation (Fig. 2*A*). One of the eight fibers tested responded only to probing of the receptive field with a blunt glass rod applying ~1000 mN of force (Fig. 2*A*, *arrow*).

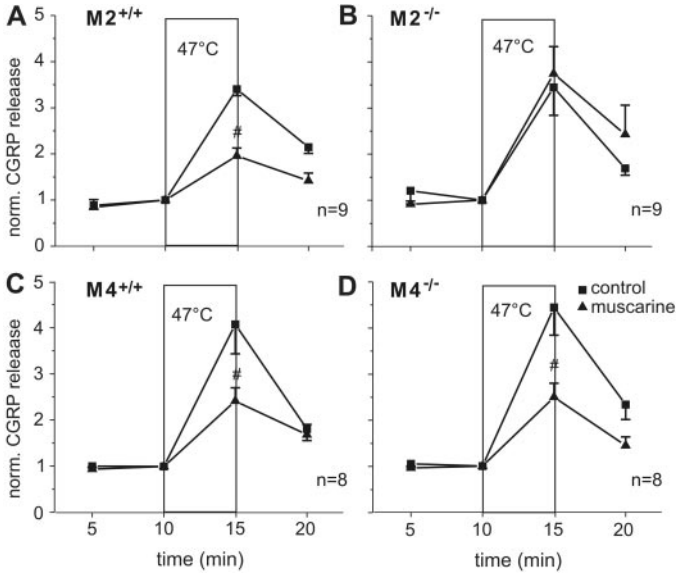

Figure 4. Muscarine effects on heat-induced CGRP release from isolated skin. Muscarine 10^{-4} M had an inhibitory effect on CGRP release in the $M2^{+/+}$ (*A*), $M4^{+/+}$ (*C*), and $M4^{-/-}$ mice (*D*) but not in the $M2^{-/-}$ mice (*B*). The *open columns* indicate the period of stimulation with heated SIF and coapplication of muscarine; *n* refers to the number of preparations from different animals. All increases over baseline were significant (Wilcoxon matched pairs test), and the *# symbols* indicate significant differences between groups (#Mann–Whitney *U* test).

M2 KO

Interestingly, many of the C-MH fibers were weakly and transiently excited during muscarine administration, showing an enhanced mean discharge rate that was significantly higher than in the other experimental groups (Fig. 3). Moreover, five of the eight C-MH fibers tested were actually sensitized against heat stimulation; the whole group showed a significant decrease of the mean heat threshold and an increase of the heat-induced discharge that was not significant (Fig. 1*A,B*). On the contrary, there was no alteration of the median von Frey threshold (Fig. 2*B*).

M4 WT

All tested fibers showed a clear and significant desensitization to heat stimulation after muscarine administration (Fig. 1*C,D*), and two units no longer responded to heat stimulation up to 46°C, but they were able to recover their heat responsiveness after a 10–20 min washout period (data not shown). In all but one of the units we also found a marked desensitization to mechanical stimulation (Fig. 2*C*), and one fiber responded only to probing the receptive field with a glass rod (~1000 mN); the same unit no longer responded to heat stimulation but was still electrically excitable (Fig. 2*C*, *arrow*).

M4 KO

Seven of eight units examined were desensitized against heat stimulation after muscarine administration, showing a significant increase in the mean heat threshold and a parallel decrease in the number of spikes (Fig. 1*C,D*). One fiber did not respond to heat stimulation up to 46°C. The median von Frey threshold was significantly increased (Fig. 2*D*).

Muscarinic effects on heat-induced CGRP release

The basal CGRP release from all experiments amounted to 93 ± 4 pg/gm fresh weight of skin; the means of the four experimental groups did not differ significantly.

Muscarine 10^{-4} M significantly reduced the mean heat-induced CGRP release by 57% in the M2 WT mice but was totally ineffective in decreasing neuropeptide release in the M2 KO mice (Fig. 4*A*,*B*). In preparations from M4 KO and M4 WT mice, muscarine suppressed heat-induced CGRP release by 56 and 59%, respectively, similar to the results obtained with M2 WT mice (Fig. 4*C*,*D*).

DISCUSSION

In the present study we have analyzed the effects of muscarine on peripheral nociceptors of mice lacking M2 or M4 receptors and of their WT littermates (Gomeza et al., 1999a,b). Muscarine treatment induced major desensitization to mechanical and noxious heat stimuli of cutaneous C-units in the WT mice, in agreement with previous data gained from the same isolated skin preparation in the rat (Steen and Reeh, 1993; Bernardini et al., 2001b). On the basis of studies with muscarinic agonists and antagonists, we recently proposed that these effects might involve activation of M2 receptors (Bernardini et al., 2001b). However, the lack of muscarinic ligands endowed with a high degree of receptor subtype selectivity left this proposal somewhat uncertain, in particular with respect to the differentiation between M2 and M4 receptors, which both couple to G-proteins of the G_i family (Wess, 1996; Caulfield and Birdsall, 1998).

In the present work we showed that the desensitizing effects of muscarine were unaltered in mice lacking the M4 receptor, excluding this muscarinic receptor subtype as a mediator of peripheral antinociception. However, in the M2 KO mice, muscarine was no longer able to cause reductions in nociceptor sensitivity. On the contrary, in these animals, muscarine induced a low-transient discharge in the C-fibers and a mild sensitization of the heat responsiveness. This effect is unlikely to be due to the repeated application of moderate heat stimuli (Reeh, 1988; Steen and Reeh, 1993; Guenther et al., 1999; Bernardini et al., 2001b) but may be caused by the presence of other muscarinic receptor subtypes, such as M1 and M3, on sensory neurons (Bernardini et al., 1999; Tata et al., 2000). Although M3 receptors were not found on rat cutaneous nerve terminals via immunocytochemistry (Bernardini et al., 2001b), such receptors are expressed in cultured rat dorsal root ganglion neurons and induce a substantial Ca^{2+} influx during activation by muscarine (Haberberger et al., 2000). An increase in intracellular Ca^{2+} can induce nociceptor sensitization (Guenther et al., 1999), and this pathway may be particularly effective in the absence of the desensitizing M2 receptors. The electrophysiological results are well supported by the neurochemical findings showing that muscarine depressed stimulated CGRP release in WT and M4 KO mice but not in M2 KO mice.

These results clearly indicate that M2 receptors are responsible for cholinergic nociceptor desensitization. There might be several possible sources of peripheral, endogenous acetylcholine. In fact, it has been demonstrated that sensory neurons themselves express choline acetyltranferase and acetylcholinesterase and are able to synthesize acetylcholine (ACh) (Tata et al., 1994). Moreover, an increasing number of reports indicates a rather widespread expression of cholinergic markers in non-neuronal cells. In particular, human keratinocytes as well as fibroblasts and glial cells synthesize and release ACh (Grando et al., 1993; Wessler et al., 1997; Buchli et al., 1999). Keratinocytes are a source of continuous ACh release in the skin (Grando et al., 1993; Nguyen et al., 2001), in the closest possible vicinity to epidermal nerve endings equipped with M2 receptors (Bernardini et al., 2001b). However, local tissue concentrations of ACh in the skin in health and disease are unknown. Nevertheless, one might speculate that nociceptor sensitivity is normally under permanent inhibitory control through tonic activation of M2 receptors, a concept that may have important pathophysiological implications.

Cholinergic desensitization of nociceptors has previously been found to be sustained and long lasting (e.g., for at least 40 min after 5 min exposure to agonists) (Steen and Reeh, 1993; Bernardini et al. 2001b). Moreover, it has been reported recently that cholinesterase blockers can prevent post-arthroscopy pain when instillated in the knee joint to increase ACh levels in the synovial fluid (Buerkle et al., 1998; Yang et al., 1998). Our present data suggest that this analgesic activity mostly likely involves ACh-mediated activation of peripheral M2 receptors. Because stimulation of M2 receptors results in reduced pain sensitivity through both peripheral (this study) and central mechanisms (Gomeza et al. 1999a), agents that can selectively activate this receptor subtype should have considerable therapeutic potential.

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