Excitatory Nicotinic and Desensitizing Muscarinic (M2) Effects on C-Nociceptors in Isolated Rat Skin

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The actions of different cholinergic agonists and antagonists were investigated on nociceptive afferents using the rat skin-saphenous nerve preparation, *in vitro*. Nicotine was able to weakly excite C-nociceptors and to induce a mild sensitization to heat stimulation (in 77% of tested fibers) in a dose-dependent manner (10^{-6} to 10^{-5} M), but it caused no alteration in mechanical responsiveness tested with von Frey hairs. Muscarine did not induce a significant nociceptor excitation, but almost all fibers exhibited a marked desensitization to mechanical and heat stimuli in a dose-dependent manner (from 10^{-6} to 10^{-4} M). The muscarinic effects could be prevented by the general muscarinic antagonist scopolamine (10^{-5} M), by the M3 antagonist 1,1-dimethyl-4-diphenylacetoxypiperidium

oxide (10^{-6} M) co-applied with the M2 antagonist gallamine (10^{-5} M), and by gallamine alone. As positive control we used the relatively M2-selective agonist arecaidine (10^{-6} to 10^{-5} M), obtaining a similar desensitizing effect as with muscarine. Finally, we performed an immunocytochemical study that demonstrated the presence of M2 but not M3 receptors in thin epidermal nerve fibers of the rat hairy skin. Altogether, these data demonstrate opposite effects of nicotinic and muscarinic receptor stimulation on cutaneous nociceptors. M2 receptor-mediated depression of nociceptive responsiveness may convey a therapeutic, i.e., analgesic or antinociceptive, potential.

Key words: acetylcholine; analgesia; cholinergic; mechanosensitivity; pain; sensitization

Acetylcholine (ACh) is the classical neurotransmitter activating nicotinic as well as muscarinic receptor subtypes. ACh is generally considered to be an algogenic agent because it has been shown to produce burning pain when applied to human skin (Keele and Armstrong, 1964; Rukwied and Heyer, 1999). Nonetheless, its role in physiological pain mechanisms has not been clearly understood. In fact, it is still unknown whether ACh appears in inflammatory exudates or under other painful conditions, but possible extraneuronal sources of ACh in the close vicinity of primary afferent terminals have meanwhile been identified. For example, in the corneal epithelial cells, high concentrations of ACh have been found that may be released after injury, and ACh has been shown to excite corneal nerve endings (Pesin and Candia, 1982; Tanellian, 1991). Regarding skin, it has been demonstrated that human keratinocytes are able to synthesize and secrete ACh. Here, ACh plays a role in regulating cell–cell attachment, but in addition, it may be released in larger amounts after cutaneous injury (Grando et al., 1993). Moreover, the ability of dorsal root ganglion (DRG) cells to synthesize ACh has been reported (Tata et al., 1994).

In the rat skin-nerve preparation and in the cornea, only unmyelinated C-fiber endings were excited by ACh and its analog carbachol, respectively (Tanellian, 1991; Steen and Reeh, 1993). The major finding was that repeated carbachol treatment of polymodal nociceptors left almost all fibers with a marked and

sustained desensitization to mechanical stimulation (Steen and Reeh, 1993). If the physiological role of ACh lay in a capacity to desensitize nociceptors, this may convey a therapeutic, i.e., analgesic or antinociceptive, potential. A selective desensitizing effect of ACh would not be expected to be mediated by the nicotinic receptor–ion channel complex but by one of the five muscarinic receptor subtypes that connect to different second messenger pathways (Mei et al., 1989).

The presence of muscarinic AChRs (mAChRs) (Bauer et al., 1994; Wanke et al., 1994; Bernardini et al., 1998; Haberberger et al., 1999; Tata et al., 1999) as well as nicotinic AChRs (nAChRs) (Boyd et al., 1991; Zoli et al., 1995; Flores et al., 1996; Liu et al., 1998) in DRG cells is established. On the other hand, it is well documented that both nicotinic (Rao et al., 1996; Bannon et al., 1998; Traynor, 1998) and muscarinic receptors (Bartolini et al., 1992; Naguib and Yaksh, 1997; Ellis et al., 1999; Gomez et al., 1999) are involved in the modulation of central nociception. Intrathecal administration of nicotinic agonists was shown to block heat nociception in the tail-flick assay (Rao et al., 1996; Bannon et al., 1998), and recent knock-out studies showed a crucial role of the muscarinic M2 and M4 receptor subtypes in mediating analgesic effects of oxotremorine administration as assessed in the hot-plate and the tail-flick tests (Ellis et al., 1999; Gomez et al., 1999).

The present electrophysiological study sought to differentiate the effects of muscarinic and nicotinic agonists and antagonists on peripheral nociceptive terminals to clarify the physiological role of ACh in nociception.

MATERIALS AND METHODS

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

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Figure 1. Original recording from two C-MH fibers of the saphenous nerve with receptive fields in isolated rat hairy skin, illustrating also the typical experimental protocol. Instantaneous discharge rates are plotted over a time scale of two different resolutions showing heat responses (32–46°C in 20 sec; gray columns) and responses to chemical stimuli (of 5 min duration); von Frey thresholds are indicated by open triangles. **A** shows nicotine sensitization to heat. **B** shows lack of muscarine-induced excitation and dose-dependent desensitization to heat and mechanical (von Frey) stimulation.

General procedures

The preparations were obtained from 62 home-bred male Wistar rats (200–380 gm) that were 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 [synthetic interstitial fluid (SIF); see below] was overlaid with paraffin oil; here the nerve was teased into smaller and smaller filaments until single-fiber unitary activity could be recorded via gold wire electrodes.

The skin was superfused with 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, and 7.6 sucrose (Bretag, 1969), thermostatically controlled at 32°C, and bubbled continuously with carbogen (95% O_2 , 5% CO_2).

Characterization of C-fibers

Receptive fields of C-fibers were located 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 M Ω) to measure conduction velocity and establish the identity of mechanically and electrically evoked impulses using the “collision technique” (Iggo, 1958). The thresholds to mechanical stimulation were tested with a set of 18 von Frey hairs calibrated from 1 to 256 mN in a geometric series ($x_i = x_{i-1} \times \sqrt{2}$). 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 rise from 32 to 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 to be “heat threshold.” For testing cold responsiveness, the ring was filled with cooled SIF at $\sim 4^\circ\text{C}$.

Chemical stimulation

The metal rings to isolate receptive fields were also used for chemical stimulations. They had inner diameters of 6.6–9.6 mm and comprised volumes of 0.3–0.6 ml that were perfused at 32°C with 2.25 ml/min to produce a turbulent flow. All solutions were bubbled continuously with carbogen.

Stock solutions (10^{-3} M) of all drugs used were kept frozen (-20°C) and were freshly diluted in SIF for every experiment. The following agents were used: arecaidine-*but-2*ynyl ester (Tocris), gallamine-triethiodide (Sigma, St. Louis, MO), muscarine chloride (Sigma), nicotine (Sigma), scopolamine hydrobromide (Sigma) and 1,1 dimethyl-4-diphenylacetoxy piperidium iodide (4-DAMP) (Sigma).

Control experiments. SIF was applied at the receptor field for 5 min before and after the mechanical and heat responsiveness were tested.

Agonist experiments. The cholinergic agonists nicotine, muscarine, or arecaidine at increasing 10^{-6} , 10^{-5} , and 10^{-4} (only muscarine) M concentrations were superfused over the receptive fields for 5 min at 10 min intervals. After each drug administration the mechanical and heat thresholds were tested (Fig. 1A,B).

Antagonist experiments. The general muscarinic antagonist scopolamine and the specific M2 antagonist gallamine were used at 10^{-5} M concentration, superfused over the receptive fields for 5 min, and in combination with muscarine 10^{-5} M for an additional 5 min. Mechanical and heat thresholds were tested before and after drug application.

Data analysis

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 response was assessed as the total number of spikes counted in the 5 min of stimulation. Statistical comparisons were made using the *t* test for dependent and independent variables. Nonparametric statistical analyses of von Frey threshold changes were made using the Wilcoxon matched pair test. Differences were considered statistically significant at $p < 0.05$. The statistical evaluations of changes in heat and mechanical (von Frey) thresholds were complicated by the fact that many fibers were so strongly desensitized after muscarinic agonist administration that heat stimulation (up to 46°C) no longer excited them, and unquantifiable probing with a glass rod was needed to stimulate them mechanically. This resulted in “missing values” that led to underestimation of the effect in the statistical analysis. For illustration, those values are indicated by arrowheads in Figures 3 and 5. In the case of heat responsiveness lost, 46°C was entered as default value for heat threshold to enable statistical comparison.

Immunohistochemistry

Specimens for immunohistochemistry were obtained from five adult rats (Wistar, Harlan Winkelmann, Borchern, Germany) of either sex that were killed by inhalation of chloroform followed by transcardial perfusion with rinsing solution containing polyvinylpyrrolidone and procainamide-HCl (Forssmann et al., 1977) and then with Zamboni's fixative (Zamboni and de Martino, 1967). Specimens of hairy skin from the hindpaws were dissected, washed in 0.1 M phosphate buffer (PB), cryoprotected in 18% sucrose in 0.1 M PB, and frozen in liquid nitrogen. Sections at a thickness of 10 μm were made using a cryostat (Jung Frigocut 1900 E, Leica, Bensheim, Germany). After blocking with PBS containing 10% normal porcine serum, 0.1% bovine serum albumin, and 0.5% Tween 20, sections were covered overnight with mixtures of primary antisera. Polyclonal M2- or M3-antisera (Biotrend, Köln, Germany) diluted in PBS/NaCl 1:1000 were combined with the monoclonal

Figure 2. Concentration–response curves for induced C-fiber excitation. Nicotine-induced discharge was of a significantly higher rate than spontaneous activity (*spa*; recorded during 5 min before chemical stimulation), and 10^{-5} M nicotine was significantly more effective than 10^{-6} M ($*p < 0.05$, *t* test). Both concentrations of nicotine induced significantly more discharge than muscarine ($*p < 0.05$, *t* test).

protein gene product (PGP) 9.5 antibody 1:400 (Biotrend). Then, sections were washed in 0.1 M PB and covered for 1 hr with a mixture of Cy-3-conjugated anti-rabbit IgG (1:3000; Dianova, Hamburg, Germany) and FITC-conjugated anti-mouse IgG (1:400; Cappel, West Chester, PA), washed out, and coverslipped in carbonate-buffered glycerol, pH 8.6. The slides were evaluated independently by two observers by epifluorescence microscopy (Olympus BX 60 F, Hamburg, Germany) using appropriate filter combinations for Cy3 (excitation filter 525–560 nm, barrier filter 570–650 nm) and FITC (excitation filter 460–490 nm, barrier filter 515–550 nm). Controls were run by omitting the first antibody.

RESULTS

We examined 88 mechanosensitive C-fibers from the rat hairy skin, *in vitro*; 73% were sensitive to mechanical and heat stimulation (C-MH), 12% were sensitive to mechanical and cold stimulation (C-MC), and 15% were high-threshold mechanosensitive (C-HTM) and did not respond to thermal stimulations.

Controls

For negative control experiments, 13 C-MH fibers ($cv = 0.44 \pm 0.10$ m/sec) were used. We found nearly no spontaneous activity during 5 min of superfusion with SIF (Fig. 2) and no changes of the sensory properties; in fact, mechanical and heat thresholds remained constant after SIF administration (Figs. 3A, 5A).

Although the population of C-fibers examined was rather heterogeneous, the differences between the median von Frey and the mean heat responses were not significant between the distinct groups of fibers tested with the various drugs.

Nicotine

A population of 19 fibers [conduction velocity (cv) = 0.57 ± 0.22 m/sec] consisting of nine C-MHs, six C-MCs, and four C-HTMs was tested with nicotine at increasing 10^{-6} to 10^{-5} M concentrations (Fig. 1A). Regardless of the fiber type, nicotine induced a significant ($p < 0.05$ compared with control period) and dose-dependent excitation with very low discharge rate; 12 of the 19 fibers responded. Seven of the nine C-MH fibers were significantly ($p < 0.05$) and dose-dependently sensitized against heat

Figure 3. Heat thresholds of C-MH fibers before and after administration of cholinergic agonists and antagonists. Arrowheads in C and D indicate fibers no longer excited up to 46°C .

stimulation, showing a decrease of the heat threshold (Fig. 3B) and an increase of the heat-induced discharge (from 12 ± 7 to 18 ± 9 and 23 ± 16 spikes after 10^{-6} and 10^{-5} M nicotine, respectively) (Fig. 4A). In the majority of the fibers the heat threshold dropped by $>3^{\circ}\text{C}$ (Table 1). On the contrary, there was no alteration of the median von Frey threshold (Fig. 5B, Table 2).

Muscarine

Twenty C-fibers ($cv = 0.59 \pm 0.20$ m/sec)—11 C-MH, 3 C-MC, 6 C-HTM—were tested with muscarine at increasing 10^{-6} , 10^{-5} , and 10^{-4} M concentrations (Fig. 1B); 7 of 20 units showed a slight, apparently dose-dependent but not significant excitation (Fig. 2). Ten of 11 C-MH units revealed a marked and dose-dependent desensitization against heat stimulation (Figs. 3C, 4B, Table 1). Most of the C-MH fibers showed an increase of the heat threshold by $>3^{\circ}\text{C}$ (Fig. 3C, Table 1) or were no longer excited by heat stimulation up to 46°C (Fig. 3C, arrows, Table 1). The increase of the heat thresholds was always accompanied by a decrease in the numbers of spikes during heat stimulation (on

Figure 4. Averaged per stimulus-time histograms of heat responses of C-MH units. Nicotine (*A*) induced a dose-dependent desensitization, lowering the threshold and increasing the mean response. Muscarine (*B*) and the M2-agonist arecaidine (*C*) induced a dose-dependent desensitization with increased threshold and reduced response. *D* illustrates the heat stimulus. In *A*, a few white columns are masked by gray ones, and in *C* three black columns vanish behind gray ones, but in all cases the differences were negligible (see Results for statistics).

average from 16 ± 9 to 10.2 ± 8 , 6.2 ± 5 , and 2 ± 1.5 spikes after increasing muscarine concentrations) as reflected in the averaged heat responses (Fig. 4*B*). Independent of fiber type, 19 of 20 C-fibers tested were significantly and dose-dependently desensi-

tized to mechanical stimulation (Fig. 5*C*). In particular, 6 of these 19 fibers responded only to probing of the receptive field with a blunt glass rod applying ~ 1000 mN of force (Fig. 5*C*, arrows, Table 2); among them, four C-MH fibers also no longer responded to heat stimulation.

Muscarinic antagonists

Seven fibers (one C-MC, one C-HTM, five C-MH; $cv = 0.39 \pm 0.08$ m/sec) were tested with the general muscarinic antagonist scopolamine (10^{-5} M) alone and in combination with muscarine at the same concentration. Neither after antagonist alone nor after co-application with muscarine did we find relevant changes of the von Frey or of the heat thresholds (Figs. 3*E*, 5*E*). These results were comparable with those of the negative control experiments.

To approach the question of the muscarinic receptor subtype involved in the desensitization, we performed the same type of antagonist experiments using a combination of the noncompetitive M2 antagonist gallamine with the competitive M3 antagonist 4-DAMP (one C-MC, six C-MH; $cv = 0.45 \pm 0.09$ m/sec), obtaining results comparable to those with scopolamine (no change of sensory properties after gallamine alone or in co-application with muscarine; data not shown). Seven (two C-HTM, five C-MH; $cv = 0.49 \pm 0.2$ m/sec) and 14 (C-MH; $cv = 0.55 \pm 0.16$ m/sec) other fibers were tested with either gallamine (10^{-5} M) or 4-DAMP (10^{-6} M), respectively. As shown in Figures 3*F* and 5*F*, gallamine was able to prevent muscarine-induced desensitization, because there were no changes of the von Frey and heat thresholds after drug superfusion. However, also on co-application of 4-DAMP with muscarine, only a slight desensitization to heat and no changes of the von Frey thresholds occurred (data not shown); this could be attributable to the fact that the subtype selectivity of 4-DAMP (Caulfield, 1993) is not confined to the M3 receptor ($K_d = 8.9$ – 9.3) but overlaps with antagonistic effects on M1 ($K_d = 8.6$ – 9.2), M2 ($K_d = 7.8$ – 8.4), M4 ($K_d = 8.4$ – 9.4), and M5 ($K_d = 8.9$ – 9.0).

Arecaidine

To further support M2 involvement in nociceptor desensitization, the M2 agonist arecaidine was tested at 10^{-6} and 10^{-5} M concentrations on eight C-MH ($cv = 0.43 \pm 0.20$) fibers. Seven of eight fibers showed a clear dose-dependent desensitization to heat stimulation: in three of them the heat threshold was increased by $>2^\circ\text{C}$ and in another two by $>3^\circ\text{C}$, and two units no longer responded to heat stimulation up to 46°C (Fig. 3*D*, Table 1). Increases in heat threshold were always accompanied by dose-dependent decreases in heat-induced discharge (on average from 22 ± 15 to 13 ± 11 and 5.6 ± 4 spikes after 10^{-6} and 10^{-5} M arecaidine, respectively) (Fig. 4*D*). In all fibers we also found a marked dose-dependent desensitization to mechanical stimulation (Fig. 5*D*): in three of eight the mechanical threshold was more than doubled, in four of eight it increased >2.8 -fold, and one fiber responded only to probing of the receptive field with the glass rod (~ 1000 mN); the same unit no longer responded to heat stimulation but was still electrically excitable (Fig. 5*D*, Table 2).

Immunohistochemistry

M2, but not M3, receptor immunoreactivity (IR) was present in PGP 9.5-positive nerve fibers at the epidermis–dermis border (Fig. 6*a,c*). Keratinocytes in the basal layer of the epidermis showed a pericellular M3-IR (Fig. 6*c*). M2-IR could be detected in the upper epidermal cell layers, whereas the deeper layers showed only a light staining (Fig. 6*a*).

Table 1. Cholinergic effects on heat thresholds

Heat threshold			Decrease		Increase		
Treatment	Fibers	No change	3°C	>3°C	↑ 3°C	>3°C	>46°C
nic	9 C-MH	3	1	5	0	0	0
mus	11 C-MH	1	0	0	1	3	6
are	8 C-MH	1	0	0	3	2	2

nic, Nicotine; mus, muscarine; are, arecaidine.

DISCUSSION

In the present study we have analyzed the different effects of muscarinic and nicotinic agonists and antagonists on cutaneous nociceptors in the isolated rat skin. Nicotine was able to excite C-nociceptors and to induce a sensitization against heat stimulation in a dose-dependent manner but no alteration in the me-

chanical responsiveness. Heat sensitization was found in 77% of the C-MH fibers tested; in 71% of these, the heat threshold dropped by >3°C. On the other hand, muscarine did not produce a significant excitation, but almost all fibers revealed a marked desensitization to mechanical (99% of the C-fibers tested) and heat (90% of the C-MH tested) stimulation in a dose-dependent way. Actually 36 and 54% of the C-MH fibers were completely desensitized to mechanical and heat stimulation, respectively. Also ~33% of the C-HTM showed a complete muscarinic desensitization against mechanical stimuli. The muscarinic effects could be completely prevented by the general muscarinic antagonist scopolamine, by the co-application of the M3 antagonist 4-DAMP together with the M2 antagonist gallamine, and by gallamine alone. These results indicated an M2 or M3 receptor involvement in the desensitization induced by muscarine. For further elucidation we used arecaidine, an M2-specific agonist; here we found the same effects as with muscarine alone. Actually 87 and 100% of the fibers were desensitized after arecaidine against heat and mechanical stimulation, respectively. Moreover, our immunohistochemical study showed the presence of M2, but not M3, receptors in the unmyelinated nerve fibers at the epidermis–dermis border.

Although acetylcholine has not been recognized as a neurotransmitter in any one of the DRG neuronal populations, it has been demonstrated that sensory neurons are able to synthesize ACh as well as choline acetyltransferase and acetylcholinesterase (Tata et al., 1994). Moreover, recent experiments demonstrate a widespread expression of the cholinergic system in non-neuronal cells. The synthesizing enzyme choline acetyltransferase, the signaling molecule ACh, and the respective receptors (nicotinic and muscarinic) are expressed in epithelial cells, e.g., in human airways, alimentary tract, and epidermis (Wessler et al., 1998, 1999). Human keratinocytes as well as fibroblasts and glial cells are able to synthesize ACh (Grando et al., 1993; Wessler et al., 1997; Buchli et al., 1999).

Neuronal nicotinic receptors belong to the superfamily of ligand-gated ion channels. They exhibit high conductance for Ca^{2+} , which may allow them to take part in synaptic mechanisms in which Ca^{2+} acts as second messenger to control other ion channels and other intracellular functions (Lindstrom et al., 1996). The presence of nicotinic receptors in sensory neurons is well documented (Boyd et al., 1991), and recent *in situ* hybridization studies have demonstrated that these receptors belong to the $\alpha 3\beta 4$ subtype (Zoli et al., 1995; Flores et al., 1996). Our study confirms the presence of functional nAChRs in sensory neurons and demonstrates that they are implicated in C-fiber excitation and heat sensitization. Nicotine-induced excitatory responses in rat sensory neurons (Sucher et al., 1990; Roberts et al., 1995), in rabbit corneal afferent nerves (Tanellian, 1991), and in rat trigeminal ganglion neurons (Liu et al., 1998) have also been demonstrated.

The activation of nicotine receptors in dorsal root ganglion

Figure 5. Mechanical thresholds tested with von Frey hairs before and after administration of cholinergic agonists and antagonists. Arrowheads in *C* and *D* indicate fibers responding only to a glass rod pressure (~1000 mN).

Table 2. Cholinergic effects of von Frey thresholds

von Frey threshold			Decrease		Increase		Glass rod (~1000 mN)
Treatment	Fibers	No change	↓ ≤3 steps	↓ >3 steps	↑ ≤3 steps	↑ >3 steps	
nic	9 C-MH	3	1	1	1	1	0
	4 C-HTM	3	0	1	0	0	0
	6 C-MC	3	0	1	2	0	0
mus	11 C-MH	0	0	0	3	4	4
	6 C-HTM	1			2	1	2
	3 C-MC	0	0	0	1	2	0
are	8 C-MH	0	0	0	3	4	1

nic, Nicotine; mus, muscarine; are, arecaidine.

(Zhong and Nurse, 1997) and in PC12 (Harkins and Fox, 1998) cells provides an important Ca^{2+} entry pathway through both Ca^{2+} -permeable nAChR and voltage-dependent Ca^{2+} channels. In addition, in C-nociceptors and DRG cells, experimental elevation of intracellular calcium levels induces an increase in the heat responses (Kress and Guenther, 1999). Altogether this evidence can easily explain the sensitization against heat stimulation that we found after nicotine administration.

Five genes that encode mAChR proteins (m1–m5) have been identified (Bonner, 1989; Bonner et al., 1988). They belong to the superfamily of G-protein-coupled receptors and activate different second messenger transduction systems, with m1, m3, and m5 (via the α subunits of the $G_{q/11}$ family) acting through the phosphoinositol cascade, whereas m2 and m4 (via G_i and G_o α subunits) mainly lower cAMP levels (Caulfield and Birdsall, 1998). Many studies reported the presence of muscarinic receptors in avian (Bernardini et al., 1998; Tata et al., 2000) and mammalian (Steen and Reeh, 1993; Bauer et al., 1994; Wanke et al., 1994; Bernardini et al., 1999) afferent neurons.

The main finding of the present study was that muscarine

treatment of C-units left them with a marked and sustained desensitization to mechanical and heat stimuli. The mechanical desensitization is in agreement with preceding data gained from the same preparation (Steen and Reeh, 1993); it was reported that the ACh analog carbachol excited C-nociceptors and at the same time produced a long-lasting (up to 45 min) desensitization to mechanical stimulation. Most of the literature regarding the action of different mediators on nociceptors deals with questions of sensitization (e.g., by inflammatory mediators). So, the literature is poor in respect to endogenous desensitizing effects, but a recent study showed that GABA_B receptors, which act through G-proteins to regulate potassium and calcium channels, also inhibit mechanosensitivity of primary afferent endings (Page and Blackshaw, 1999). M2 receptors inducing desensitization can be explained by the fact that apart from lowering the intracellular cAMP concentration, they also gate a low-threshold voltage-operated K^+ channel that builds up a hyperpolarizing force (Pan and Williams, 1994). The activation of these K^+ channel can also explain the lack of excitation that we found after muscarinic agonist administration. In the skin–nerve preparation application

Figure 6. Immunocytochemical detection of M2 but not M3 ACh receptors in rat cutaneous nerve fibers. Immunoreactivity for M2R, M3R, and PGP 9.5 at the epidermis–dermis border of the rat skin. Some nerve fibers showed immunoreactivity for M2R and PGP 9.5 (arrowheads in *a, b*), whereas M3R immunoreactivity could not be observed in PGP 9.5 nerve fibers at the epidermis–dermis border (arrows in *c, d*). Note the strong M2R-IR of the upper epidermal and the strong M3R-immunoreactivity of the basal epidermal layer. Scale bar, 40 μm .

of cAMP, analogs increased the sensitivity for both heat and mechanical stimulation (Kress et al., 1996). So, the opposite, lowering intracellular cAMP (through G_i and/or G_o), could contribute to explain the desensitization of nociceptors.

Our results clearly show that the M2 muscarinic subtype is implicated in nociceptor desensitization, and the expression of this subtype in sensory neurons is well documented (Bernardini et al., 1999; Haberberger et al., 1999; Tata et al., 1999). In addition we are showing the presence of M2-IR in nerve fibers in the epidermis of rat hairy skin; this confirms previous immunohistochemical studies in the same preparation (Haberberger and Bodenbenner, 2000).

On the other hand, the involvement of both nicotinic and muscarinic receptors in the modulation of central nociception is well documented. Intrathecal administration of nicotinic agonists has been shown to block heat nociception in the tail-flick assay (Rao et al., 1996; Bannan et al., 1998); among them, epibatidine, is the most powerful. Moreover, the antinociceptive effect of epibatidine was not blocked by the opioid antagonist naloxone, suggesting a therapeutic action different from that of morphine and a potentially novel therapeutic mechanism of providing pain relief (Traynor, 1998). Muscarinic receptors also seem to be implied in central antinociceptive mechanisms (Bartolini et al., 1992; Naguib and Yaksh, 1997). The latest research, using knockout mice, found a crucial role of M2 and M4 receptor subtypes in mediating analgesic effects as assessed in the hot-plate and tail-flick tests after oxotremorine administration, which does not exclude the involvement of peripheral nociceptors (Ellis et al., 1999; Gomeza et al., 1999).

In conclusion, the desensitizing muscarine effect was stronger and more general, blunting mechanical and heat sensitivity, than the sensitizing and excitatory nicotinic action. These findings are in full agreement with the previously reported mixed effect of carbachol, weakly exciting and desensitizing nociceptors to mechanical stimulation (Steen and Reeh, 1993). Overall desensitization by endogenous ACh may be the reason why postsurgical patients with joint pain gain benefit from intra-articular instillation of cholinesterase blockers increasing local ACh levels (Yang et al., 1998). In the skin, keratinocytes are a likely source of continuous ACh release (Grando et al., 1993), in the closest possible vicinity to epidermal nerve endings equipped with M2 receptors. Thus, one might speculate that nociceptive mechanosensitivity is normally under permanent inhibitory control that may wane when the keratinocytes are damaged, resulting in disinhibition and mechanical hyperalgesia.

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