M2-Receptor Subtype Does Not Mediate Muscarine-Induced Increases in $[\text{Ca}^{2+}]_i$ in Nociceptive Neurons of Rat Dorsal Root Ganglia

RAINER HABERBERGER,¹ ANDREAS SCHOLZ,² WOLFGANG KUMMER,¹ AND MICHAELA KRESS³

¹Institute for Anatomy and Cell Biology and ²Institute for Physiology, Justus-Liebig-University Giessen, D-35385 Giessen; and ³Department of Physiology and Experimental Pathophysiology, University of Erlangen-Nürnberg, D-91054 Erlangen, Germany

Received 9 August 1999; accepted in final form 9 June 2000

Haberberger, Rainer, Andreas Scholz, Wolfgang Kummer, and Michaela Kress. M2-receptor subtype does not mediate muscarine-induced increases in $[\text{Ca}^{2+}]_i$ in nociceptive neurons of rat dorsal root ganglia. J Neurophysiol 84: 1934–1941, 2000. Multiple muscarinic receptor subtypes are present on sensory neurons that may be involved in the modulation of nociception. In this study we focused on the presence of the muscarinic receptor subtypes, M2 and M3 (M2R, M3R), in adult rat lumbar dorsal root ganglia (DRG) at the functional and transcriptional level (immunohistochemistry). After 1 day in culture exposure of dissociated medium-sized neurons (20–35 μm diam) to muscarine was followed by rises in $[\text{Ca}^{2+}]_i$ in 76% of the neurons. The $[\text{Ca}^{2+}]_i$ increase was absent after removal of extracellular calcium and did not desensitize after repetitive application of the agonist. This rise in $[\text{Ca}^{2+}]_i$ may be explained by the expression of M3R, which can induce release of calcium from internal stores via inositoltriphosphate. Indeed the effect was antagonized by the muscarinic receptor antagonist atropine as well as by the M3R antagonist, 4-diphenylacetoxyn-N-(2 chloroethyl)-piperidine hydrochloride (4-DAMP). The pharmacological identification of M3R was corroborated by RT-PCR of total RNA and single-cell RT-PCR, which revealed the presence of mRNA for M3R in lumbar DRG and in single sensory neurons. In addition, RT-PCR also revealed the expression of M2R, which did not seem to contribute to the calcium changes since it was not prevented by the M2 receptor antagonist, gallamine. Immunohistochemistry demonstrated the presence of M2R and M3R in medium-sized lumbar DRG neurons that also coexpressed binding sites for the lectin I-B4, a marker for mainly cutaneous nociceptors. The occurrence of muscarinic receptors in putative nociceptive I-B4-positive neurons suggests the involvement of these acetylcholine receptors in the modulation of processing of nocicepti stimuli.

INTRODUCTION

Acetylcholine (ACh) is a potent algogen that produces burning pain when applied to the human suction blister base or applied iontophotically to human skin (Magerl et al. 1990; Steen and Reeh 1993; Vogelsang et al. 1995). Although widespread excitatory effects have been reported to explain this algesic action, these effects were frequently postulated to be secondary to vasodilation (Akoev 1981; Diamond 1959). Possible sources of ACh in the close vicinity of primary afferent nerve terminals have been identified, but at present it is not known if ACh at all appears in the inflammatory environment to excite nociceptors. In the cornea, epithelial cells contain a high concentration of ACh and ACh excited corneal nerve endings (Pesin and Candia 1982; Tanelian 1991). In the skin, keratinocytes synthesize, store, and secrete the substance (Grando et al. 1993) and, cutaneous unmyelinated nociceptors respond to application of ACh both via nicotinic and muscarinic receptors in an in vitro model (Steen and Reeh 1993). While the excitatory effect of nicotinic receptor activation is easily explained by the opening of cation channels and consequent depolarization of the neuron, nociceptor activation via muscarinic ACh receptors is somewhat surprising. Even more so since in contrast to the excitatory action in the periphery, the intrathecal application of muscarinic agonists lead to a prolonged analgesia, which indicates the presence of inhibitory muscarinic effects in the CNS (Abram and Winne 1995; Beazard and Morris 1993). These opposing effects may be explained by the differential expression of receptor subtypes. Pharmacologically, muscarinic receptors can be subdivided into four subtypes, whereas five different muscarinic receptors genes (M1–M5) have been described (Caulfield and Birdsall 1998; Felder 1995). The M1-, M3-, and M5-receptor subtypes are coupled to the inositoltriphosphate/diacyl glycerol (IP3/DAG) pathway resulting in release of Ca$^{2+}$ from internal stores and activation of protein kinase C (PKC) while M2- and M4-receptor subtype activation results in the inhibition of adenylate cyclase activity (Felder 1995). Functional studies in cultured DRG neurons demonstrated the coupling of muscarinic receptor activation to the inhibition of voltage-activated calcium channels (Formenti and Candia 1991; Wanke et al. 1994) and to the calcium dependent stimulation of nitric oxide synthase (NOS) and consecutive cyclic GMP (cGMP) production (Bauer et al. 1994). None of the previous studies has attempted to identify and localize the muscarinic receptor subtypes that contribute to these functional changes in DRG neurons. Since both suggest an involvement of calcium ions, it was the aim of the present study to investigate the effects of muscarine on the intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) in DRG neurons. For this purpose, the calcium indicator dye Fura-2 was nondisruptively loaded into the neurons, and calcium concentration was monitored microfluorimetrically in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The muscarinic antagonists atropine, gallamine, and 4-DAMP were used to pharmacologically identify the receptor subtypes involved. For detection of the mRNA, we used reverse transcription-polymerase chain reaction (RT-PCR) and single-cell RT-PCR. Immunohistochemical and histochemical techniques allowed the further classification of the neurons. Double labeling of M2R/M3R with I-B4 identified putative nociceptive neurons.

**METHODS**

**Cell culture**

Details of dissociation procedures have been published elsewhere (Zeilhofer et al. 1996). Briefly, lumbar DRG (L₁–L₅) were harvested from adult Wistar rats of either sex (100–160 g) that had been killed by asphyxiation with 100% CO₂. After dissection, ganglia were transferred into Dulbecco’s modified eagle medium (DMEM, Gibco, Karlsruhe, Germany) supplemented with 25 mg/500 ml gentamycin (Sigma, Deisenhofen, Germany). The connective tissue was removed and ganglia were treated with collagenase (0.28 U/ml in DMEM, 75 min, Boehringer Mannheim, Germany) and trypsin (25,000 U/ml in DMEM, 12 min, Sigma). After dissociation, the cell suspension was centrifuged at 2000 g and finally resuspended in supplemented culture medium. After plating on cover slips coated with poly-l-lysine (200 μg/ml, Sigma) cultures were kept in serum free TNB 100 medium (Biochrom, Berlin) supplemented with penicillin/streptomycin (both from Gibco) and 100 ng/ml nerve growth factor (Alomone Labs, Jerusalem, Israel) at 37°C in a humid atmosphere containing 5% CO₂.

**Calcium measurement in isolated DRG neurons**

Recordings of the intracellular free calcium concentration were performed in isolated neurons between 20 and 36 h in primary culture. External solution consisted of (in mM) 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (Merck, Darmstadt, Germany) at pH 7.3 adjusted with NaOH. For calcium measurements, neurons were loaded nondisruptively with 3 μM Fura-2 (Molecular Probes, Leiden, Netherlands). Background-corrected fluorescent images were taken with a slow scan CCD camera system with fast monochromator (PTI, Monmouth Junction, New Jersey) coupled to an Axiovert microscope with ×40 fluorol oil-immersion objective (Zeiss, Jena, Germany). Fura-2 was excited at 340 and 380 nm wavelengths (λ) and fluorescence was collected at λ > 420 nm at a frequency of 1 Hz with equal exposure time at each wavelength (200 ms). [Ca²⁺]₀ was calculated as previously published (Zeilhofer et al. 1996), and calibration constants obtained in vitro were Rₚ = 0.44, Rₚₛ = 8.0, and Kₑff = 1.2 μM. For application of chemicals, a fast 10-channel perfusion system with common outlet was used. Solenoids were controlled manually from a switch board (Dittert et al. 1998). To determine the source of [Ca²⁺]₀, increase, five cells were exposed to muscarine in calcium-free extracellular solution consisting of 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 EGTA (all from Sigma), and 10 HEPES (Merck) at pH 7.3 adjusted with NaOH. Chemicals (atropine, muscarine, 4-DAMP, gallamine) were purchased from Biotrend (Köln, Germany). Intraindividual comparisons were performed using the Wilcoxon matched pairs test and differences were considered significant at P < 0.05.

**RT-PCR**

For RT-PCR, lumbar DRG and pieces of large intestine that served as positive control of five Wistar rats were quick-frozen in RNazol (WAK-Chemie, Bad-Homburg, Germany) and homogenized after thawing using a turrax. The total RNA was isolated using the RNazol reagent according to the recommended protocol. Contaminating DNA was removed using DNase (1 U/μg total RNA, Gibco-BRL, Life Technologies GmbH, Karlsruhe, Germany) in the presence of 20 μM Tris-HCl (pH 8.4), 2 mM MgCl₂, and 50 mM KCl at 15 min at 25°C. Equal amounts of RNA were reverse transcribed in the presence of (in mM) 3 MgCl₂, 75 KCl, 50 Tris-HCl, pH 8.3, 10 dithiothreitol, and 0.5 dNTPs (Gibco-BRL) and 25 μg oligo(dT) (MWG Biotech, Ebersberg, Germany), with 200 U of Superscript RNase H⁻ reverse transcriptase (Gibco-BRL) for 50 min at 42°C. For the PCR reaction 4 μl buffer II, 4 μl MgCl₂, 1 μl dNTP (10 mM each), 0.4 μl (2 U) AmpliTaq Gold polymerase (all reagents from Perkin Elmer), and 1 μl of each primer (20 μM, M2R, M2R Genbank Accession No. J03025 forward primer 5’AGCCCGCGAAAATCGT-GAA3’ position 1534; reverse primer 5’GACATTGTATGCGCC-CCAC3’, position 1666, product 132 bp, M2R Genbank Accession No. M16407, forward primer 5’ACCAATCTCTCGGCAGACAA3’, position 1364; reverse primer 5’GCGACATCTTCTTCCGCTT3’, position 1485, product 121 bp, MWG Biotech) were mixed. Cycling conditions for PCR were 10 min at 95°C, 35 cycles of 45 s at 94°C, 60 s at 58°C, and 45 s at 73°C followed by 7 min at 73°C. Control reactions for RT-PCR included the absence of the RT reaction before PCR and the absence of template that was replaced by water. Control reactions showed no amplification products.

**Single-cell RT-PCR**

Neurons were identified after establishing the whole cell configuration by sodium currents elicited with voltage steps (150 ms, E = +20 mV) from a holding potential of −80 mV. Cytoplasm of these neurons was aspirated under visual control into a patch pipette (3–9 MΩ) filled with 6 μl sterile filtered recording solution [in (mM) 151 KCl, 10 NaCl, 0.5–3 EGTA, and 10 HEPES, pH 7.2] (Scholz et al. 1998b). The pipette contents were subsequently ejected into a RNase-free 0.2 ml tube containing 10 μl lysis buffer (Kummer et al. 1998). Sterile gloves were worn and the capillaries used were fire-polished directly before the experiment on a microforge. RT of one aliquot (7 μl) was performed after heating the tubes (10 min, 70°C) and cooling on ice for 5 min. The other aliquot was diluted in 10 μl sterile water and served as the control without RT. The RT-mix contained 1 μl dNTP-mix (10 mM each), 0.8 μl random hexamer (50 μM), 0.5 μl RNase inhibitor (10 U), 0.8 μl murine leukemia virus reverse transcriptase.

**Table 1. Antisera, lectin, and secondary reagents**

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Host Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antisera; antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2 receptor (457–466)</td>
<td>—</td>
<td>1 : 1500</td>
<td>Biotrend, Köln, Germany</td>
</tr>
<tr>
<td>M3 receptor (580–589)</td>
<td>—</td>
<td>1 : 1000</td>
<td>Biotrend</td>
</tr>
<tr>
<td>Lectin and secondary reagents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>Biotin</td>
<td>1 : 200</td>
<td>Amersham Buchler, Braunschweig, Germany</td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>FITC</td>
<td>1 : 500</td>
<td>Organon Teknika, Eppelheim, Germany</td>
</tr>
<tr>
<td>Lectin 1-B4</td>
<td>Biotin</td>
<td>1 : 50</td>
<td>Sigma, Deisenhofen, Germany</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>Cy-3</td>
<td>1 : 200</td>
<td>Amersham Buchler</td>
</tr>
</tbody>
</table>
tase, EC 2.7.7.49 (all reagents Perkin Elmer), and H₂O added to the total RNA to a final volume of 17.5 μl. The steps for the RT were 10 min 20°C, 60 min 43°C, and 5 min 99°C. Subsequent time release PCR (Perkin Elmer) was performed using three 5 μl aliquots of the RT mix at 2 min 45 s at 95°C, 60 cycles of 45 s at 94°C, 60 s at 58°C and 45 s at 73°C followed by 7 min at 73°C. Three 5 μl aliquots without RT were added to the PCR mix. Aliquots were run on a 2% agarose gel buffered with Tris-acetate EDTA containing ethidium bromide. As a control for the presence of cDNA, we used primers corresponding to the housekeeping gene, phosphobilinogen deaminase (PBGD) (Fink et al. 1999) and for glycerin aldehyde 3-phosphat dehydrogenase (GAPDH) (Haberberger et al. 1999). The PCR products were tested for their identity by cycle sequencing (AbiPrism Cycler, MWG Biotech). Control reactions for single-cell RT-PCR included the absence of the RT reaction before PCR and the absence of template that was replaced by water. Control reactions showed no amplification products.

Immunohistochemistry

Five young adult Wistar rats of either sex were deeply anesthetized and perfused transcardially with polyvinylpyrrolidone- and procainamide HCl-containing rinsing solution (Forssmann et al. 1977) followed by 300 ml Zamboni’s fixative (Zamboni and de Martino 1967). For the embedding in polyethylenglycol (PEG), animals were perfused and tissues removed as previously described (Haberberger et al. 1999). Ganglia were dehydrated (4 x 10 min in 80% EtOH, 2 x 15 min in 100% EtOH, 3 x 10 min in dimethylsulphoxide and 2 x 10 min in 80% EtOH) and incubated at 55°C in PEG (mw 1,000) followed by embedding in PEG (mw 1,450, Anderson et al. 1997). The PEG blocks were sectioned at 6 μm with a tetramer (Jung, Heidelberg, Germany). Histochemistry and immunohistochemistry were performed at free-floating sections. Sections were incubated overnight at room temperature with polyclonal rabbit antisera raised against amino acid residues 457–466 of the human M2R or against the amino acid residues 580–589 of the human M3R or biotinylated

**FIG. 1.** Muscarine induced rises in [Ca²⁺], in 80% of small- to medium-sized dissociated dorsal root ganglion (DRG) neurons. B: repeated application of 10⁻⁶ M muscarine did not induce significant tachyphylaxis of the response in the neuron depicted in A. C: the average responses of 7 neurons.

**FIG. 2.** Muscarine-induced increases in [Ca²⁺] were completely inhibited by the nonsubtype selective muscarinic receptor antagonist atropine. B: the neuron depicted A responded to 10⁻⁶ M muscarine with a rise in [Ca²⁺], which was blocked by 10⁻⁶ M atropine. The average effects are depicted in C; *, the significant reduction by atropine (P < 0.01).

**FIG. 3.** Muscarine-induced rises in [Ca²⁺], were not inhibited by the allosteric M2 inhibitor gallamine. B: specimen of a single DRG neuron (see arrow in A) and average results from 9 neurons (C).

**FIG. 4.** Muscarine-induced calcium responses were significantly blocked by the M3 receptor antagonist 4-DAMP. B: specimen of a single DRG neuron that is depicted in A. C: the average inhibition by 4-DAMP; *, the significant block (P < 0.01).
I-B4-lectin (Table 1) Secondary reagents were fluoresceinisothiocyanate (FITC)-conjugated anti-rabbit IgG from goat and streptavidin conjugated to Cy-3 (Table 1). Control sections, exposed to M2R or M3R antiserum that had been preabsorbed with the corresponding synthetic antigen (20–100 µg antigen/ml diluted antiserum; antigen from Biotrend), showed no immunolabeling. For each muscarinic receptor subtype, two sections (at least 50 µm apart) from one ganglion were used for image analysis (ScionImage, Scion, Las Vegas, NV). Only cells with clear visible nuclei were used.

Dissociated sensory neurons after 20–36 h in primary culture were fixed using Zamboni’s fixative. The unspecific binding sites were blocked with PBS, containing 10% normal swine serum, 0.1% bovine serum albumin, and Tween-20 for 1 h. Subsequent incubation was performed as previously described. Sections and cells were examined under epifluorescence (BX60 microscope, Olympus, Hamburg, Germany) using appropriate filter combinations for Cy-3 (excitation filter 525–560 nm, barrier filter 570–650 nm) and FITC (excitation filter 460–490 nm, barrier filter 515–550 nm).

RESULTS

Exposure of dissociated neurons (20–35 µm diam) to muscarine for 30 s (in 1 µM concentration) increased [Ca^{2+}]_i (Fig. 1). Application of 10^{-6} M muscarine was followed by an increase of [Ca^{2+}]_i by 334 ± 65 (SE) nM in 76% neurons (13/17 cells). The [Ca^{2+}]_i increase showed no significant tachyphylaxis, but there was a tendency to be less pronounced after the second and the third stimulation with the agonist in the concentration of 10^{-6} M (Fig. 1). The effect of muscarine was antagonized by the muscarinic receptor antagonist atropine (10^{-6} M, P < 0.01) and by the M3R-antagonist 4-DAMP (1).
mM, P, 0.01), whereas the allosteric M2-receptor antagonist gallamine (10−6 M) showed no significant effect on the increase in [Ca2+]i (Figs. 2–4). The [Ca2+]i increased by 153 ± 24 nM after muscarine application and 106 ± 35 nM after application of muscarine together with gallamine (Fig. 3). No rise in [Ca2+]i was observed when the neurons were exposed to muscarine in the absence of extracellular calcium (n = 5), which suggests calcium influx as a possible source of the increase.

The presence of M2R and M3R protein was demonstrated by means of immunohistochemistry. Sensory neurons in lumbar DRG showed immunoreactivity (IR) for both receptor subtypes (Fig. 5). M2R- and M3R-IR neurons were distributed throughout the ganglion. The intensity of immunoreactivity for both receptor subtypes varied over a wide range. Intense intracellular M2R-IR occurred in somata and axonal processes of small- to medium-sized neurons (20–35 μm diam). M2R-IR was present in 41% (153/373 cells) of lumbar DRG neurons. The vast majority of the cells (97%, 149/153 cells) had diameters between 20 and 30 μm. Most of these M2R-IR neuronal profiles (83.6%, 128/153 cells) showed also I-B4 labeling, but I-B4-positive cells without M2R-IR were also present (Figs. 5 and 6). The labeling with I-B4 varied from intense to weak or absent. Both, intensely and weakly labeled cells expressed M2R-IR. Intense M3R-IR was mainly present in somata of small- to medium-sized neurons, whereas axonal processes showed no or only faint staining. The M3R-IR was present in about 90% (350/385 cells) of the perikarya including I-B4-positive neurons (33.2%, 128/390 cells) of all neuronal profiles (Figs. 5 and 6). Smooth muscle cells of intraganglionic blood vessels expressed intense M3R-IR but no M2R-IR (Fig. 5).

Sensory neurons (20–35 μm diam) expressed M2R-IR and M3R-IR after 20–36 h in culture (Fig. 7). Double labeling showed M2R-IR/M3R-IR and I-B4 binding sites in single sensory neurons (Fig. 7). The M2R-IR was present intracellularly. Binding sites for I-B4 were found in the plasma membrane with and without additional staining of the Golgi apparatus (Fig. 7). Occasionally M2R-IR was present in I-B4-negative neurons (Fig. 7). I-B4-positive neurons without immunoreactivity for M2R were also present (Fig. 5). Analysis of the mRNA of M2R and M3R showed the presence of both muscarinic receptor types in the cDNA of rat lumbar DRG. No difference occurred between RT-PCR and time-release RT-PCR using total RNA of lumbar DRG (Fig. 8). The expected

**FIG. 8.** RT-PCR analysis for M2R- and M3R-mRNA expression in rat DRG tissue. No difference occurred between RT-PCR (35 cycles) and time-release RT-PCR (65 cycles) using total RNA of lumbar DRG. Amplicons were M2R (132 bp), M3R (121 bp), and the house keeping genes glyceraldehyde 3-phosphat dehydrogenase (GAPDH, 299 bp) and phosphobilinogen deaminase (PBGD, 128 bp). Amplification products were absent in controls of time-release RT-PCR without reverse transcriptase (ϕ RT) or template (ϕ template).

μM, P < 0.01), whereas the allosteric M2-receptor antagonist gallamine (10−6 M) showed no significant effect on the increase in [Ca2+]i, (Figs. 2–4). The [Ca2+]i, increased by 153 ± 24 nM after muscarine application and 106 ± 35 nM after application of muscarine together with gallamine (Fig. 3). No rise in [Ca2+]i was observed when the neurons were exposed to muscarine in the absence of extracellular calcium (n = 5), which suggests calcium influx as a possible source of the increase.

The presence of M2R and M3R protein was demonstrated by means of immunohistochemistry. Sensory neurons in lumbar DRG showed immunoreactivity (IR) for both receptor subtypes (Fig. 5). M2R- and M3R-IR neurons were distributed throughout the ganglion. The intensity of immunoreactivity for both receptor subtypes varied over a wide range. Intense intracellular M2R-IR occurred in somata and axonal processes of small- to medium-sized neurons (20–35 μm diam). M2R-IR was present in 41% (153/373 cells) of lumbar DRG neurons. The vast majority of the cells (97%, 149/153 cells) had diameters between 20 and 30 μm. Most of these M2R-IR neuronal profiles (83.6%, 128/153 cells) showed also I-B4 labeling, but I-B4-positive cells without M2R-IR were also present (Figs. 5 and 6). The labeling with I-B4 varied from intense to weak or absent. Both, intensely and weakly labeled cells expressed M2R-IR. Intense M3R-IR was mainly present in somata of small- to medium-sized neurons, whereas axonal processes showed no or only faint staining. The M3R-IR was present in about 90% (350/385 cells) of the perikarya including I-B4-positive neurons (33.2%, 128/390 cells) of all neuronal profiles (Figs. 5 and 6). Smooth muscle cells of intraganglionic blood vessels expressed intense M3R-IR but no M2R-IR (Fig. 5).
amplicons with 132 bp (M2R) and 121 bp (M3R) in length were amplified and subsequent sequencing (ABI-Prism) showed identity of the product with the published rat M2R and M3R sequences (Fig. 8) (Bonner et al. 1987). Control PCR without RT or template showed no products. Single-cell RT-PCR analysis of eight lumbar DRG neurons of the rat showed the presence of M2R and M3R mRNA in four single sensory neurons. Cytosol was aspirated from typically small- to medium-sized neurons, 20–35 μm in diameter. For the amplification time-release RT-PCR was used (Fig. 9). To exclude aspiration from satellite cells, the neurons were identified by sodium currents elicited with voltage steps in the whole cell configuration (Fig. 9). Amplicons with the expected size for M2R (132 bp), M3R (121 bp), or both receptor subtypes were present in single sensory neurons (Fig. 9). Control PCR without template or RT showed no products.

**DISCUSSION**

This study demonstrates for the first time that muscarine induces rise in \([Ca^{2+}]_i\), in rat sensory neurons. The muscarinic-mediated increase was seen in about 80% of small- to medium-sized neurons (20–35 μm diam). The response was mediated by the M3R but not the M2R subtype and did not exhibit significant tachyphylaxis. These functional data were corroborated by the isolation of M3R but also M2R mRNA in single DRG neurons as well as by positive M3R and M2R immunostaining of sensory neurons in vitro and in situ.

In rat lumbar sensory neurons, muscarine induced rise in \([Ca^{2+}]_i\), that was similar to the responses shown in chick parasympathetic ciliary ganglion cells where muscarine increased \([Ca^{2+}]_i\), between 186 and 476 nM (Sorimachi et al. 1995). In five cells, muscarine was applied in calcium-free solution, and under these conditions no rise in \([Ca^{2+}]_i\), was observed. This may suggest calcium influx via activation of muscarinic receptors as the source of the calcium increase. M2R have been described in rat DRG neurons by means of immunohistochemistry and in situ hybridization (Haberberger et al. 1999; Tata et al. 1999). The M2R-antagonist gallamine, however, did not inhibit the response and none of the second-messenger pathways connected to M2R are reported to increase calcium levels but rather contribute to a decrease.

The inhibition of the evoked rises in \([Ca^{2+}]_i\), by administration of the muscarinic receptor-antagonist atropine and the M3R-antagonist 4-DAMP suggested the involvement of M3R, which is coupled to activation of phospholipase C and the production of IP₃ and DAG. IP₃ acts on intracellular receptors followed by release of calcium from internal stores. In the present study, the rise in \([Ca^{2+}]_i\), fully depends on the presence of extracellular Ca²⁺. Therefore this may be due to calcium influx from extracellular space. We abstain from any speculation on the nature of this pathway, but the M3-receptor subtype may be involved in this effect. Because of the absence of real subtype selectivity of muscarinic antagonists, we cannot exclude the involvement of M1R or M5R. In a variety of neurons such changes in intracellular calcium concentration regulate important signaling pathways like the activity of phospholipases, adenylate cyclases, or guanylate cyclase (Felder 1995). Similarly, calcium may exert a variety of effects in nociceptive neurons: first, inflammatory mediators like Bradykinin and ATP, and algogens like capsaicin increase \([Ca^{2+}]_i\), in sensory neurons (Kress and Guenther 1999). Such rises in \([Ca^{2+}]_i\), that were similar to those observed in the present study, induced heat sensitization of nociceptors (Guenther et al. 1999). Therefore the activation of muscarinic receptors may cause similar changes in the sensitivity of sensory neurons. Second, Huang and Neher (1996) showed a calcium-dependent release of substance P (SP) from the perikarya of dissociated DRG neurons of similar size as in the present study. The increase in \([Ca^{2+}]_i\), evoked by muscarine could, therefore also stimulate the exocytosis of neuropeptides, suggesting a role of ACh in modulating either the axon reflex response in the target tissue or transmission at the central terminal in the dorsal horn. Accordingly ACh increased \([Ca^{2+}]_i\), and greatly facilitated the electrically evoked \(^{[3]}H\)-aspartate efflux through M3-receptors in cultured cerebellar granule cells of the rat (Beani et al. 1997). Third, ion channels could be modulated by the elevated \([Ca^{2+}]_i\). It was shown that \([Ca^{2+}]_i\) release from intracellular stores in cultured neonatal rat DRG neurons activated calcium-activated chloride conductances (Ayar and Scott 1999). Such \([Ca^{2+}]_i\) release might also activate calcium-activated potassium conductances like the BKₓ channel in DRG neurons, which influenced the action potentials and the refractory period between action potentials (Scholz et al. 1998a). Fourth, the rise in \([Ca^{2+}]_i\), may play a role in signaling pathways like the activation of phospholipases or NOS (Felder 1995; Hu and Fakahany 1993). Both, muscarinic receptors and NOS have been described in rat DRG by autoradiography, immunohistochemistry (M2R, M3R), histochemistry (NOS), and pharmacological methods (M2R, M3R, M4R, NOS) (Aimi et al. 1991; Aley et al. 1998; Haberberger et al. 1999; Wamsley et al. 1981; this study). The NOS product nitric oxide has a variety of modulatory effects on nociception including production and facilitation of hyperalgesia (Aley et al. 1998).

The muscarine-induced increase in \([Ca^{2+}]_i\), observed in the present material is apparently in contrast to previous studies on dissociated paratracheal neurons, pyramidal cells, and dissociated DRG neurons describing an inhibition of high-voltage activated \(Ca^{2+}\)-channels through activation of muscarinic receptors (M2R or M4R) (Murai et al. 1998; Stewart et al. 1999; Wanke et al. 1994). However, such an inactivation of voltage-activated calcium channels can be calcium dependent (for review, see Levitan 1999) and, therefore may be the consequence of stimulation of M3R and subsequent increase in \([Ca^{2+}]_i\).

Although muscarinic receptor antagonists lack a true subtype selectivity (Caulfield and Birdsall 1998), the presence of M3R is likely since the functional results presented here were corroborated by the demonstration of M3 receptor mRNA and protein in small- to medium-sized neurons by means of RT-PCR and immunohistochemistry. The cell diameters of M3R-positive neurons from rat lumbar DRG in the present study are very similar to those of muscarinic receptor immunoreactive profiles in thoracic DRG of the rat and in chick DRG (Bernardini et al. 1998; Haberberger et al. 1999). In addition, their size corresponds well to the size of cells investigated by microfluorometric calcium measurement. Small- to medium-sized neurons represent the perikarya of thinly or nonmyelinated afferent neurons (Snider and McMahon 1998).

Not only M3R-mRNA but also M2R-mRNA was found in some of these neurons, and this corresponds well with results from in situ hybridization, which showed M2R-mRNA in
medium-small neurons in rat DRG (Tata et al. 1999). The finding of M2R- in addition to M3R-mRNA is in agreement with present immunohistochemical detection of muscarinic M2R- and M3R-protein in small- to medium-sized neurons in situ, in sections of lumbar DRG, and, in dissociated neurons. M3R-IR was found in 90% of lumbar neurons including I-B4-positive cells, which were M2R-IR too. Therefore it can be concluded that at least the subpopulation of I-B4-positive neurons contain M2R- and M3R-protein. The presence of multiple muscarinic receptor-subtypes in single neurons has previously been demonstrated in cultured pyramidal neurons from rat somatosensory cortex where the activation of different muscarinic receptors in single neurons resulted in an inhibition of N-, P- and L-type currents (see the preceding text) (Stewart et al. 1999).

Nociceptive neurons can be subdivided in two classes. One class depends on the presence of nerve growth factor and expresses the neuropeptides SP and CGRP (Snider and McMahon 1998). The other class is sensitive to glial cell derived neurotrophic factor and binds the plant lectin I-B4 (Bennett et al. 1996; Molliver et al. 1995). I-B4 is a marker of thinly myelinated and unmyelinated C-fiber afferent neurons that project mainly to the skin and, to a lesser extent to viscera, that terminate in the spinal cord (Bennett et al. 1996; Molliver et al. 1995; Petruska et al. 1997; Penderleith and Snow 1993). The two groups of neurons show partial overlap since Wang and co-workers (1994) demonstrated SP- and/or CGRP-IR in a subpopulation of I-B4-positive cells. SP-positive cells were present in weakly labeled I-B4 neurons, whereas intensely I-B4-labeled cells showed no SP/CGRP-IR (Wang et al. 1994). We observed that M2R- and M3R-IR were present in intensely as well as weakly I-B4-stained cells. In addition to the strongly immunoreactive M2R sensory neurons I-B4-negative cells were observed with minor but visible immunoreactivity for M2R and M3R. Therefore it cannot be excluded that M2R and M3R were, in addition to their occurrence on I-B4-positive cells, also present on SP-containing sensory neurons.

It was not possible to use both, M2R- and M3R-antisera together for the detection of both receptor proteins in single neurons. However, about 84% of M2R-IR neurons were I-B4 positive, and all I-B4-positive neurons showed M3R-IR. Therefore we can conclude that both receptor subtypes were present in I-B4-positive neurons. I-B4-positive neurons were small- to medium-sized. The mRNA for both receptors was detectable in single neurons of this size, suggesting the presence of both receptor proteins on individual sensory neurons. The presence and localization of muscarinic M2- and M3R-mRNA and protein in rat lumbar putative nociceptive DRG sensory neurons suggest the involvement of different muscarinic receptor subtypes in sensory nociceptive signal transduction and processing.

The authors thank A. Wirth-Huecking, I. Izydorczyk, and M. Bodenbenner for expert technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 547, C2 and SFB 353, A10) and the Sanderstiftung (96.058.1).

REFERENCES


