Prejunctional \( M_1 \) facilitory and \( M_2 \) inhibitory muscarinic receptors mediate rat bladder contractility

ALAN S. BRAVERMAN,1 IRA J. KOHN,1 GARY R. LUTHIN,2 AND MICHAEL R. RUGGIERI1,3

Departments of 1Urology and 2Pharmacology, Temple University School of Medicine, Philadelphia 19140; and 3Allegheny University of the Health Science, Philadelphia, Pennsylvania 19102

Braverman, Alan S., Ira J. Kohn, Gary R. Luthin, and Michael R. Ruggieri. Prejunctional \( M_1 \) facilitory and \( M_2 \) inhibitory muscarinic receptors mediate rat bladder contractility. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R517–R523, 1998.—Subtype-selective muscarinic antagonists effects on carbachol-induced and electric field-stimulated contractility of rat bladder were compared in vitro. Schild plot analysis of cumulative carbachol dose-response curves in the presence of antagonists was consistent with \( M_3 \)-mediated bladder contractions. However, nerve-evoked contractions were inhibited 15% at 30 Hz \( (P < 0.01) \) by 10 nM pirenzepine \( (M_2 \)-selective antagonist), whereas 10 nM methoctramine \( (M_2 \)-selective antagonist) increased these contractions by 17% at 30 Hz \( (P < 0.01) \). Identical doses had no effect on carbachol-induced contractions, indicating prejunctional \( M_1 \) facilitory and \( M_2 \) inhibitory receptors. \( M_1 \) receptors could not be identified by subtype-selective antibodies, nor could the \( M_1 \) transcript be identified by Northern hybridization. However, \( M_1, M_2, M_3, \) and \( M_4 \) transcripts were identified in rat bladder using the reverse transcriptase-polymerase chain reaction, providing support for the existence of the \( M_1 \) subtype. In conclusion, strong evidence is provided for the existence of prejunctional \( M_1 \) facilitory and \( M_2 \) inhibitory receptors. \( M_1 \) receptors mediated electric field-stimulated release of \(^{3}H\)acetlycholine from nerve terminals in the rat urinary bladder (16). Recent studies, on the other hand, suggest that no \( M_1 \) receptors are present in the rat urinary bladder (18). Immunological studies using subtype-specific antibodies revealed the existence of \( M_2 \) and \( M_3 \) receptor subtypes \( (25, 26) \) but not the \( M_1, M_4, \) or \( M_5 \) subtypes. Furthermore, Northern blot hybridization has identified mRNA encoding the \( M_2 \) and \( M_3 \) but not other subtypes of muscarinic receptors in the rat urinary bladder \( (16) \). Recent studies, on the other hand, show that low doses of PZP and oxotremorine inhibit electric field-stimulated release of \(^{3}H\)acetlycholine from nerve terminals in the rat urinary bladder \( (20, 21) \). These prejunctional receptors inhibited by PZP appeared to be active only during high-frequency electrical stimulation, whereas the action of the receptors activated by oxotremorine predominated during low-frequency stimulation \( (21) \).

To delineate the function of the different muscarinic receptor subtypes in bladder contractility, we measured the effect of several subtype-selective muscarinic antagonists on both direct muscle stimulation by carbachol and on nerve-evoked contractions induced by electric field stimulation. In addition, we used reverse transcriptase-polymerase chain reaction \( (RT-PCR) \) to identify the muscarinic receptor subtype mRNA transcripts \( (M_1-M_5) \) expressed in the rat urinary bladder. As such, unlike a previous report that characterized the muscarinic receptor subtypes involved in carbachol-induced contraction \( (26) \), this is the first report that combines both molecular and pharmacological methods to identify and functionally classify the muscarinic receptor subtypes involved in nerve-evoked contraction of the rat urinary bladder.

METHODS

Materials. The following drugs or chemicals were obtained from the sources indicated: carbachol and atropine from Sigma \( (St. Louis, MO) \) and methoctramine, 4-DAMP, 4-DAMP mustard, and p-F-HHSiD from Research Biochemicals International \( (Natick, MA) \).

Muscle strips. Urinary bladders were removed from 200- to 250-g male Sprague-Dawley rats \( (Ace Animals, Boyertown, PA) \) euthanized by decapitation. The urinary bladder body (tissue above the ureteral orifices) was dissected free of the serosa and surrounding fat. Muscarinic receptors are only associated with smooth muscle in the rat urinary bladder, as demonstrated by autoradiography \( (13) \), and no differences in contractility were seen when the mucosa was removed \( (published observations) \); therefore, no further dissection was performed. The bladder was divided in the mid-sagittal plane and then cut into four longitudinal smooth muscle strips \( (–4 \times 10 \text{ mm}) \). The muscle strips were then stretched slowly to achieve a final isometric tension of 1 g in tissue baths containing 15 ml of modified Tyrode solution \( ([in mM] \text{ NaCl}, 2.7 \text{ KCl}, 0.4 \text{ NaH}_{2}\text{PO}_{4}, 1.8 \text{ CaCl}_{2}, 0.5 \text{ MgCl}_{2}, 23.8 \text{ NaHCO}_{3}, \text{ and } 5.6 \text{ glucose}) \) and equilibrated with 95% \( \text{O}_{2} \) and 5% \( \text{CO}_{2} \) at 37°C for 30 min.
Carbachol dose response. After equilibration to the bath solution, bladder strips were incubated for 30 min in the presence or absence of antagonist. Dose-response curves were derived from the peak tension developed after cumulative addition of carbachol (10 nM to 460 µM) added at 0.01 volume of the bathing solution. Cumulative dosing of carbachol as opposed to single doses was used based on the findings of Durant et al. (8), who showed no differences in tension when comparing these dosing regimens. Each strip was used for only one dose-response curve. Each concentration of antagonist was tested on 3–15 strips. Dose ratios were determined based on the average 50% effective concentration (EC50) values derived from dose-response curves of 16 antagonist-free strips performed in parallel with antagonist-treated strips. An EC50 value was determined for each strip via a Hill transformation of the data. The EC50 values determined in the presence of antagonist were used to generate Schild plots to calculate PA2 values for each antagonist.

Frequency response. Bladder strips as prepared above were desensitized to purinergic stimulation by the addition of two doses of 30 µM βγ-methylene adenosine triphosphate separated by a 30-min rest period. The frequency-response curves shown in Fig. 2 were obtained with the continuous presence of βγ-methylene adenosine triphosphate (final concentration 60 µM) in the bathing solution. To ensure that the purinergic response was continually desensitized, subsequent to some experiments, we measured the contractile response to electric field stimulation after the addition of 10 µM atropine. This combination of purinergic desensitization and atropine inhibited >90% of the electric field-stimulated response, confirming the continued desensitization of the purinergic component of contraction throughout the experiment. Nerve-evoked contractions were induced by electric field stimulation generated by a solid-state square-wave stimulator (model S88, Grass Instruments, Quincy, MA) interfaced through a stimulus power booster (Stimu-Splitter II, Med-Lab Instruments, Loveland, CO) to maintain the amplitude, duration, and shape of the stimulus signal, which is transmitted simultaneously to 12 tissue baths in parallel. The 2.5-cm-long serpentine-shaped platinum electrodes are situated parallel to the long axis of the muscle strips ~1.25 cm apart in 15-ml organ baths (Radnoti Glass Technology, Monrovia, CA). The contractile response resulting from a submaximal (70% of maximum) electric field stimulation of 8 V at 1-ms duration at increasing frequencies (between 1 and 60 Hz) was recorded for each muscle strip, with a 4-min recovery period between each change in frequency. In preliminary experiments, no significant reduction in electric field-induced contractility was observed by multiple stimulations separated by a 4-min recovery period. Two stimulation paradigms were tested. In the first, stimulation at each frequency was continuous until peak tension was reached (5–10 s). In the second paradigm, 100 shocks were given at each frequency. The bladder strips were then incubated with or without antagonist for 30 min and then re-stimulated. In an attempt to use minimal quantities of drugs, 3-ml tissue baths were constructed with platinum electrodes ~2 cm apart at the top and bottom of the long axis of the muscle strip. Identical stimulation paradigms were performed using this electrode configuration (except that 12 V at 1-ms pulse duration was used, because this gave a contraction of 70% of maximum).

RT-PCR. Total RNA was isolated from rat bladder tissue using an RNA isolation kit (Stratagene, La Jolla, CA). Total RNA (10 µg) was reverse transcribed using oligo-dT primers. The reverse-transcribed products were screened for the presence of m1–m5 cDNA by PCR. PCR was carried out with pfu polymerase (Stratagene, La Jolla, CA) using two sets of oligonucleotide primers designed to be specific for the m1 receptor and a single set of oligonucleotide primers for m2–m5 receptors (Table 1). PCR reactions were performed on a DNA Pacer (Belco Products, Vineland, NJ) and consisted of an initial cycle of 95°C for 5 min and 62°C for 5 min followed by 30 cycles of 95°C for 5 min, 62°C for 3 min, 72°C for 3 min, and a final cycle of 72°C for 10 min. The reaction products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed.

Statistical and data analysis. Results are reported as means ± SE. The contractility data curves displayed in Figs. 1 and 2 were generated by a curve-fitting program (Origin, MicroCal Software, Northampton, MA) based on a sigmoidal fit of the data. Statistical analysis was performed by analysis of variance with a post hoc Scheffe’s test (GB-STAT, Dynamic Microsystems, Silver Spring, MD). The slopes of Schild plots were analyzed for difference from unity using the 95% confidence interval.

RESULTS

Carbachol response. Schild analysis of the shift in the carbachol dose-response curve for each of the muscarinic receptor antagonists revealed a dose-dependent competitive inhibition of bladder muscle contraction. PZP (1 nM-10 µM, PA2 = 7.1, slope = 0.83, not significantly different from unity) inhibited carbachol-stimulated muscle contractions at a concentration consistent with an M3 receptor directly mediating muscle contraction, as previously shown using Meth (PA2 = 6.1, slope = 0.86, not significantly different from unity), 4-DAMP, and f-P-HHbicide (26). Carbachol dose-response curves and Schild plots for PZP and Meth are shown in Fig. 1.

Electric field response. Two electric field-stimulation paradigms were used to characterize the effect of PZP and Meth on nerve-mediated contractions. Two electric field-stimulation paradigms were used to characterize the effect of PZP and Meth on nerve-mediated contractions.
trode configurations were also tested. Both stimulation paradigms gave essentially identical results, and only the data obtained from the second paradigm, when strips were allowed to contract maximally to each frequency of stimulation, are presented. Electric field stimulation in the presence of various concentrations of PZP, Meth, 4-DAMP, and p-F-HHSiD revealed a dose-dependent inhibition of nerve-mediated contraction of bladder smooth muscle (Fig. 2). 4-DAMP and p-F-HHSiD inhibited both carbachol and electric field-stimulated muscle contractions at the same concentrations. However, 10 nM PZP, a dose that had no effect on carbachol-induced muscle contractions, significantly (P < 0.01) inhibited electric field-stimulated contractions at frequencies >2 Hz. Also, 10 nM Meth, a dose that also had no effect on carbachol-stimulated muscle contractions, significantly (P < 0.01) increased electric field-stimulated contractions over control at ≥8 Hz.
The M₁ receptor-mediated facilitation as a percentage of the predrug contraction appeared to be greater at frequencies between 2 and 8 Hz. However, in terms of actual grams of tension difference, the M₁-mediated facilitation was greater at frequencies >8 Hz. The M₂ receptor-mediated inhibition appeared to be greater at frequencies between 8 and 20 Hz in terms of both percentage and actual grams of tension differences (Fig. 3).

Interestingly, when the stimulating electrodes were at the top and bottom of the long axis of the muscle strip, no significant M₁ facilitation could be observed. At 20 Hz, both control and 10 nM PZP-treated strips reached 89.6% of the predrug maximum. It appears that the orientation of the electric field is also an important stimulation parameter.

RT-PCR. Oligo-dT primers were used in the RT reaction. This allowed direct amplification of RT products without further purification of the cDNA and potential loss of RT products before PCR. The use of oligo-dT primers also reduced the RT products expected to be produced from rRNA, and we felt that to identify low-abundance mRNAs, any method to selectively increase RT products from mRNA would be important. We were unable to amplify RT products directly from the RT reaction mix using random nine-nucleotide primers. With oligo-dT primers, we were able to identify transcripts for the m₁–m₄, but not the m₅, muscarinic receptor in bladder RNA preparations (Fig. 4). Several groups, including ours, have previously been unable to detect m₁ or m₄ receptors in this tissue by Northern blot hybridization, radioligand binding, or immunoprecipitation (16, 18, 25, 26). Negative controls (no RT) yielded no products, confirming lack of DNA contamination in the RNA samples.

DISCUSSION

Previous studies have demonstrated that high-affinity PZP binding sites are not present in the rat urinary bladder (18). Other studies using subtype-specific antibodies failed to identify m₁ receptors in this tissue by immunoprecipitation (25, 26) or by immunohistochemical means (not shown). Immunoprecipitation analysis revealed that the m₂ receptor accounts for between 80 and 90% of total rat bladder muscarinic receptors, with the m₃ receptor accounting for ~10% (25, 26). Northern blot analysis has also failed to identify m₁ receptor mRNA in the rat urinary bladder (16). These findings are consistent with a low density of m₁ receptors in the rat bladder and may explain our inability to localize the m₁ receptor to specific cell types.

Recent studies suggest that prejunctional M₁ facilitory and M₂ inhibitory receptors in the rat urinary bladder modulate acetylcholine release (20, 21). In these studies, M₁-mediated facilitation of acetylcholine release was seen only during high-frequency continuous electrical stimulation. Acetylcholine release was reduced by 85% in the presence of 1 µM atropine (a nonselective muscarinic receptor antagonist) and by 70% in the presence of 50 nM PZP, implicating the M₁ subtype in this response. Somogyi et al. (21) concluded that both M₁ and M₂ receptors are present prejunctionally and that activation of M₁ receptors leads to an increase in acetylcholine release, whereas activation of M₂ receptors leads to a decrease in acetylcholine release from nerve terminals in the rat urinary bladder. A recent study by Tobin and Sjögren (23) also supports this localization and function of muscarinic receptor subtypes in the rabbit urinary bladder.

We have previously shown that the affinity of a series of muscarinic antagonists to inhibit direct muscarinic receptor stimulation by carbachol is most consistent with m₃ receptors mediating rat bladder smooth muscle contraction (26). Different results were observed in the present study for inhibition of electric field-stimulated contractions. These contractions are considered to be primarily nerve evoked, because they are effectively (>95%) blocked by 0.1 µM tetrodotoxin (19). They are also considered to be due to the release of acetylcholine by nerves, because in our experimental design, all postjunctional purinergic responses were desensitized. Although nerves releasing other neurotransmitters or peptides may be present in the rat urinary bladder, >90% of the contractile response to electric field stimulation is blocked with a combination of atropine and purinergic desensitization (data not shown); therefore, the sum net effect of these other transmitters is <10% of the maximum contraction.

Fig. 3. Effect of frequency on M₂ inhibition or M₁ facilitation of rat urinary bladder contractility. A: average percent modulation of rat urinary bladder strip contractility to electrical stimulation of 8 V, 2–60 Hz, 1-ms pulse duration, produced by 10 nM PZP and 10 nM Meth, and, for time controls, run simultaneously without antagonist. Data are expressed as average percent modulation = SE. %Modulation = 100 × (postdrug contraction in grams – predrug contraction in grams)/predrug contraction in grams for each frequency. B: actual difference in grams = SE of postdrug contractions minus predrug baseline contractions for time control, 10 nM PZP, and 10 nM Meth.
Prejunctional receptors modulating acetylcholine release would not be expected to affect direct muscle stimulation due to carbachol. Although these prejunctional receptors may be activated by carbachol, in the absence of nerve stimulation, there is not enough acetylcholine released from nerve terminals to affect contraction, and, although prejunctional facilitatory autoreceptors are activated, contractility is not enhanced. Therefore, the modulatory effects of prejunctional receptors will only be seen with nerve-evoked contractions.

Figure 2 shows that at a dose of 10 nM, the M1-selective antagonist PZP significantly decreased nerve-evoked contractility. Conversely, 10 nM of the M2-selective antagonist Meth significantly increased nerve-evoked contractions. Figure 1 shows that these doses of the two antagonists had no effect on carbachol-induced contractions, suggesting that these receptors are prejunctional. Unlike Somogyi et al. (20, 21), who reported facilitation only at high frequencies and inhibition at low frequencies, our results indicate both facilitation and inhibition at both low and high frequencies (Fig. 3). These differences could be due to differences in the electric stimulation parameters used in these studies. Whereas we stimulated submaximally at 8 V at a pulse duration of 1 ms (1–60 Hz), the results reported by Somogyi et al. (20, 21) were obtained with a stimulation of 100 V and a 0.25-ms duration (0.4 and 10 Hz). As an example, using the stimulation paradigm of Somogyi et al. (20, 21), 10 Hz resulted in a maximal contraction, whereas, using our conditions, 10 Hz resulted in a contraction of ∼60% of maximum.

Our results correlate the changes in acetylcholine release seen by Somogyi et al. (21) with changes in smooth muscle contractility in the rat urinary bladder. In general agreement, Somogyi et al. (21) reported a 70% reduction in acetylcholine release with 50 nM PZP. Our results, a 15% reduction in rat bladder contractility at 30-Hz stimulation in the presence of 10 nM PZP, are similar to those of Tobin and Sjögren (23), who reported a 10% reduction in rabbit bladder contractility to the same dose of PZP. These findings provide functional evidence for prejunctional M1 facilitatory and M2 inhibitory receptors (i.e., on nerve) and postjunctional M3 receptors mediating bladder muscle contraction. The prejunctional receptors may be localized to the prejunctional synaptosomal plasma membrane or may be located on the axon of the parasympathetic nerve, as is the case with α2-adrenoceptor on dog mesenteric nerve axons (5). The M1 receptors that enhance acetylcholine release may serve to ensure complete bladder emptying during micturition, whereas the M2 receptors may act in an autoinhibitory fashion to stop the release of acetylcholine from nerve endings and end the contraction.

Whereas a postjunctional muscle plasma membrane location of M1 and M2 receptors in bladder cannot be ruled out by these experiments, if they are in this location, antagonist affinities in our experimental paradigms indicate that they do not participate in transducing the contraction. Other data obtained by selective alkylation of M3 receptors with 4-DAMP mustard suggest that the M2 receptor may be involved in blocking the increase in cAMP, and hence relaxation, induced by isoproterenol activation of β-adrenergic receptors, thereby participating in contraction (4, 8, 9).

The frequency-response curves seen in Fig. 2 appear to indicate that these antagonists are noncompetitive in nature. However, it is the concentration of acetylcholine in the vicinity of the postjunctional receptors that determines contractility. The acetylcholine in our experimental paradigm is released by parasympathetic nerves, is relatively short lived due to breakdown by acetylcholinesterase, and cannot be increased without limit as is the case with carbachol concentration-effect curves. The stimulus used to elicit the release of acetylcholine is an electric field with varying frequency. It is possible that this stimulus is not able to evoke the release of enough acetylcholine to overcome the inhibitory affects of the antagonists; hence the antagonism would appear noncompetitive. Another complicating factor is that these antagonists have both pre- and postjunctional effects at higher concentrations.

To reconcile our inability to identify bladder m1 receptors using subtype-selective antibodies or m1 receptor mRNA by Northern hybridization with the evidence presented by Somogyi et al. (21) and herein, we used a highly sensitive molecular means (RT-PCR) to identify m1, m2, m3, and m4 receptor mRNA in the rat urinary bladder (Fig. 4). Although this is a highly sensitive technique, we cannot localize the mRNA to prejunctional sites. Although these results do not prove the presence of prejunctional receptors, they are consistent with the premise that there are prejunctional m1 and m2 receptors. However, in the case of the rat urinary bladder, which does not contain intramural ganglion cells (11), the identified mRNA must originate from other cells in the bladder, be axonal in origin, or both. If the mRNA is derived solely from nonneuronal cells in the bladder, then the RT-PCR results would not support the proposed model. However, it is possible that the axons of the nerves innervating the bladder contain mRNA encoding the m1 and m2 receptor sub-
types. A recent review (22) describes both mRNA localization and protein synthesis occurring within dendritic processes of neurons and polynucleosomes located in proximal axonal segments. Axonal localization of mRNA has been described for both invertebrates (6, 24) and vertebrates, specifically in rats (14, 17), along with protein synthesis occurring in the squid giant axons (12) and the axons of neurons regenerating in culture (15). Therefore, our demonstration of the presence of mRNA coding for presynaptic receptors in the rat urinary bladder may indicate that this mRNA is axonal in origin. In conjunction with the functional studies we describe, this constitutes strong evidence for presynaptic M1 and M2 receptor subtypes in rat bladder.

The function of the m4 receptor in the rat urinary bladder, if present, is unclear. In a recent study, Alberts (1) argued for the existence of presynaptic M4 inhibitory receptors in the guinea pig urinary bladder based on the correlation of the EC50 values of 20 muscarinic antagonists for increasing electrically stimulated acetylcholine release with published affinity values of these antagonists for M1-M4 receptors. The presence of M4 receptors, however, remains inconclusive because of the inability of the available antagonists to distinguish between M2 and M4 receptor subtypes. We were unable to detect the presence of M5 receptor mRNA using m5-specific primers and RT-PCR. The RT reaction used to produce the cDNA library was performed using oligo-dT primers to synthesize cDNA from mRNA. In contrast to the results reported here, when random primers were used to prime the RT reaction, no m1 PCR amplification product was observed from either rat or human (unpublished observation) cDNA prepared from urinary bladder RNA as previously described (30). The reason for the failure to identify m1 mRNA using the random primers is unclear, although it may be related to either a lower efficiency of RT using random primers or to random primers carried over from the RT reaction inhibiting the PCR reaction. Either way, care must be exercised in analyzing negative results obtained with RT-PCR if only one type of primer is used. This observation may help clarify some contradictory results obtained using RT-PCR when different types of oligonucleotide primers are used in the RT reaction.

In conclusion, the present study, based on both functional and molecular studies, indicates the presence of presynaptic M1, facilitory and M2 inhibitory receptors and postjunctional M3 receptors in the rat bladder. The physiological significance of presynaptic modulatory receptors in the bladder requires further investigation. It remains to be determined if these receptors are colocalized to the same nerves and under exactly what conditions in vivo these receptors come into play. Functional characterization of the muscarinic receptor subtypes in human bladder may allow for the clinical application of subtype-selective agents in the treatment of a variety of voiding dysfunctions while potentially minimizing the side effects of current cholinergic-based therapy.

We gratefully acknowledge the technical assistance of Sharon Filer-Maerten.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants RO1-DK43333 (M. R. Ruggieri and G. Luthlin) and RO1-DK 39086 (M. R. Ruggieri).

Address for reprint requests: M. R. Ruggieri, Temple Univ. School of Medicine, Dept. of Urology Research, 3400 North Broad St., Philadelphia, PA 19140.

Received 16 May 1997; accepted in final form 5 November 1997.

REFERENCES


Address for reprint requests: M. R. Ruggieri, Temple Univ. School of Medicine, Dept. of Urology Research, 3400 North Broad St., Philadelphia, PA 19140.


