Subtypes of muscarinic receptors regulating gallbladder cholinergic contractions

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Parkman, Henry P., Anthony P. Pagano, and James P. Ryan. Subtypes of muscarinic receptors regulating gallbladder cholinergic contractions. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1243–G1250, 1999.—The aim of this study was to determine the functional role of muscarinic receptor subtypes regulating gallbladder cholinergic contractions. Electrical field stimulation (EFS; 16 Hz) produced contractile responses of guinea pig gallbladder muscle strips in vitro that were inhibited by 1 µM tetrodotoxin (2 ± 2% of control) and 1 µM atropine (1 ± 1% of control), indicating activation of intrinsic cholinergic nerves. Exogenous ACh (5 µM)-induced contractions were inhibited by atropine (1 ± 1% of control) but not tetrodotoxin (102 ± 1% of control), indicating a direct effect on smooth muscle. The M1 receptor antagonist pirenzepine (10 nM) had no effect on ACh-induced contractions but inhibited EFS-induced contractions by 11 ± 3%. The M2 antagonist methoctramine (10 nM) had no effect on ACh-induced contractions but augmented EFS-induced contractions by 5 ± 2%. The M3 antagonist 4-DAMP (10 nM) inhibited ACh-induced contractions by 14 ± 4% and EFS-induced contractions by 22 ± 5%. In conclusion, specific M1, M2, and M3 receptors modulate gallbladder muscle contractions by regulating ACh release from cholinergic nerves and mediating the contraction. Cholinergic contractions are mediated by M3 receptors directly on the smooth muscle. M2 receptors are on cholinergic nerves and function as presynaptic inhibitory autoreceptors. M1 receptors are on cholinergic nerves and function as presynaptic facilitatory autoreceptors.

The intrinsic cholinergic innervation of the gallbladder is important for gallbladder contractility and emptying. The muscarinic receptor antagonist atropine decreases meal-stimulated gallbladder emptying (9). Cholecystokinin (CCK), the gastrointestinal hormone that mediates meal-stimulated gallbladder emptying, has been shown to facilitate ACh release from gallbladder neurons (14, 27). Furthermore, the gallbladder contractile effect of CCK infusion in vivo is inhibited by atropine (11). Evidence for the intrinsic cholinergic nerves of the gallbladder has been provided by electrical field stimulation (EFS), which stimulates intrinsic nerves, causing release of ACh and cholinergically mediated contractions (18, 28). Recently, immunohistochemical studies have verified the presence of intrinsic cholinergic neurons in the gallbladder (23). The ACh released from these intrinsic nerves will act on smooth muscle muscarinic receptors, causing gallbladder contraction.

There are multiple different muscarinic receptor subtypes that have been identified using molecular (m1, m2, m3, m4, and m5) and pharmacological (M1, M2, M3, and M4) techniques (10). The muscarinic receptor subtype(s) mediating cholinergic contractions in the gallbladder is controversial. Different studies have suggested involvement of the M1, M2, M3, and M4 subtypes (4, 15, 16, 22, 24).

Most tissues, however, actually contain a mixture of receptor subtypes that may have different locations and may serve distinct physiological functions (6). Besides the smooth muscle muscarinic receptor that mediates smooth muscle contractions, cholinergic neurons in many tissues contain presynaptic muscarinic receptors on nerve terminals that serve to limit (“inhibitory autoreceptor”) or enhance (“excitatory autoreceptor”) the release of ACh from cholinergic nerves (10).

The aim of this study, therefore, was to determine the functional roles of different muscarinic receptor subtypes in regulating gallbladder cholinergic contractions.

METHODS

Gallbladder muscle strip preparation. Guinea pigs (Ace Animals, Boyertown, PA) weighing 400–450 g were fasted overnight before study. The gallbladder was removed after the guinea pig had been killed by CO2 asphyxiation. The gallbladders were opened along the longitudinal axis and rinsed with Krebs bicarbonate buffer (composition in mM: 120 NaCl, 4.6 KCl, 2.5 CaCl2, 1.2 MgCl2, 22 NaHCO3, 1.2 NaH2PO4, and 11.5 glucose; oxygenated with 95% O2 and 5% CO2; pH 7.4). Gallbladder muscle strips were prepared as previously described (18). Full-thickness strips (2 × 10 mm) containing mucosa, smooth muscle, and serosa were cut along the longitudinal axis of the gallbladder. The strips were suspended in 10-ml organ baths containing Krebs bicarbonate buffer (temperature 37°C). Muscle tension was measured along the longitudinal axis using an isometric force transducer (FT.03C; Grass Instruments, Astro-Med, W. Warwick, RI). The muscle strips were suspended between platinum electrodes placed adjacent and parallel to the long axis of the muscle strip. These were connected to an electric stimulator (model SD9; Grass Instruments). After a 30-min equilibration period, the preparations were stretched until the contractile force to 100 µM ACh was maximal (Lmax).

Experimental protocols. EFS with 1–16 Hz, 100 V, 0.5 ms pulse width duration (square wave), and 30-s train duration parameters were used to activate the intrinsic nerves. Our previous studies have shown that EFS produces a frequency-dependent contractile response that is primarily mediated by cholinergic nerves, since the contractile response is inhibited by 1 µM tetrodotoxin or 1 µM atropine (18). Exogenous ACh was also used to directly contract the gallbladder smooth muscle. For these studies, a submaximal concentration of ACh (5 µM) was used, which gives ~68% of the maximal response to 100 µM ACh (19). This concentration was chosen so that augmentation or inhibition of the contractile response...
could be detected. The contractile response to 16 Hz EFS is similar in amplitude to the contractile response to 5 μM ACh (18).

For the receptor antagonist experiments, graded concentrations (10 nM to 10 μM) of selective muscarinic antagonists were tested against the response of EFS (16 Hz) and exogenous ACh (5 μM). Initial responses to EFS or exogenous ACh in normal Krebs solution were used as the control responses. After 20 min of washing in normal Krebs solution, the antagonist was administered, followed in 10 min by a repeat EFS or addition of exogenous ACh in the continued presence of the antagonist.

At the end of the experimental protocols, the muscle strips were incubated for 10 min in 1 μM tetrodotoxin with repeat EFS with 16 Hz to ensure that there were no responses with EFS, verifying that only neural responses were elicited.

In additional experiments, the effects of muscarinic agonists on gallbladder muscle contractility were determined. After reaching L_{max} and equilibrating for 30 min, increasing concentrations of bethanechol or McN-A-343 were applied over the range 10^{-8} to 10^{-3} M. At least 15 min of washing between doses was allowed for reequilibration to basal tone. For experiments with channel blockers (tetrodotoxin and ω-conotoxin GVIA), an initial response to the agonist in normal Krebs solution was used as the control response. After 20 min of washing in normal Krebs solution, tetrodotoxin and ω-conotoxin GVIA was administered, followed in 5 min by a repeat application of the control dose of agonist in the continued presence of the channel blocker.

Compounds. ACh chloride, hexamethonium chloride, the nonspecific muscarinic antagonist atropine sulfate, and tetrodotoxin were obtained from Sigma Chemical (St. Louis, MO). The following muscarinic receptor subtype antagonists were used: pirenzepine dihydrochloride, an M_{1} receptor antagonist (Sigma Chemical); methoctramine hydrochloride, an M_{2} receptor antagonist (Research Biochemicals International, Natick, MA); 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methiodide, an M_{3} receptor antagonist (Research Biochemicals International, Natick, MA); and tropicamide, an M_{4} receptor antagonist (Sigma Chemical). Bethanechol and the M_{1} receptor agonist McN-A-343 were obtained from Research Biochemicals International. Telenzepine dihydrochloride, a putative M_{1} receptor antagonist, and p-fluoro hexahyro-sila-difenidol hydrochloride (p-F-HHSID), a putative M_{3} receptor subtype antagonist, were obtained from Research Biochemicals International.

Data analysis. Data are expressed as means ± SE of results obtained from 4–15 muscle strips. Student’s t-test was used to determine if the effects of receptor antagonists on EFS- or ACh-induced contractions were significantly different from the control responses. For experiments using receptor antagonists, each preparation served as its own control, and the amplitude of contraction after incubation with the receptor antagonist was compared with the amplitude of contraction in normal Krebs solution immediately preceding incubation with the antagonist. P < 0.05 was considered statistically significant.

RESULTS

General observations. EFS of the gallbladder muscle strips produced frequency-dependent contractile responses of the gallbladder muscle strips, as previously reported (18). Repeat stimulations at 16 Hz gave reproducible contractions; the second stimulation-induced contraction was 99.9 ± 1.2% of the initial stimulation (see Table 1). EFS-induced contractions (16 Hz) were inhibited by either 1 μM tetrodotoxin (2 ± 2% of control; P < 0.01) or 1 μM atropine (1 ± 1% of control; P < 0.01) but not by 100 μM hexamethonium (102 ± 3% of control), indicating activation of intrinsic cholinergic nerves, as shown in our previous study (18).

The gallbladder muscle strip contractile response to repeat exogenous administrations of a submaximal concentration of ACh (5 μM) was reproducible; the second ACh-induced contraction was 104 ± 3% of the first administration. The gallbladder muscle strip contractile response to exogenous 5 μM ACh was inhibited by 1 μM atropine (1 ± 1% of control) but not by 1 μM tetrodotoxin (102 ± 1% of control) or hexamethonium (101 ± 4% of control), suggesting that ACh is acting primarily directly on the smooth muscle to cause contraction.

Effect of muscarinic receptor antagonists to 5 μM ACh and 16 Hz EFS. In these experiments, graded concentrations (10 nM to 10 μM) of selective M_{1}, M_{2}, M_{3}, and M_{4} muscarinic antagonists were tested against the contractile response of EFS and exogenous ACh (see Figs. 1–3). The nonspecific muscarinic antagonist atropine was the most potent antagonist in inhibiting both ACh-induced and EFS-induced gallbladder contraction (see Figs. 1 and 2). At 0.1 μM, atropine inhibited 5 μM ACh-induced contractions by 82 ± 6% (P < 0.01) and EFS (16 Hz)-induced contractions by 74 ± 10% (P < 0.01). Higher concentrations of atropine produced nearly complete inhibition of either ACh- or EFS-induced contractions. The effect of the M_{3} receptor antagonist 4-DAMP was similar to that of atropine in inhibiting both ACh- and EFS-induced contractions (see Figs. 1–3).

High doses of the selective muscarinic subtype antagonists (1–10 μM) inhibited the contractile response to both ACh and EFS (see Figs. 1 and 2), probably through nonspecific muscarinic blockade. At 1 μM, each of the putative muscarinic receptor subtype antagonists inhibited the contractile effects of ACh and EFS: pirenzepine, 31 ± 4% inhibition of ACh-induced contractions and 44 ± 7% inhibition of EFS-induced contractions; methoctramine, 33 ± 2% inhibition of ACh-induced contractions and 13 ± 5% inhibition of EFS-induced contractions; 4-DAMP, 99 ± 1% inhibition of ACh-induced contractions and 87 ± 3% inhibition of EFS-induced contractions; tropicamide, 60 ± 5% inhibition

| Table 1. Effect of low-dose muscarinic subtype antagonists (10 nM) on exogenous ACh-induced and EFS (16-Hz)-induced guinea pig gallbladder contractions |
|---------------------------------|---------------|---------------|
|                                 | ACh (5 μM)    | EFS (16 Hz)   |
| Control (for repeat stimulations) | 104 ± 3       | 100 ± 1       |
| Atropine (nonspecific M antagonist) | 91 ± 3*       | 82 ± 5*       |
| Pirenzepine (M_{1} receptor antagonist) | 103 ± 1       | 89 ± 3*†      |
| Methoctramine (M_{2} receptor antagonist) | 97 ± 2        | 105 ± 2*†     |
| 4-DAMP (M_{3} receptor antagonist) | 86 ± 4*       | 78 ± 5*       |
| Tropicamide (M_{4} receptor antagonist) | 102 ± 3       | 100 ± 1       |

Data are presented as percent of control response and are reported as means ± SE. EFS, electrical field stimulation; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine. *P < 0.05 vs. control response; †P < 0.05 vs. effect of antagonist on 5 μM ACh.
of ACh-induced contractions and 62 ± 5% inhibition of EFS-induced contractions.

Lower concentrations (10–100 nM) of the putative selective muscarinic antagonists provided insight into the location and function of the different receptor subtypes. The M1 selective receptor antagonist pirenzepine (10 nM) had no effect on ACh-induced contractions but significantly inhibited EFS (16 Hz)-induced contractions by 11 ± 3% (P < 0.01; see Fig. 4A). The M2 antagonist methoctramine (10 nM) had no effect on ACh-induced contractions but augmented EFS (16 Hz)-induced contractions by 5 ± 2% (P < 0.05; see Fig. 4B). The M3 antagonist 4-DAMP (10 nM) significantly inhibited both ACh-induced contractions by 14 ± 4% (P < 0.01) and EFS (16 Hz)-induced contractions by 22 ± 5% (P < 0.01; see Fig. 4C). The M4 antagonist tropicamide (10 nM) had no effect on ACh- or EFS (16 Hz)-induced contractions (see Table 1).

Several other putative receptor subtype antagonists were tested. Telenzepine, a putative M1 receptor subtype antagonist, gave similar results as pirenzepine. Telenzepine, at a concentration of 10 nM, inhibited the effects of EFS (16 Hz)-induced contractions by 6.2 ± 3.0% (P < 0.05), whereas it did not have an effect on exogenous ACh-induced contractions. p-F-HHSiD, a putative M3 receptor subtype antagonist, gave similar results as 4-DAMP, except that these inhibitory effects of p-F-HHSiD were only seen at high concentrations (1 µM). p-F-HHSiD (1 µM) inhibited both 5 µM ACh-induced contractions (14 ± 4% inhibition; P < 0.05) and EFS (16 Hz)-induced contractions (33 ± 12% inhibition; P < 0.05).

Effect of muscarinic receptor antagonists on different frequency stimulation with EFS. Additional experiments were performed to test the effects of the putative muscarinic receptor subtype antagonists against a range of different EFS frequencies (1–16 Hz; see Table 2). The M3 receptor antagonist 4-DAMP (10 nM) inhibited EFS contractions to a similar degree, by 50–70%, in response to a range of stimulation frequencies (1–16 Hz). The M2 receptor antagonist methoctramine (10 nM) augmented EFS contractions, expressed as a percentage of the baseline response, more prominently for lower-frequency (1–4 Hz) stimulation than for higher-frequency (8–16 Hz) stimulation. The M1 receptor antagonist pirenzepine inhibited EFS-induced contractions preferentially for higher frequencies (16 Hz) than for lower frequencies (1–4 Hz).
DISCUSSION

The inhibition of meal- and CCK-induced gallbladder emptying by atropine demonstrates that cholinergic nerves and muscarinic receptors are important in mediating physiological gallbladder emptying (9, 11). This has been directly shown by the demonstration that CCK facilitates cholinergically mediated synaptic input to gallbladder neurons (1, 14).

The specific subtypes of muscarinic receptors that mediate gallbladder contraction and emptying in response to cholinergic stimulation have been controversial. In vitro studies using putative specific receptor subtype antagonists to carbachol-induced contractions and to receptor binding of radiolabeled scopolamine have suggested that the muscarinic receptor on guinea pig gallbladder smooth muscle is the M3 subtype (8, 13, 22, 24). This has also been recently suggested in studies using putative receptor subtype agonists (8). However, it must be recognized that, at the present time, there are no truly “selective” agonists for the muscarinic subtypes, and, consequently, it is difficult to assign a muscarinic subtype on the basis of agonist affinity (7).

Other in vitro studies, also studying the effect of putative receptor subtype antagonists on the contractile response to exogenous application of muscarinic agonists (carbachol), have suggested that the M4 receptors mediate cholinergic guinea pig gallbladder contractions (16, 17). More recent studies in isolated feline gallbladder muscle cells have suggested that ACh contracts the gallbladder by both M2 and M3 receptors (4). In vivo studies in animals and humans have shown that gallbladder emptying after a meal or CCK is reduced by M1 receptor antagonists (15, 29). Thus the muscarinic receptor subtypes mediating cholinergic gallbladder contractions are not clear; there is evidence from different studies for involvement of each of the M1, M2, M3, and M4 subtypes in mediating the gallbladder contractions.

Most tissues contain a mixture of receptor subtypes that have different locations and may serve distinct functions (6). This also appears true for the gallbladder, as recent semiquantitative analysis in human gallbladders with PCR for mRNA demonstrated a muscarinic subtype expression in the order m2 > m3 > m4 > m1 (12). Furthermore, cholinergic neurons in many tissues contain modulatory presynaptic muscarinic receptors on nerve terminals that serve to limit (inhibitory autoreceptor) or enhance (excitatory autoreceptor) the release of ACh from cholinergic nerves (10). Whether regulatory autoreceptors are present in the gallbladder on cholinergic neurons has not been addressed in prior studies. The presence of presynaptic muscarinic autoreceptors may explain some of the conflicting data on the type of muscarinic receptor subtypes mediating gallbladder contraction that are reported in the literature.

EFS of the guinea pig gallbladder produces a contractile response that is, as expected, inhibited by tetrodotoxin. The EFS-induced contractions are also inhibited
by atropine, suggesting that the contractile response of
the gallbladder is from activation of intrinsic cholinergic nerves (18). In this present study, we took advan-
tage of these cholinergic contractions induced by EFS
in the guinea pig gallbladder to investigate the role of
specific muscarinic receptor subtypes in mediating
muscle contractility in response to the release of endog-
enous ACh from the intrinsic cholinergic nerves of the
gallbladder. We indirectly assessed ACh release from
the intrinsic cholinergic nerves by comparing the ef-
fects of antagonists on EFS-induced cholinergic contrac-
tions mediated by endogenous release of ACh with their
effects on exogenous ACh-induced contractions.

Our studies suggest that M1, M2, and M3 receptor
subtypes are present, and each may have a specific
functional role (see Fig. 5). The M3 receptor antagonist
4-DAMP inhibited both contractile responses to exog-
enous ACh and to EFS. This suggests that the M3
receptors are present on the smooth muscle and medi-
ate cholinergic-induced contractions. The M2 antago-
nist methoctramine had no effect on ACh-induced con-
tractions but augmented EFS-induced contractions.
This suggests that M2 receptors are present on choliner-
gic nerves and function normally when activated to
inhibit ACh release from the neurons; that is, they

Table 2. Effect of muscarinic subtype antagonists on
different frequencies of EFS-stimulated gallbladder
muscle strip contractions

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<th>Frequency, Hz</th>
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<th>2</th>
<th>4</th>
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<tr>
<td>Pirenzepine</td>
<td>104±13</td>
<td>112±19</td>
<td>111±14</td>
<td>92±7</td>
<td>93±3†</td>
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<td>(10 nM)</td>
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<tr>
<td>Methoctramine</td>
<td>275±53*</td>
<td>218±51*</td>
<td>141±13†</td>
<td>136±11†</td>
<td>119±9†</td>
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<td>(10 nM)</td>
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<tr>
<td>4-DAMP</td>
<td>30±30†</td>
<td>33±24†</td>
<td>49±13†</td>
<td>38±6†</td>
<td>44±11†</td>
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<td>(10 nM)</td>
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Results are expressed as the percent of the control stimulation
response at the same frequency and are reported as means ± SE from
3–5 muscle strips from different animals. *P < 0.01 and †P < 0.05 vs.
control response.

Fig. 5. Summary schematic illustrating the location and function of
the muscarinic receptor subtypes suggested from the results of this
study.
function as presynaptic inhibitory autoreceptors. The M₁ selective receptor antagonist pirenzepine had no effect on ACh-induced contractions but significantly inhibited EFS-induced contractions. This suggests that M₁ receptors are located on cholinergic nerves and function normally when activated to increase release of ACh from the nerves; that is, they function as presynaptic excitatory autoreceptors. Thus specific M₁, M₂, and M₃ muscarinic receptors may modulate gallbladder smooth muscle contraction by regulating ACh release from cholinergic nerves and by also mediating the contraction. In the gallbladder, the M₁ receptor may enhance ACh release postprandially and enable complete gallbladder emptying, whereas the M₂ receptor may act as an autoinhibitory receptor to stop release of ACh from nerve endings and end contraction (25).

In this study, the effects of presynaptic autoreceptor blockade with muscarinic subtype antagonists were small, giving only 5–10% alteration in the contractile response. Part of this was due to the fact that low concentrations (10 nM) of the antagonists were used. At higher concentrations, where more blockade might have been expected, each of the muscarinic receptor subtype-specific antagonists used appeared to have nonspecific muscarinic effects, probably from M₂ antagonist actions to inhibit the smooth muscle contractile response. This poses a potential limitation to these studies that measured gallbladder contractility and indirectly inferred events about presynaptic muscarinic receptors regulating ACh release, since the postsynaptic smooth muscle response is also mediated through muscarinic receptors (26). Another way to study presynaptic autoreceptors is to directly measure ACh release from the intrinsic nerves (21). The direct measurement of ACh release does not rely on a distant endpoint such as muscle contraction, which may be influenced by multiple factors, including the effects of other neurally released neurotransmitters.

Studies using receptor binding and molecular probes suggest that smooth muscle, in general, contains both M₂ and M₃ receptors (10). Functionally, however, muscle contraction is mainly due to M₂ receptors, as shown in other tissues (10) and in this study in the guinea pig gallbladder. The smooth muscle M₁ receptor can be brought out by special experimental techniques. In cat gallbladder isolated myocytes, neither an M₂ or M₃ receptor antagonist alone inhibited the contractile response to carbachol, but the combination did, suggesting cholinergic contractions of gallbladder muscle are through stimulation of both M₂ and M₃ receptors (4). In these studies, the M₂ receptors were linked to calcium influx, activation of phospholipase D, and a protein kinase C-dependent pathway, whereas the M₃ receptor was preferentially associated with activation of phospholipase C, intracellular calcium release, and a calmodulin-dependent pathway (4). A role for the M₂ receptor in the contractile response of smooth muscle has also been demonstrated when cAMP levels are increased such as under β-adrenergic receptor activation (7). This may provide a mechanism for contractions in smooth muscle inhibited by the sympathetic nervous system. Interestingly, under several pathological conditions, M₂ smooth muscle receptors are able to upregulate. In the urinary bladder under normal conditions, M₃ receptors mediate the contractile response; however, after denervation of the muscle, a smooth muscle M₂ receptor appears to increase to complement the M₃ receptor in mediating cholinergic bladder contractions during neural dysfunction (3). At the lower esophageal sphincter, under normal conditions, M₃ receptors mediate the contractile response; however, in the presence of acute acid-induced esophagitis, a second muscarinic contractile pathway is mediated by M₂ receptors (20).

Our results suggesting smooth muscle M₂ receptors, prejunctional M₂ inhibitory autoreceptors, and prejunctional M₃ facilitatory autoreceptors are similar to those observations in the rat urinary bladder where presynaptic receptors have also been detected (2). In the urinary bladder, it has been suggested that activation of the different excitatory and inhibitory presynaptic autoreceptors may be stimulation frequency dependent. M₁-mediated facilitation of ACh release is seen during high-frequency continuous electrical stimulation or with treatment with the cholinesterase inhibitor phystostigmine with subsequent increased concentrations of endogenous ACh (21). In contrast, M₂ autoinhibition is seen predominately at low-frequency stimulation (21). In our studies, one frequency of EFS was predominately used (16 Hz). This stimulation frequency, which gives a contractile response equivalent to a submaximal concentration of ACh (5 µM), was chosen so that augmentation or inhibition of the contractile response could be detected. When a range of different frequencies was used, the gallbladder was found to be similar to the rat urinary bladder; that is, the M₂ receptor antagonist methoctramine (10 nM) augmented EFS contractions, expressed as a percentage of the control response, better for lower-frequency stimulation than for higher-frequency stimulation. Alternatively, the M₁ receptor antagonist pirenzepine inhibited EFS-induced contractions preferentially for higher frequencies than for lower frequencies. Thus, in the gallbladder, the M₁ presynaptic facilitatory autoreceptor is activated with higher-frequency stimulation, whereas the M₂ presynaptic inhibitory autoreceptor is activated more prominently at lower-frequency stimulation. The higher-frequency stimulation in vitro may be analogous to what occurs with postprandial gallbladder emptying, when cholinergic activity is increased. In addition, it has been suggested further, from studies in the urinary bladder, that the excitatory (M₁) and inhibitory (M₂) autoreceptors may have different locations on the presynaptic nerves such that the presynaptic receptor may be localized to the presynaptic synaptosomal plasma membrane or may be located more proximally on the axon of the cholinergic nerves at a further distance from the source of ACh release and thereby receive much less ACh during cholinergic nerve stimulation (21).

Our studies with the M₄ receptor antagonist tropicamide did not reveal a role for the M₄ subtype. Only at high concentrations (10 µM) did tropicamide have a slight
inhibitory effect on both ACh- and EFS-induced contractions. At these doses, each of the putative subtype antagonists had the same effect and probably represents nonspecific, possibly M3 receptor, inhibition. The presence of an M4 receptor is difficult to detect because of the inability of available antagonists to adequately distinguish between the M2 and M4 receptor subtypes (5).

The results of these studies have important ramifications for possible treatment of gallbladder dysfunction. Functional characterization of the muscarinic receptor subtypes in the gallbladder may allow for the clinical application of subtype-selective agents in the treatment of gallbladder motor dysfunction while potentially minimizing the side effects of current nonspecific cholinergic-based therapy from both cholinergic agonists and cholinergic agonists on other tissues, especially the urinary bladder and iris. For example, can cholinergic antagonists and cholinergic agonists on other tissues, especially the urinary bladder and iris. For example, can cholinergic antagonists and cholinergic agonists on other tissues, especially the urinary bladder and iris. For example, can cholinergic antagonists and cholinergic agonists on other tissues, especially the urinary bladder and iris. For example, can cholinergic antagonists and cholinergic agonists on other tissues, especially the urinary bladder and iris. For example, can cholinergic antagonists and cholinergic agonists on other tissues, especially the urinary bladder and iris. For example, can cholinergic antagonists and cholinergic agonists on other tissues, especially the urinary bladder and iris.

In summary, our studies suggest that specific M1, M2, and M3 muscarinic receptor subtypes modulate gallbladder smooth muscle contraction by regulating release of ACh from cholinergic nerves and by mediating the smooth muscle contraction. Cholinergic contractions of the guinea pig gallbladder are primarily mediated by M3 receptors directly on the gallbladder smooth muscle. M2 receptors are on cholinergic nerves and function as prejunctional inhibitory autoreceptors. M1 receptors are on cholinergic nerves and function as prejunctional facilitatory autoreceptors.

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