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Functional Role for the M_2 Muscarinic Receptor in Smooth Muscle of Guinea Pig Ileum

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SUMMARY

A functional role for the M_2 muscarinic receptor in smooth muscle contraction was investigated in isolated guinea pig ileum. Contractile responses to the muscarinic agonist oxotremorine-M (oxo-M) were measured in isolated ilea that had been pretreated with histamine (0.32 μM) and isoproterenol (0.64 μM) to achieve conditions of elevated cAMP. The resulting concentration-effect curve was biphasic, consisting of high (0–50 nM) and low (>50 nM) potency components. The reversible M_2-selective antagonist AF-DX 116 ([[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3*b*][1,4]benzodiazepine-6-one) (1 and 10 μM) shifted this curve in a manner that was inconsistent with competitive antagonism at a single receptor site; the high affinity component was significantly blocked, whereas there was little effect on the low affinity portion of the curve. To inactivate the M_3 muscarinic receptors selectively, ilea were incubated with the irreversible M_1/M_3-selective muscarinic antagonist 4-DAMP mustard [*N*-(2-chloroethyl)-4-piperidinyldiphenylacetate] (40 nM) for 1 hr in the presence of AF-DX 116 (1 μM) and were then washed extensively. Under these conditions, the contractile responses to oxo-M, in the presence of histamine and isoproterenol or forskolin, were antagonized by AF-DX 116 (1 μM) in a manner consistent with that mediated by an M_2 receptor. AF-DX 116 caused 6.6- and 11-fold increases in the EC_{50} value for oxo-M for ilea pretreated with isoproterenol and forskolin, respectively, and a significant increase in the Hill coefficient in both cases. Under basal conditions, AF-DX 116 caused only a 1.34-fold increase in the EC_{50} value and no change in the Hill coefficient. In addition, under basal conditions 4-DAMP mustard treatment shifted the oxo-M contractile response curve to the right approximately 20-fold. However, when histamine was present in combination with isoproterenol or forskolin 4-DAMP mustard treatment shifted the concentration-effect curves for oxo-M to the right only about 3.5-fold. Oxo-M produced an M_3-mediated stimulation of phosphoinositide hydrolysis in the longitudinal muscle of rat ileum with an EC_{50} value of 30 μM. 4-DAMP mustard (10 nM; 1 hr) prevented this response, resulting in a 6.6-fold increase in the EC_{50} value with a 65% reduction of the maximal response. In contrast, this treatment blocked M_2-mediated inhibition of isoproterenol-stimulated adenylate cyclase with only a 2-fold increase in EC_{50}, without affecting maximum inhibition. A more selective blockade of the M_3-mediated response was achieved by incubation of the longitudinal muscle slices with 4-DAMP mustard (40 nM) for 1 hr in the presence of AF-DX 116 (1 μM), followed by extensive washing. Under these conditions, the EC_{50} value for phosphoinositide hydrolysis was shifted 2.5-fold, with an 80% reduction in the maximum response. However, no change was observed in the ability of oxo-M to inhibit adenylate cyclase with this treatment. Our results demonstrate that 4-DAMP mustard can be used to inactivate M_3-mediated responses selectively and that contractile responses of guinea pig ileum can be elicited not only by activation of phosphoinositide-coupled M_3 receptors but also through activation of the more abundant cAMP-inhibitory M_2 receptors.

Many smooth muscle-containing tissues, including the gastrointestinal tract, urinary bladder, respiratory tract, and vasculature, consist of coexisting populations of M_2 and M_3 muscarinic receptors. It is well known that contractile responses are mediated via the M_3 muscarinic receptor (1–3); however, this subtype comprises only 20% of the total muscarinic receptors in smooth muscle (1, 4, 5). The function of the M_2 receptor, which accounts for the remaining 80% of the receptors, remains unknown.

Muscarinic acetylcholine receptors can be classified into four subtypes (M_1-M_4) based on their pharmacological properties or affinity profiles for various antagonists. M_1 muscarinic receptors have high affinity for pirenzepine ($M_1 > M_4 \geq M_3 > M_2$) (6–8) and are mainly present in neuronal tissues. The M_2 muscarinic receptors, characterized by their high affinity for AF-DX 116 (11[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3*b*][1,4]benzodiazepine-6-one)

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ABBREVIATIONS: HHSiD, hexahydrosiladifenidol; 4-DAMP, *N*,*N*-dimethyl-4-piperidinyldiphenylacetate; 4-DAMP mustard, *N*-(2-chloroethyl)-4-piperidinyldiphenylacetate; KRB, Krebs Ringer bicarbonate; oxo-M, oxotremorine-M.

($M_2 > M_1 \geq M_4 > M_3$) (7, 9, 10), account for most, if not all, of the binding sites in mammalian heart. The antagonists HHSiD and 4-DAMP have high affinity for the M_3 muscarinic receptor, which triggers responses in smooth muscle and exocrine glands, but also show affinity for the M_1 and M_4 receptor subtypes ($M_3 \geq M_1 \geq M_4 > M_2$) (7, 11). Himbacine exhibits high affinity for M_4 receptors as well as M_2 receptors ($M_2 \geq M_4 > M_1 > M_3$) (7, 12). Molecular biological studies, however, have revealed five genes for the muscarinic receptor (m_1-m_5) (13). Based on the binding properties of the expressed recombinant muscarinic receptors and the distribution of their mRNA, it appears that the molecularly defined receptor subtypes m_1, m_2, and m_3 represent the pharmacologically defined M_1, M_2, and M_3 subtypes, respectively (14–17).

In guinea pig ileum, the presence of both M_2 and M_3 receptors has been demonstrated (4). Studies on the signaling mechanisms of the M_2 and M_3 muscarinic receptors present in rat ileum have shown that the M_2 subtype is coupled to the inhibition of adenylate cyclase, whereas the M_3 receptor mediates phosphoinositide hydrolysis, resulting in calcium mobilization and contraction (1). Recent studies from our laboratory have shown that, in whole-cell preparations of rat ileum, M_2 receptor activation specifically opposes increased levels of cAMP induced by β-adrenergic and forskolin stimulation of adenylate cyclase but has no effect on basal levels of cAMP or on cAMP levels stimulated by prostaglandin E_1, prostaglandin E_2, and 2-chloroadenosine (18). Because increased levels of cAMP have been shown to relax or inhibit smooth muscle contraction (19), it has been suggested that inhibition of cAMP accumulation by M_2 muscarinic receptor activation may lead to an inhibition of relaxation induced by other receptors (1, 18). Thus, there may be two ways to elicit muscarinic contraction of smooth muscle, i.e., a direct M_3 receptor-mediated contraction via hydrolysis of phosphoinositides and an indirect M_2 receptor-mediated inhibition of the relaxation induced by other receptors (disinhibition of contraction).

Determination of the relative contribution of each subtype to contractile function is hampered by the lack of agonists with marked selectivity for one subtype over the other. An alternative approach to exploring one subtype is to use a nonselective agonist and use the available "selective" antagonists to block one subtype while leaving the other site open for activation. This is the rationale behind the use of the compound 4-DAMP mustard, which we used to inactivate the M_3 receptor subtype selectively while leaving the M_2 subtype intact.

In the present study, we investigated a functional role for the M_2 muscarinic receptor by using the irreversible muscarinic antagonist 4-DAMP mustard to inactivate M_3-mediated phosphoinositide hydrolysis selectively, while M_2-mediated inhibition of cAMP accumulation remained intact. We then performed contractile studies on guinea pig ileum to demonstrate that M_2 receptor activation causes indirect contraction, presumably by opposing relaxation elicited by isoproterenol and forskolin.

Materials and Methods

Formation of the aziridinium ion of 4-DAMP mustard. 4-DAMP mustard goes through two sequential reactions in aqueous solution at neutral pH. The first of these is cyclization to its reactive aziridinium ion and the second is hydrolysis of the aziridinium ion to the stable alcohol product. In all experiments, 4-DAMP mustard (10 μM) was incubated in 10 mM phosphate buffer, pH 7.4, at 37° for 30 min, to allow formation of the reactive aziridinium ion, and then placed immediately on ice until use.

Phosphoinositide hydrolysis. Male Sprague-Dawley rats (150–250 g) were sacrificed by decapitation, and their ilea were rapidly dissected out. The longitudinal muscle was removed by the method of Rang (20) and placed immediately in KRB buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose) gassed with O_2/CO_2 (19:1). The muscle strips were cross-chopped at 350 μm with a McIlwain tissue chopper, washed extensively, and equilibrated at 37°. The slices from three rats were incubated at 37° in a volume of 100 ml of KRB buffer with the aziridinium ion of 4-DAMP mustard, by itself or in combination with AF-DX 116 (1 μM), for 1 hr and then for an additional 10 min in the presence of 0.05 M Na_2SO_3. Slices were washed extensively to remove AF DX-116 and inactivated 4-DAMP mustard. Control slices were incubated in the same manner except for exposure to 4-DAMP mustard and AF DX-116. The tissue was incubated for 90 min in a final volume of 4 ml of KRB buffer containing 40 μCi of *myo*-[^{3}H]inositol. After this labeling phase, the tissue was washed three times and the slices were allowed to settle in fresh KRB buffer containing LiCl (10 mM). Aliquots (50–100 μl) of sedimented tissue slices were pipeted into small tubes containing KRB buffer (0.35 ml), LiCl (10 mM), and various concentrations of oxo-M. The incubation with agonist lasted 30 min at 37°. The incubations were stopped by the addition of 1.13 ml of chloroform/methanol (1:2, v/v). The accumulated inositol phosphates were extracted and separated by the method of Berridge *et al.* (21). Water (0.37 ml) and chloroform (0.37 ml) were added to separate aqueous and organic phases. The tubes were centrifuged, and an aliquot (1 ml) of the aqueous phase was added to another tube containing 2 ml of water. This tube was centrifuged to sediment residual chloroform, and essentially all of the aqueous phase (2.9 ml) was applied to an anion exchange column consisting of 1 ml of Dowex AG 1-X8 (100–200 mesh). The column was washed with four aliquots of water (5 ml) to remove [^{3}H]inositol, which was discarded. Total [^{3}H]inositol phosphates were eluted from the column with 2.5 ml of 1 M ammonium formate/0.1 M formic acid, and the amount of radioactivity was determined by using a scintillation counter. An aliquot (0.2 ml) of the organic phase was counted and used as a measurement of [^{3}H]inositol incorporation into phospholipids. The amount of [^{3}H]inositol phosphate formed (cpm) is expressed as a percentage of the total amount of radioactivity in the organic phase plus the inositol phosphate fraction, to correct for minor variations in the amount of tissue added to each assay.

cAMP accumulation. Slices of the longitudinal muscle of the ileum were prepared from three rats as described above and were gassed continuously with O_2/CO_2 (19:1). The slices were incubated for 45 min at 37° in 15 ml of KRB buffer with [^{3}H]adenine (40–60 μCi), to label endogenous ATP. Slices were washed to remove extracellular [^{3}H]adenine and were incubated again for 15 min. Slices were incubated for 1 hr at 37° with the aziridinium ion of 4-DAMP mustard alone or in combination with AF-DX 116 (1 μM). Slices were incubated for an additional 10 min with 0.01 M Na_2SO_3 and were then washed four times to remove AF-DX 116 and inactivated 4-DAMP mustard. Aliquots (50–100 μl) of gently packed tissue slices were incubated for 10 min at 37° in KRB buffer (0.7 ml) containing isoproterenol (1 μM), 3-isobutyl-1-methylxanthine (0.5 mM), and various concentrations of oxo-M. Reactions were stopped by the addition of 0.2 ml of trichloroacetic acid (30%, w/v) and homogenization with a Polytron. The tubes were centrifuged, and the [^{3}H]cAMP and [^{3}H]ATP were separated from the supernatant fraction using the double-chromatography method described by Salomon *et al.* (22). Approximately 2000 cpm of [^{32}P]cAMP were added as an internal standard. The supernatant was applied to a cation exchange column (1.25 ml of Dowex AG 50W-X4, 200–400 mesh) and washed twice with 1.25 ml of water. This eluate was collected, and the radioactivity was measured to determine the incorporation of [^{3}H]adenine into [^{3}H]ATP. The Dowex column was positioned over a column of neutral alumina (0.6 g), and the [^{3}H]cAMP was eluted onto

the alumina column with 5 ml of water. The [^{3}H]cAMP was eluted from the alumina with 4 ml of imidazole/0.1 M HCl, pH 7.5. These fractions were collected and the radioactivity was measured to determine the amount of accumulated [^{3}H]cAMP. The [^{3}H]cAMP values were corrected for recovery of the internal standard and are expressed as a percentage of the amount of incorporation of [^{3}H]adenine, to correct for minor variations in the amount of tissue added to each assay.

Isolated ileum. Male guinea pigs were sacrificed by CO_2 asphyxiation and the whole ileum was rapidly removed. Ileal segments (2–3 cm) were mounted longitudinally in an organ bath containing KRB buffer at 37° gassed with O_2/CO_2 (19:1). Isometric contractions were measured with a force displacement transducer and recorded on a polygraph. The ileum was equilibrated for 40 min at a resting tension of 0.5 g. Three test doses of the muscarinic agonist oxo-M were added to the bath to ensure reproducibility of the preparation. Ilea that did not achieve >60% of maximum with each test dose were discarded. After each test dose the ileum was washed with fresh KRB buffer and incubated for 5 min. To calculate an EC_{50} value for oxo-M, six to 10 concentrations of the compound, spaced geometrically every 0.33 log units, were added cumulatively to the bath, and contractile responses were measured. After an EC_{50} value for oxo-M was obtained, the ileum was washed three times and incubated for 30 min before additional measurements were made. In some experiments, tissues were incubated with the aziridinium ion of 4-DAMP mustard (40 nM) for 1 hr in the presence of AF-DX 116 (1 μM) and then for an additional 10 min in the presence of Na_2SO_3 (0.5 mM). Tissues were washed extensively to remove AF-DX 116 and inactivated 4-DAMP mustard. When present, the antagonist AF-DX 116 was incubated with the ileum for 20 min before contractions were measured.

Data analysis. The EC_{50} values of oxo-M (concentration of oxo-M required for half-maximal response) for contraction, inhibition of cAMP accumulation, and stimulation of phosphoinositide hydrolysis were estimated by nonlinear regression analysis of the data according to increasing or decreasing logistic equations, as described previously (1). This regression analysis also yielded estimates of the Hill coefficient. To obtain a more accurate estimate of the increase in the EC_{50} value caused by a given antagonist, the EC_{50} values were calculated by regression analysis with the maximal responses fixed to the same value, provided that the antagonist had no significant effect on the maximal response. The EC_{20} and EC_{80} values were calculated by interpolation with the best fitting logistic equation.

The proportion of receptors inactivated by 4-DAMP mustard in the phosphoinositide hydrolysis assay was estimated by a modification of the method of Furchgott described previously (23). Because 4-DAMP mustard did not decrease the maximal inhibition of cAMP accumulation induced by oxo-M, the percentage of alkylation of receptors was calculated using the relationship described by Paton (24), % occupancy = $(DR - 1/DR) \times 100$, where DR denotes the ratio of the EC_{50} value of oxo-M measured in the presence of the antagonist divided by that measured in the absence of the antagonist.

Compounds. 4-DAMP mustard was synthesized in our laboratory as described previously (25). Radiolabeled chemicals were obtained from ICN Biochemicals (Costa Mesa, CA). All other drugs were obtained from Sigma Chemical Co. (St. Louis, MO), except for AF-DX 116, which was acquired from Boehringer Ingelheim Pharmaceuticals (Ridgefield, Connecticut).

Results

Isolated ileum. Previous studies demonstrated that the M_3 receptor mediates contractions of isolated guinea pig ileum (1, 11). To verify the lack of contribution of the M_2 receptor to the contractile response under standard (basal) conditions, we measured the potency with which the M_2-selective antagonist AF-DX 116 blocked contractions of guinea pig ileum elicited by the nonselective agonist oxo-M. Oxo-M contracted the ileum with an EC_{50} value of 35 nM and a Hill coefficient of 1.81. AF-DX 116 at concentrations of 1 and 10 μM caused simple, parallel, rightward shifts of the concentration-effect curve of 2.3- and 18-fold, respectively, with Hill coefficients of 1.90 and 1.96, respectively (Fig. 1; Table 1). The calculated K_b values (see equation below) for AF-DX 116 at 1 and 10 μM were approximately the same (0.74 and 0.59 μM, respectively). These values are consistent with those expected for an M_3 response.

Because previous work from our laboratory demonstrated that the M_2 receptor specifically opposes the increase in cAMP elicited by the β-adrenergic agonist isoproterenol (18), we investigated whether the M_2 receptor contributes to the oxo-M contractile response in the presence of isoproterenol. If the M_2 receptor prevents the inhibitory effect of isoproterenol on M_3-mediated contractions, it might be expected that, in the presence of isoproterenol, oxo-M would trigger a contraction through both the M_2 and M_3 receptors and the potency of AF-DX 116 for blocking oxo-M-induced contractions would be enhanced in the presence of isoproterenol. Dose-dependent contractions in response to oxo-M were measured in tissues in the absence and presence of a single concentration of isoproterenol (1 μM). Isoproterenol caused a 2.1-fold increase in the EC_{50} value of the curve, with no change in maximal response (Fig. 2). Contractile responses to oxo-M were also measured in the presence of isoproterenol and AF-DX 116. At concentrations of 1 and 10 μM, AF-DX 116 caused 3.1- and 11.4-fold increases in EC_{50} value, respectively, relative to that measured in the presence of isoproterenol alone. The calculated K_b values (see equation below) for AF-DX 116 at 1 and 10 μM were

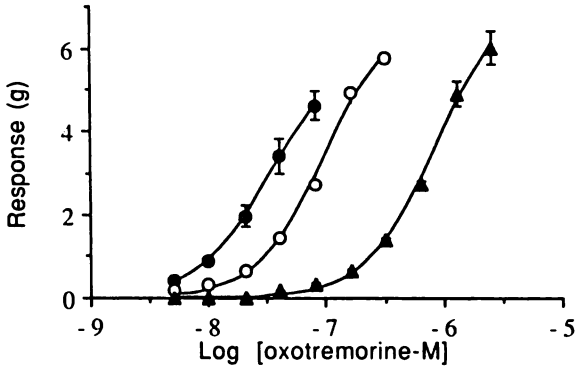

Fig. 1. Effects of AF-DX 116 on the concentration-effect curve for oxo-M in isolated guinea pig ileum, measured under standard basal conditions. Contractile responses were measured in the absence (●) and presence of 1 μM (○) and 10 μM (▲) AF-DX 116. Each *point* represents the mean ± standard error of the contractile response from six separate experiments.

TABLE 1

Hill coefficients and EC_{50} values for oxo-M-induced contractile responses in the absence and presence of AF-DX 116

The parameter estimates were calculated from the data shown in Figs. 1 and 3.

Conditions	Tissue treatment		
	Control	1 μM AF-DX 116	10 μM AF-DX 116
Basal			
n_H	1.81 ± 0.12	1.90 ± 0.07	1.86 ± 0.06
EC_{50} (nM)	34.8	81.9	623
Histamine/isoproterenol			
n_H	0.90 ± 0.10	1.84 ± 0.29[a]	1.89 ± 0.13[b]
EC_{50} (nM)	63.6	146	751

[a] Significantly different from control, $p < 0.001$.
[b] Significantly different from control, $p < 0.02$.

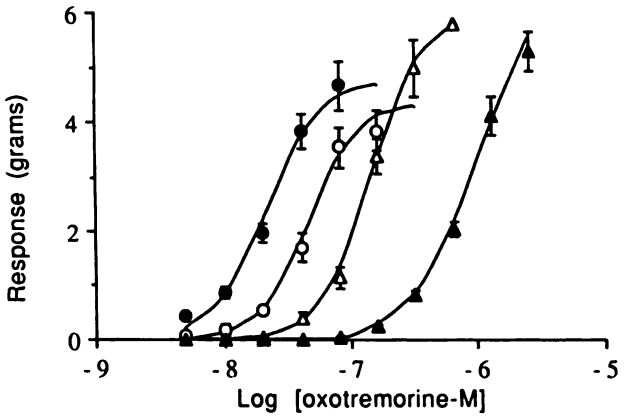

Fig. 2. Effects of AF-DX 116 on oxo-M-induced contractile responses of guinea pig ileum, measured in the presence of isoproterenol. Contractile responses were measured in the presence of isoproterenol (1 μM) alone (○) or in combination with 1 μM (△) or 10 μM (▲) AF-DX 116. ●, Control. Each *data point* represents the mean ± standard error of the contractile response from eight separate experiments.

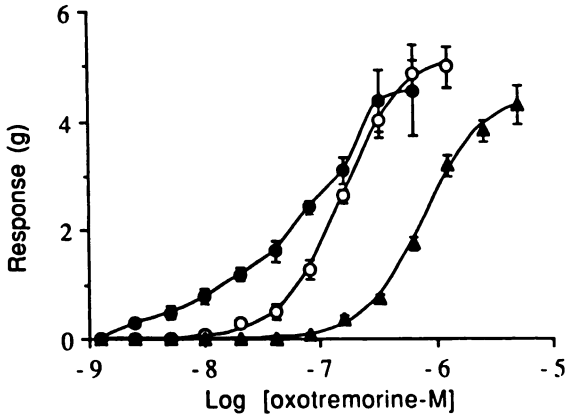

Fig. 3. Effects of AF-DX 116 on the oxo-M concentration-effect curve in isolated guinea pig ileum, measured in the presence of histamine and isoproterenol. Contractile responses to oxo-M were measured in the presence of histamine (0.32 μM) and isoproterenol (0.64 μM) (●). Similar measurements were repeated in the presence of 1 μM (○) and 10 μM (▲) AF-DX 116. By itself, histamine typically caused a contraction that was 50–70% of the maximal contraction induced by oxo-M. Each *data point* represents the mean ± standard error of the contractile response from six to eight separate experiments.

approximately the same (0.48 and 0.96 μM, respectively). These values were not significantly different from those observed under standard basal conditions (see above); thus, AF-DX 116 did not display enhanced potency in these experiments. It is possible that the M_2 and M_3 receptors are differentially localized and that activation of the M_2 receptor does not produce interaction with or disinhibition of contractions elicited by the M_3 receptor. Therefore, in the following experiments, we introduced a heterologous contractile agent, histamine.

The following experiments were designed to determine whether M_2 receptor activation can cause an indirect contraction by preventing the relaxing effects of isoproterenol on histamine-induced contractions. This mechanism is referred to as "M_2-mediated disinhibition of contraction." Experimental conditions consisted of measuring contractile responses to oxo-M in the presence of a heterologous contractile agent (histamine) and in the presence of isoproterenol, which is analogous to a physiological state of increased sympathetic activity. The tissues were first contracted with a submaximal concentration of histamine (0.32 μM) and then relaxed back to base-line levels with isoproterenol (0.64 μM). These compounds remained in the organ bath while contractile responses to oxo-M were measured relative to base-line levels. The resulting concentration-effect curve was biphasic, consisting of high (0–50 nM) and low (>50 nM) potency components. Nonlinear regression analysis of the data revealed an overall EC_{50} value of 64 nM and a Hill coefficient of 0.91 (Fig. 3). AF-DX 116, at concentrations of 1 and 10 μM, shifted this curve in a manner that was inconsistent with competitive antagonism at a single receptor site. At a concentration of 1 μM AF-DX 116, the high potency component (EC_{20}) was shifted 9.4-fold, whereas there was little effect (1.5-fold shift) on the low potency portion of the curve (EC_{80}) (Fig. 3). A 10-fold higher concentration of AF-DX 116 (10 μM) shifted the EC_{20} value 55-fold and the EC_{80} value 10-fold. This greater effect of AF-DX 116 on the high potency portion of the concentration-effect curve of oxo-M caused a steepening of the curve, which was quantified by estimation of the Hill coefficient. The resulting Hill coefficients for the curves in the presence of 1 and 10 μM AF-DX 116 were 1.84 and 1.89, respectively (compared with a control value of 0.91) (see Table 1). This observation suggests that more than one type of receptor is mediating contraction and that AF-DX 116 can discriminate between them. The expected shifts of M_2- and M_3-mediated responses caused by AF-DX 116 can be predicted using the following relationship: $K_b = [B]/(DR - 1)$, in which K_b denotes the dissociation constant of the antagonist, $[B]$ denotes the concentration of the antagonist, and DR (dose ratio) denotes the ratio of the EC_{50} value of oxo-M measured in the presence of the antagonist divided by that measured in the absence of the antagonist. Using K_b values for AF-DX 116 that have been reported elsewhere (1 and 0.1 μM for M_2 and M_3 receptors, respectively) (1), we would predict that 1 μM AF-DX 116 would cause 11- and 2-fold shifts of M_2- and M_3-mediated responses, respectively, and 10 μM AF-DX 116 would cause 101- and 11-fold shifts of M_2- and M_3-mediated responses, respectively. In cases where two different receptors contribute to the response, there is no simple way to apply the above equation to determine the K_b value for the two sites without knowing the relationship between occupancy and response. Nevertheless, there is general agreement between the shifts that we observed for the EC_{20} and EC_{80} responses and those predicted according to the above equation for M_2- and M_3-mediated responses. Therefore, we conclude that the high potency component of contraction is mediated via the M_2 receptor, whereas the low potency component is mediated through the M_3 receptor.

Effects of 4-DAMP mustard on M_2- and M_3-mediated second messenger pathways. To demonstrate an M_2 effect on contraction more clearly without interference from the M_3 receptor, we attempted to inactivate the M_3 receptor with the irreversible M_3-selective antagonist 4-DAMP mustard. Experiments were performed to analyze the effects of 4-DAMP mustard on M_2-mediated inhibition of adenylate cyclase and M_3-mediated phosphoinositide hydrolysis in slices of the longitudinal muscle of rat ileum. Oxo-M caused a concentration-dependent accumulation of inositol phosphates, with an EC_{50} value of 2.55 μM and maximal conversion of 22.4%. Ileal slices were incubated with 4-DAMP mustard (10 nM) at 37° for 1 hr and then washed extensively. This treatment caused a 6.5-fold

increase in the EC_{50} value for oxo-M, with a 60% decrease in the maximum response (Fig. 4a; Table 2). This effect corresponded to 94% alkylation of the muscarinic receptors, as estimated by the method of Furchgott (see Materials and Methods).

Oxo-M caused a concentration-dependent inhibition of isoproterenol-stimulated accumulation of cAMP in slices of the longitudinal muscle of the ileum, a response mediated via the M_2 receptor (18). The maximal inhibition induced by oxo-M was 25%, with an EC_{50} value of approximately 21.7 nM. Ileal slices were incubated with 4-DAMP mustard (10 nM) at 37° for 1 hr and were washed extensively. The resulting inhibition curve was shifted to the right by approximately 2.1-fold, with no change in the maximum inhibition (Fig. 4b; Table 2). The fraction of receptors occupied by 4-DAMP mustard under these conditions was calculated to be 52%, according to the relationship described by Paton (24). Thus, by itself 4-DAMP mustard caused a selective inactivation of M_3 receptors (94%), relative to M_2 receptors (52%); however, greater selectivity was achieved using the conditions described below.

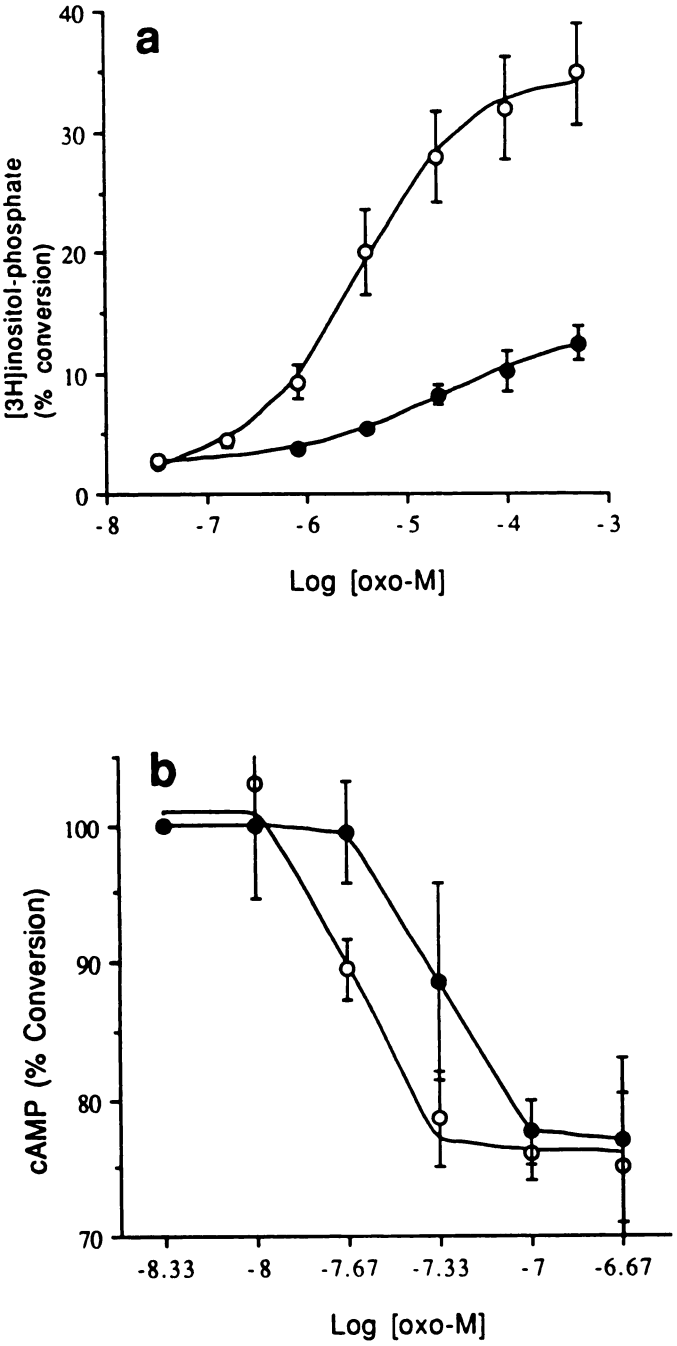

Fig. 4. Effects of 4-DAMP mustard on M_3-mediated phosphoinositide hydrolysis (a) and on M_2-mediated inhibition of cAMP accumulation (b) in the longitudinal muscle of rat ileum. Slices of the longitudinal muscle were incubated in the absence (○) and presence (●) of the aziridinium ion of 4-DAMP mustard (10 nM) at 37° for 1 hr and were then washed extensively before oxo-M-induced responses were measured. In b, cAMP levels were stimulated by 1 μM isoproterenol. *Data points* represent the mean ± standard error of the response from at least four experiments, each done in triplicate.

TABLE 2

Effects of 4-DAMP mustard on oxo-M-induced stimulation of phosphoinositide hydrolysis and inhibition of cAMP accumulation

The parameter estimates were calculated from the data shown in Figs. 4 and 5.

Treatment	Phosphoinositide hydrolysis		Adenylate cyclase inhibition	
	EC_{50}	E_{max}	EC_{50}	I_{max}
	μM	% of conversion	nM	% of inhibition
Control	3.55	31.6	21.7	25
10 nM 4-DAMP mustard	22.8	11.1	46.2	23
Control	1.36	44.8	30.0	33.7
40 nM 4-DAMP mustard[a]	4.30	10.2	31.1	33.2

[a] Incubation with 4-DAMP mustard in the presence of 1 μM AF DX-116.

Previous binding studies from our laboratory with homogenates of rat heart and submaxillary gland showed that M_3 receptors in the gland can be selectively blocked when incubated with 40 nM 4-DAMP mustard for 1 hr in the presence of the reversible M_2-selective antagonist AF-DX 116 (1 μM) (25). AF-DX 116 protects the M_2 receptors from alkylation by 4-DAMP mustard and can subsequently be washed off the receptor, leaving the M_3 receptors irreversibly blocked. This treatment was used in rat ileum to inactivate M_3-mediated responses more selectively. In these experiments, oxo-M stimulated phosphoinositide hydrolysis in control ilea with an EC_{50} value of 1.34 μM and a maximal effect of 27.1% conversion of phosphoinositides into inositol phosphates. Treatment of the ilea with 4-DAMP mustard (40 nM) and AF-DX 116 (1 μM), followed by extensive washing, caused a 2.67-fold increase in the EC_{50} value of oxo-M and an accompanying 72% reduction in maximum accumulation of inositol phosphates (Fig. 5a; Table 2). Using Furchgott's method, this effect corresponds to an 88% alkylation of receptors in the ilea. Similar 4-DAMP mustard treatment had no effect on the ability of oxo-M to inhibit cAMP accumulation. In control experiments oxo-M caused a maximal 33.7% inhibition of isoproterenol-stimulated cAMP accumulation, with an EC_{50} value of 30.0 nM, and in 4-DAMP mustard-treated tissues oxo-M induced a maximal 33.2% inhibition of cAMP accumulation, with an EC_{50} value of 31.1 nM (Fig. 5b; Table 2). These experiments show that M_3-mediated responses can be selectively prevented by pretreatment with 4-DAMP mustard in the presence of AF DX-116, while the M_2 pathway remains unaffected.

Contractile studies with 4-DAMP mustard-treated tissues. To further elucidate a role for the M_2 receptor, isolated guinea pig ilea were treated with 4-DAMP mustard under conditions that inactivated M_3 muscarinic receptors selectively

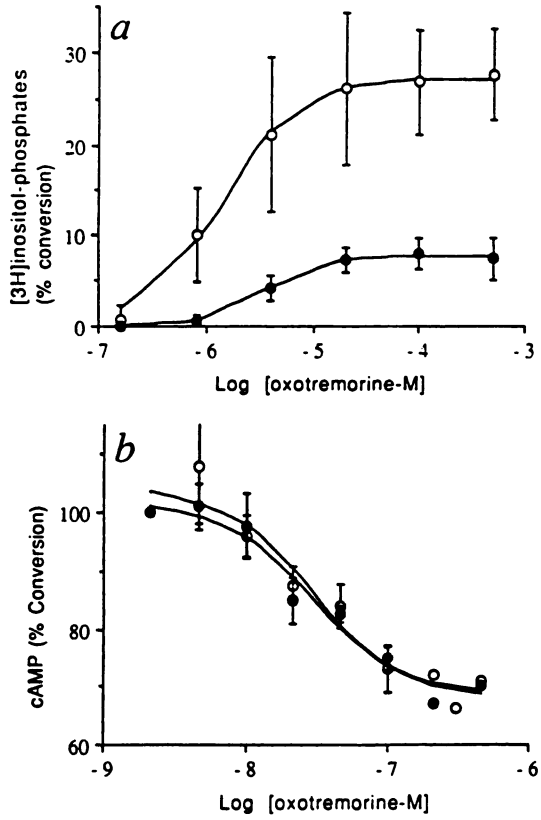

Fig. 5. Selective antagonism of M_3-mediated phosphoinositide hydrolysis (a) over M_2-mediated inhibition of cAMP accumulation (b) by 4-DAMP mustard treatment in the longitudinal muscle of rat ileum. Slices of the longitudinal muscle were incubated with the aziridinium ion of 4-DAMP mustard (40 nM) at 37° for 1 hr in the presence of AF-DX 116 (1 μM) (●). Slices were then washed extensively to remove the AF-DX 116 and unreacted 4-DAMP mustard before oxo-M-induced responses were measured. ○, Control responses. Each *data point* represents the mean ± standard error of the response from at least four experiments, each done in triplicate.

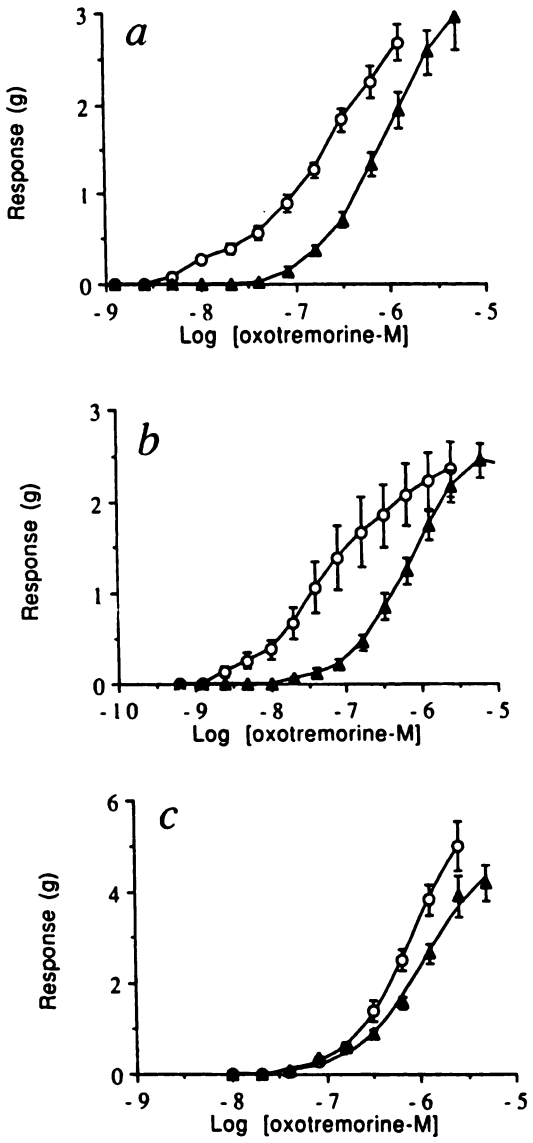

Fig. 6. Effects of AF-DX 116 on the contractile responses to oxo-M in 4-DAMP mustard-treated ilea under conditions of elevated cAMP (a and b) and under basal conditions (c). All contractile measurements were made in tissues that had been incubated at 37° in the presence of the aziridinium ion of 4-DAMP mustard (40 nM) and AF-DX 116 (1 μM) and washed extensively. a, Contractile responses to oxo-M were measured in the presence of histamine (0.32 μM) and isoproterenol (0.64 μM). ○, Control; ▲, 1 μM AF-DX 116. b, Contractile responses to oxo-M were measured in the presence of histamine (0.32 μM) and forskolin (10 μM). ○, Control; ▲, 1 μM AF-DX 116. c, Basal contractile responses were measured in the presence of KRB buffer alone (○) or in the presence of 1 μM AF-DX 116 (▲). Each *data point* represents the mean ± standard error of eight to 10 separate experiments.

(see above). Whole ilea were incubated with 4-DAMP mustard (40 nM) for 1 hr in the presence of AF-DX 116 (1 μM). Tissues were incubated for an additional 10 min with Na_2SO_3 (0.05 mM) and were washed extensively. After 4-DAMP mustard treatment, ilea were contracted with a submaximal concentration of histamine (0.32 μM) and relaxed back to base-line levels with isoproterenol (0.64 μM) or forskolin (10 μM). Contractile responses to oxo-M were measured relative to base-line levels, while these compounds remained in the organ bath (Figs. 6, a and b, and 7, a and b). In the experiments where isoproterenol was used (Fig. 6a), the oxo-M concentration-effect curve had an EC_{50} value of 461 nM and a Hill coefficient of 0.84. AF-DX 116 (1 μM) caused an 6.6-fold increase in the EC_{50} value and an increase in the Hill coefficient to 1.48. When forskolin was used (Fig. 6b), the EC_{50} value for oxo-M was 58.5 nM, with a Hill coefficient of 0.84. AF-DX 116 caused an 11-fold increase in the EC_{50} value of oxo-M and an increase in the Hill coefficient to 1.27. Oxo-M was more potent in contracting the ileum when forskolin was used as the relaxant; however, we have no adequate explanation for this observation at the present time. The K_b values for AF-DX 116 in the experiments with isoproterenol and forskolin were 0.17 and 0.10 μM, respectively, which are not much different from that expected for a pure M_2 response (i.e., $K_b = 0.1$ μM).

In contrast, AF-DX 116 was much less potent at antagonizing the contractile response to oxo-M in 4-DAMP mustard-treated tissues in the absence of isoproterenol or forskolin (basal conditions) (Fig. 6c). After similar 4-DAMP mustard treatment, the oxo-M contractile response curve had an EC_{50} value of 588 nM and a Hill coefficient of 1.67. AF-DX 116 (1 μM) caused only a 1.34-fold increase in the EC_{50} value, without affecting the Hill coefficient. The calculated K_b value of AF-DX 116 under these conditions was 2.9 μM, similar to that expected for an M_3 response. Thus, after inactivation of approximately 90% of the M_3 receptors in the ileum, oxo-M could still elicit

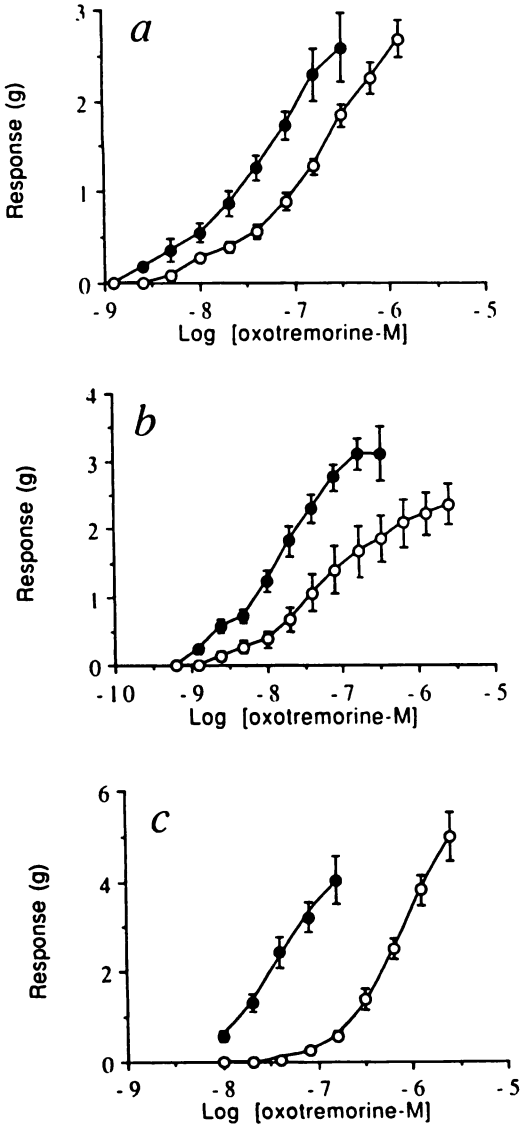

Fig. 7. Effects of 4-DAMP mustard treatment on the contractile responses to oxo-M, measured under conditions of elevated cAMP (a and b) and under basal conditions (c). Contractile responses were measured in control ilea (●) and ilea that had been treated with 4-DAMP mustard as described for Fig. 6 (○). The responses to oxo-M were measured in the absence of drugs (basal conditions) (c) and in the presence of histamine (0.32 μM) and isoproterenol (0.64 μM) (a) or histamine (0.32 μM) and forskolin (10 μM) (b). *Data points* indicate the mean ± standard error of six separate experiments.

contraction via the M_3 receptor under basal conditions, albiet with greatly reduced potency. In addition, 4-DAMP mustard treatment shifted the control oxo-M contractile response curve to the right about 20-fold (Fig. 7c) under these basal conditions. However, when histamine was present in combination with isoproterenol or forskolin, 4-DAMP mustard treatment shifted the concentration-effect curves of oxo-M to the right only about 3.5-fold (Figs. 7, a and b).

Discussion

This study demonstrates a functional role for the M_2 receptor in contraction of guinea pig ileum. Previously, isolated guinea pig ileum has been widely used as a standard assay for M_3 receptor activity, whereas the function of the abundant M_2 receptor has been completely unknown, despite the significant progress made in sorting out its signaling mechanism (1, 18). We have shown that, in the presence of histamine and isoproterenol or forskolin, selective activation of the M_2 receptor causes contraction of the ileum. The nature of this response was defined by the potency with which the M_2-selective antagonist AF-DX 116 and the M_1/M_3-selective antagonist 4-DAMP antagonized contractile responses to oxo-M. After 4-DAMP mustard treatment, AF-DX 116 caused 6.6- and 11-fold increases in the overall EC_{50} value of oxo-M in the isoproterenol- and forskolin-treated tissues, respectively, which are greater than that expected for antagonism of M_3-mediated basal contractions (i.e., 2-fold; see Fig. 6a) and similar to that expected for an M_2 effect (i.e., 10-fold; see above). These results indicate a substantial role for the M_2 receptor in contraction under these conditions. Also, in tissues that were pretreated with histamine and isoproterenol or forskolin, 4-DAMP mustard caused only a 3.5-fold increase in the overall EC_{50} value, with no decrease in the maximal contraction. This result contrasts sharply with that observed under basal conditions, where 4-DAMP mustard caused a large 20-fold increase in the EC_{50} value, with no change in the maximum response. Collectively, these results indicate that, under conditions of elevated cAMP or in the presence of histamine and isoproterenol or forskolin, a large portion of the muscarinic response is mediated through the 4-DAMP mustard-insensitive M_2 receptor. Thus, there appears to be a relative shift in the receptors contributing to the overall contractile response. The most likely mechanism for this response is that the M_2 receptor prevents the relaxing effects of isoproterenol and forskolin and allows histamine to contract the gut (M_2-mediated disinhibition of contraction).

Ishii and Kato (26) have shown that when guinea pig ileum is stimulated by maximal concentrations of both muscarinic and histamine agonists the contractile response is dominated by the stimulation of the muscarinic receptors. In their experiments, the effects of histamine (10 μM) were masked by methacholine (10 μM) and had little effect on the measured contraction. In our study, 0.32 μM histamine was used to precontract the tissue before relaxation to base-line levels with isoproterenol or forskolin. When oxo-M is added to tissues with fully functional M_3 receptors, at a concentration that is below the threshhold for contractions induced by the M_3 receptor (≤5 nM), the M_2 receptors may become activated, causing a disinhibition of the contraction induced by histamine. This hypothesis is supported by the fact that contractile responses are observed at lower concentrations under our experimental conditions of elevated cAMP than under basal conditions. The disinhibition of the histamine-induced contractions, however, becomes masked as increased concentrations of oxo-M are added to the bath and the responses become mediated via the M_3 receptors. In tissues in which the majority of the M_3 receptors have been inactivated by 4-DAMP mustard, the M_2-mediated disinhibition of histamine-induced contractions becomes more pronounced, especially at higher concentrations of oxo-M. This hypothesis explains why inactivation of M_3 receptors with 4-DAMP mustard did not have a large effect on contraction under conditions where cAMP was elevated by isoproterenol and forskolin. It also explains why these contractions were more greatly inhibited by the M_2-selective antagonist AF-DX 116.

In our contractile studies, the heterologous contractile agent

histamine was present continuously throughout the length of the experiment (approximately 10 min), which raises the question of receptor desensitization. Contractile responses in guinea pig ileum have been shown to be cross-desensitized by muscarinic agonists and histamine (27, 28). Eglen and Whiting (27) showed that the concentration-effect curve for carbachol for contraction of guinea pig ileum was shifted to the right and depressed after the tissue was exposed to 10 μM histamine for 60 min. In our guinea pig ileum preparation, pre-exposing the tissue to 0.32 μM histamine for 10 min had no effect on the EC_{50} value for oxo-M or the maximal contraction in response to oxo-M (data not shown). However, there appeared to be a slight decrease in the contractile responses to low concentrations of oxo-M (<5 nM). Nonetheless, in our protocol muscarinic receptor desensitization is not pertinent to our demonstration of an M_2-mediated contraction. The shifts caused by AF-DX 116 are independent of desensitization, because histamine was present in the control incubations as well.

The effects of AF-DX 116 on oxo-M-induced contractile responses in the presence of a single concentration of isoproterenol were also examined. In this case, AF-DX 116 (1 and 10 μM) caused similar shifts of the oxo-M concentration-effect curve (3.1- and 11.4-fold increases in EC_{50}, respectively), compared with those observed when contractile responses were measured in the presence of AF-DX 116 (1 μM) under basal conditions (2.3- and 18-fold increases in EC_{50}, respectively). At this time we have no clear explanation for why we did not observe the expected enhanced potency of AF-DX 116, but it could be that there is compartmentalization of the receptors so that the M_2 receptors disinhibit contractions elicited by histamine receptors but not by M_3 receptors. It is also possible that a greater M_2 effect might occur at higher concentrations of isoproterenol. In addition, it is important to point out that this experiment tests for the ability of AF-DX 116 to discriminate between an M_3-mediated response and a response elicited by both M_2 and M_3 receptors. A small contribution of the M_2 receptor, therefore, would be difficult to detect in this experiment. The development of selective M_2 muscarinic agonists should provide a useful means of further analyzing the role of M_2 receptors in smooth muscle and their possible potentiation of M_3 responses.

A recently published report by Fernandes *et al.* (29) stated that M_2 muscarinic receptors inhibited isoproterenol-induced relaxation of canine tracheal smooth muscle. Based on the experimental design of their study, however, it is unclear how their conclusions could have been inferred from their observed results. They demonstrated that, in tissues that had been precontracted with methacholine, AF-DX 116 (0.01–1 μM) shifted the dose-response curves for isoproterenol and forskolin up to 4.8-fold to the left. These antagonist-induced shifts, however, cannot be related quantitatively to M_2 receptor antagonism, because the authors were not measuring competitive antagonism at the M_2 receptor. The more potent relaxation in response to isoproterenol observed in the presence of AF-DX 116 could have resulted from antagonism at the M_3 receptor site, despite the authors' claim that they increased the agonist concentration "up to 2-fold" higher in the presence of the antagonist. They also showed that HHSiD had no effect on the isoproterenol dose-response curve; however, they used only a single concentration of 0.01 μM HHSiD, which did not span the effective concentration range for the M_3 receptor. K_i values up to 0.15 μM have been reported for HHSiD acting at the M_3 receptor (2).

We have also demonstrated that 4-DAMP mustard can cause a selective irreversible blockade of the M_3 receptors in the ileum (94%), relative to the M_2 receptors (52%). Assuming a large receptor reserve, it can be estimated that this selective antagonism would cause 17- and 2.1-fold shifts in the EC_{50} values of M_3- and M_2-mediated responses, respectively. This degree of selectivity is in general agreement with that predicted from a previous study on the binding properties of 4-DAMP mustard (25). A more selective inactivation of M_3-mediated phosphoinositide hydrolysis over M_2-mediated inhibition of adenylate cyclase was achieved by incubating slices of the longitudinal muscle of rat ileum with 40 nM 4-DAMP mustard in the presence of AF DX-116 (1 μM). AF DX-116 is a reversible M_2-selective antagonist that can be washed off the receptor after incubation with 4-DAMP mustard, leaving the M_3 receptors irreversibly blocked. This 4-DAMP mustard treatment blocked phosphatidylinositol bisphosphate hydrolysis with 88% alkylation of the M_3 muscarinic receptors in the ileum but did not affect the ability of oxo-M to inhibit adenylate cyclase, an M_2-mediated effect. This result is in excellent agreement with that reported previously for radioligand binding studies (25). It was shown that similar treatment with 4-DAMP mustard and AF-DX 116 caused an 86% alkylation of the M_3 muscarinic receptors in the gland but only a 7% alkylation of the M_2 receptors in the heart.

The receptor protection protocol was used to assess the effects of M_3 receptor inactivation on the biochemical responses and contractile measurements. However, a potential caveat to correlating the data from the second messenger studies with the data obtained from the contractile studies is that the former were measured in rat ileum and the latter in guinea pig ileum. Guinea pig ileum, however, contains the same composition of M_2 and M_3 muscarinic subtypes as does rat ileum (1, 4), and these subtypes have been shown to couple similarly to the inhibition of adenylate cyclase and the stimulation of phosphoinositide hydrolysis, respectively (30, 31). Therefore, we do not feel that the species difference has a significant bearing on the overall results.

In the present study we used 4-DAMP mustard to inactivate the M_3 muscarinic receptors selectively, while leaving the M_2 receptors functionally intact. Under these conditions we have shown that the contractile response can be dominated by the M_2 receptor. The most likely explanation for these results is that the M_2 receptor can cause an indirect contraction by preventing smooth muscle relaxation induced by forskolin and the β-adrenergic receptor. This mechanism also explains why, under so-called basal conditions, the M_2 receptor has no influence on contraction. A physiological function for the M_2 receptor, however, still remains uncertain. It is possible that the M_2 receptors do not receive parasympathetic innervation; thus, they may not regulate contraction *in vivo*. They may, however, be a good target for drug therapy, where activation of the M_2 receptors could increase smooth muscle tone by opposing the inhibitory sympathetic influence. The development of M_2-selective agonists could prove to be very useful in the treatment of gastrointestinal disorders and glaucoma, because the agonists would lack the side effects characteristic of excessive M_3 receptor activation (i.e., pupillary constriction, salivation, and diarrhea).

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