0022-3565/98/2841-0269$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS Vol. 284, No. 1

JPET 284:269–277, 1998 *Printed in U.S.A.*

Contractile Roles of the M_2 and M_3 Muscarinic Receptors in the Guinea Pig Colon[1]

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Accepted for publication September 22, 1997 This paper is available online at http://www.jpet.org

ABSTRACT

The contractile roles of the M_2 and M_3 muscarinic receptors were investigated in guinea pig longitudinal colonic smooth muscle. Prior treatment of the colon with N-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP mustard) (40 nM) in combination with [[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3b][1,4]benzodiazepine-6-one (AF-DX 116) (1.0 μM) caused a subsequent, irreversible inhibition of oxotremorine-M-induced contractions when measured after extensive washing. The estimate of the degree of receptor inactivation after 2 hr (97%) was not much greater than that measured after 1 hr (95%), which suggests that both 4-DAMP mustard-sensitive and -insensitive muscarinic subtypes contribute to the contractile response. Pertussis toxin treatment had no significant inhibitory effect on the control contractile response to oxotremorine-M, but caused an 8.8-fold increase in the EC_{50} value measured after a 2-hr treatment with 4-DAMP mustard. These results suggest that, after elimination of most of the M_3 receptors with 4-DAMP mustard, the contractile response can be mediated by the pertussis toxin-sensitive M_2 receptor. After pertussis toxin treatment, the kinetics of alkylation of muscarinic receptors in the colon were consistent with a single, 4-DAMP mustard-sensitive, M_3 receptor subtype mediating the contractile response. When measured after a 2-hr treatment with 4-DAMP mustard and in the presence of histamine (0.30 μM) and either forskolin (10 μM) or isoproterenol (0.60 μM), the contractile responses to oxotremorine-M were pertussis toxin-sensitive and potently antagonized by the M_2 selective antagonist, AF-DX 116. Collectively, our results indicate that the M_2 receptor elicits contraction through two mechanisms, a direct contraction and an indirect contraction by preventing the relaxant effects of cAMP-generating agents.

Muscarinic receptors are expressed abundantly in smooth muscle throughout the gastrointestinal tract in a manner that approximates a three-to-one mixture of the M_2 and M_3 subtypes (see Ehlert *et al.*, 1997). Muscarinic agonists elicit contraction through the M_3 receptor under standard conditions (*i.e.,* no other contractile or relaxant agents present) in smooth muscle ranging from the esophagus to ileum. It is known that the M_3 receptor stimulates phospholipase C-β causing inositol-1,4,5-trisphosphate accumulation and calcium mobilization. The extent to which the contractile response depends on this burst of calcium and how this calcium interacts with other transduction mechanisms remains to be determined.

The M_2 muscarinic receptor has been shown to mediate a pertussis toxin-sensitive inhibition of adenylyl cyclase activity in the ileum and colon (Candell *et al*., 1990; Zhang and Buxton, 1991; Thomas and Ehlert, 1994). This effect opposes the increase in cAMP elicited by forskolin and isoproterenol. In gastrointestinal smooth muscle, forskolin and isoproterenol elicit relaxation through cAMP. The M_2 receptor has been shown to cause an indirect contraction in the ileum by preventing the relaxant effects of forskolin and isoproterenol on histamine-induced contractions (Thomas *et al.*, 1993; Thomas and Ehlert, 1994; Reddy *et al*., 1995; Ostrom and Ehlert, 1997). In the ileum, therefore, muscarinic agonists are known to have a dual effect on contraction, a direct M_3-mediated contraction and an indirect M_2-mediated inhibition of relaxation.

Muscarinic receptors have also been shown to induce a nonselective cation conductance in the longitudinal smooth muscle of the guinea pig ileum (Inoue, 1991). This conductance is pertussis toxin-sensitive (Inoue and Isenberg, 1990a; Unno *et. al*., 1995), which suggests that the M_2 receptor may be coupled to the nonselective cation conductance. The nonselective cationic conductance is enhanced by calcium in the guinea pig ileum and jejunum (Inoue and Isenberg, 1990b; Pacaud and Bolton, 1991), and calcium is an absolute requirement in the canine colon (Cole *et al*., 1989; Lee *et al*.,

Received for publication March 3, 1997.
[1] This work was supported by National Institutes of Health grant NS 30882.
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ABBREVIATIONS: AF-DX 116, [[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3b][1,4]benzodiazepine-6-one; 4-DAMP mustard, N-(2-chloroethyl)-4-piperidinyl diphenylacetate; KRB, Krebs-Ringer Bicarbonate Buffer; p-F-HHSiD, para-fluoro-hexahydrosiladiphenidol; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

1993). This calcium requirement could be provided by M_3 receptor-mediated calcium mobilization. Therefore, it is possible that both the M_2 and M_3 muscarinic receptors mediate the nonselective cation conductance in gastrointestinal smooth muscle. Accordingly, a recent pharmacological analysis suggests that both the M_2 and M_3 receptors cooperate to induce the nonselective cation conductance (Bolton and Zholos, 1997)

In the present study, experiments were conducted to determine the contractile role of muscarinic receptor subtypes in the guinea pig colon. Our results show that the M_3 receptor elicits a pertussis toxin-insensitive contractile response under standard conditions. However, after 4-DAMP mustard treatment the standard contractile response was pertussis toxin-sensitive, which suggests a role for the M_2 receptor. We also show that M_2 receptors can prevent the relaxant effects of forskolin and isoproterenol on histamine-induced contractions.

Materials and Methods

***In vivo* pertussis toxin treatment.** In some experiments, male Hartley guinea pigs (300–400 g) were injected i.p. with 100 μg/kg body weight pertussis toxin 3 days before being sacrificed for the experiments.

Cyclic AMP accumulation. Cyclic AMP accumulation was measured in slices of the guinea pig colon by a modification of the procedure described by Daly *et al.* (1981). Male Hartely guinea pigs (300–400 g) were asphyxiated with CO_2 followed immediately by exsanguination. A 6- to 8-cm segment of colon was quickly dissected 1 cm distal to the cecum and placed in ice-cold 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 segment of colon was cut longitudinally to expose the mucosa, which was removed by a modification of the method described by Diener *et al*. (1989). The resulting colonic segments (including the longitudinal and circular muscles) were prepared and [^{3}H]cyclic AMP was measured as described previously (Thomas *et al*., 1993).

Contractile measurements. Male Hartley guinea pigs were sacrificed, and the colon was harvested as described above. The colon was cut into segments 1 to 2 cm in length. Each segment was rapidly cleaned with KRB buffer to remove its contents, connected to a force transducer and mounted longitudinally in a organ bath containing 50 ml of KRB buffer at 37°C gassed with O_2/CO_2 (19:1). The colon segments were allowed to equilibrate for 40 min at a resting tension equivalent to a load of 1.5 g (optimal pretension was determined after constructing a pretension *vs*. force of contraction curve) before measuring isometric contractions with a force transducer and polygraph. A test dose of either histamine or oxotremorine-M (highly efficacious muscarinic agonist) was then added to each bath. Once each tissue reached a sustained contraction, each bath was washed with KRB buffer and allowed to incubate 5 min before the addition of two more test doses. These three test doses were used to ensure reproducibility of the preparations. Segments of colon that did not contract to at least 60% of that elicited by 100 mM KCl were discarded. After the last 5-min incubation, the KRB buffer was replaced with 50 ml of Ca^{++} free KRB buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 10 mM glucose). The colon was incubated in Ca^{++} free media for 10 min to inhibit myogenic contraction and cause full relaxation. During this period, a resting tension of 1.5 g was maintained. Subsequently, the Ca^{++} free KRB buffer was replaced with 50 ml of K^+-deficient KRB buffer (124 mM NaCl, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose) to inhibit spontaneous contractions. After addition of the K^+-deficient KRB buffer, a large contraction was observed which declined to resting tension within 7 to 10 min. A cumulative agonist concentration-response curve was then measured by adding 9 to 18 geometrically spaced (0.33 log unit) concentrations of oxotremorine-M to each of the organ baths. The EC_{50} value was determined from this curve as described below. The K^+-deficient KRB buffer was washed from the bath, and 50 ml of KRB buffer was added. Colon segments were allowed to incubate for 30 min before any further measurements were made. The above-mentioned procedure (excluding three test doses) was repeated before each EC_{50} measurement made with the same tissue. Some colon segments were incubated with 40 nM aziridinium ion of 4-DAMP mustard for 1 hr in the presence of 1.0 μM of AF-DX 116 to alkylate M_3 muscarinic receptors selectively (Thomas *et al*., 1993). The tissues were then washed with KRB buffer, and the aziridinium ion of 4-DAMP mustard (40 nM) and AF-DX 116 (1.0 μM) were added again for an additional hour (*i.e.,* total incubation time of 2 hr). The colon segments were washed thoroughly to remove AF-DX 116 and any unreacted 4-DAMP mustard. In all experiments, 4-DAMP mustard was first converted to its aziridinium ion by incubation for 30 min at 37°C in 10 mM $NaKPO_4$, pH 7.4, as described previously (Thomas *et al*., 1992).

Data analysis. The percent inhibition of agonist-stimulated cAMP accumulation elicited by oxotremorine-M (I_c) was calculated by first subtracting out basal cAMP accumulation (B) before calculating the percent inhibition:

$$I_c = \left(1 - \frac{O - B}{A - B}\right) \times 100 \quad (1)$$

In this equation, O represents cAMP accumulation in the presence of the agonist and oxotremorine-M, and A represents cAMP accumulation in the presence of the agonist alone.

The concentration of oxotremorine-M eliciting half-maximal contraction (EC_{50}) was estimated by nonlinear regression analysis according to an increasing logistic equation as described previously (Candell *et al*., 1990).

The following procedures were used to estimate the EC_{50} value of oxotremorine-M in experiments in which the antagonist, AF-DX 116, was investigated. If AF-DX 116 had no significant effect on the maximum response, the EC_{50} value was calculated by regression analysis, sharing the same estimate of the maximum response between the control and AF-DX 116-treated concentration-response curves. In some instances, AF-DX 116 caused an increase in the maximal response to oxotremorine-M. We have no clear explanation for this effect of AF-DX 116. Because the estimate of the K_B values of AF-DX 116 depends on how the EC_{50} value is calculated, we used two methods to estimate the EC_{50} in the presence of AF-DX 116 and calculated the corresponding K_B values as described below. First, the EC_{50} values of the control ($EC_{50\ A}$) and AF-DX 116-treated ($EC_{50\ B}$) curves were estimated independently as described above. Second, the concentration of oxotremorine-M eliciting a response in the presence of AF-DX 116 ($EC_{50\ C}$) equivalent to that elicited at its EC_{50} value in the absence of AF-DX 116 ($EC_{50\ A}$) was estimated. In this paper these two estimates of the EC_{50} value for oxotremorine-M in the presence of AF-DX 116 are reported as a range (*i.e.,* $EC_{50\ C}$–$EC_{50\ B}$) together with the corresponding range of K_B values.

The dissociation constant (K_B) of the antagonist, AF-DX 116, was estimated from the shift that it caused in the oxotremorine-M concentration-response curve:

$$K_B = [B]/(CR - 1) \quad (2)$$

In this equation, [B] represents the concentration of AF-DX 116, and CR corresponds to the concentration ratio (the ratio of the EC_{50} value of oxotremorine-M measured in the presence of the antagonist divided by that measured in the absence of the antagonist). The K_B values were also recorded as a range of values corresponding to the EC_{50} value used in the calculation ($EC_{50\ C}$ or $EC_{50\ B}$).

The amount of receptor inactivation was estimated by a modification of the method of Furchgott (1966) as described previously (Ehlert, 1986). Equieffective doses of oxotremorine-M were obtained from the concentration-response curves before and after treatment with 4-DAMP mustard. These estimates were fitted to the following equation by nonlinear regression analysis:

$$\text{Log } A = \text{Log}([A'K_A q]/[K_A + (1 - q)A']) \quad (3)$$

In this equation, A and A' correspond to the concentration of oxotremorine-M eliciting equivalent contraction before and after treatment with 4-DAMP mustard, and q denotes the proportion of inactivated receptors.

Significance values (P value) were calculated by using the paired t test and are reported in the text were appropriate.

Materials. The drugs and chemicals used in this investigation were obtained from the following sources: islet-activating protein (pertussis toxin), LIST Biological Laboratories, Campbell, CA; [^{3}H]adenine, ICN Biochemicals, Costa Mesa, CA; AF-DX 116, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT; SKF 38393 and Oxotremorine-M, Research Biochemicals Incorporated, Natick, MA; 4-DAMP mustard was synthesized in our laboratory as described previously (Thomas *et al*., 1992); and all remaining drugs and chemicals were from Sigma Chemical Company, St. Louis, MO.

Results

Effect of oxotremorine-M on agonist stimulated cAMP accumulation. Zhang *et al.* (1991) have shown that the nonselective muscarinic agonist carbachol elicited a concentration-dependent decrease in adenylate cyclase activity stimulated by 10 μM forskolin in homogenates of canine colonic circular smooth muscle cells. This response was antagonized by atropine in a competitive manner and was pertussis toxin-sensitive. To determine which cAMP-stimulating agents the muscarinic receptor opposed in the guinea pig colon, we measured the ability of 10 μM oxotremorine-M to inhibit the cAMP accumulation elicited by 1 μM isoproterenol, 10 μM forskolin, 10 μM SKF 38393 or 10 μM cicaprost. Figure 1, shows that 10 μM oxotremorine-M inhibited the cAMP response to isoproterenol, forskolin, SKF 38393 and cicaprost by 31% (P = .03), 24% (P = .01), 100% (P = .002) and 4.6% (P = .05), respectively. A cursory investigation of the effects of prostaglandin D_2, prostaglandin E_2, serotonin, methoxy tryptamine, dimaprit, dopamine and secretin showed that these agonists caused 1.2- to 1.7-fold increase in cAMP and that oxotremorine-M inhibited these responses by 0% to 13%. These agonists were not investigated further.

Effect of 4-DAMP mustard treatment and AF-DX 116 on contractions elicited by oxotremorine-M under standard conditions. Previous studies on guinea pig ileum (Candell *et al.*, 1990), gastric fundus (Del Tacca *et al.*, 1990), the longitudinal muscle of the esophagus (Eglen and Whiting, 1988) and the circular muscle of the lower esophageal sphincter (Sohn *et al.*, 1993) have demonstrated that the M_3 receptor mediates contractions in these gastrointestinal smooth muscles under standard conditions. To determine whether the M_3 receptor also elicits the contractile response in the guinea pig colon, we measured the ability of the subtype selective antagonists AF-DX 116, p-F-HHSiD and pirenzipine, to shift the oxotremorine-M-contractile response curve to the right. We estimated the K_B values of the antagonists from these rightward shifts as described under "Materials and Methods." These K_B values are listed in table 1

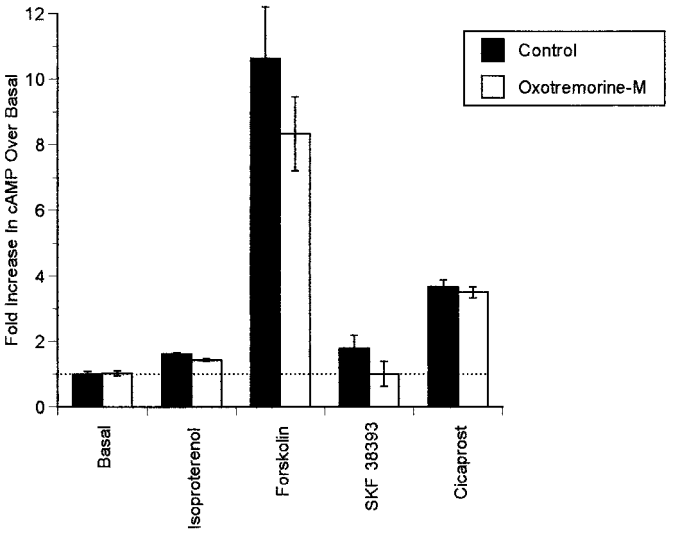

Fig. 1. Effect of oxotremorine-M on basal and agonist-stimulated cAMP formation in guinea pig colon. Colonic slices were incubated with isoproterenol (1 μM), forskolin (10 μM), SKF 38393 (10 μM) and cicaprost (10 μM) in the absence (closed bars) and presence (open bars) of oxotremorine-M (10 μM). The fold increase in cAMP over basal levels was determined as described under "Materials and Methods." Each bar represents the mean fold increase in cAMP ± S.E.M. of seven (forskolin), four (isoproterenol) and three (SKF 38393, cicaprost) experiments performed in triplicate.

together with the binding affinities (K_D values) of the same antagonists measured in Chinese hamster ovary cells transfected with the M_2 and M_3 subtypes of the muscarinic receptor (see Esqueda *et al.*, 1996 and Ehlert *et al.*, 1997). These binding experiments were carried out in a HEPES-buffered KRB solution similar to that used in our contractile studies. Keeping the composition of the buffer the same for the binding and functional experiments is important because the binding properties of muscarinic receptors are modulated by ionic strength (Pedder *et al.*, 1991). It can be seen that the K_B values of AF-DX 116, p-F-HHSiD and pirenzipine agree with their respective K_D values for the M_3 receptor, but not with those of the M_2 receptor (see table 1). There was also a lack of congruence between the antagonist K_B values of AF-DX 116, p-F-HHSiD and pirenzipine and their respective K_D values for the M_1 (6.24, 7.08, 7.77), M_4 (6.96, 7.08, 7.23) and M_5 (5.29,6.26, 6.55) subtypes, as reported by Esqueda *et al.* (1996) and Ehlert *et al.* (1997). We conclude that the M_3 receptor mediates the contractile response to oxotremorine-M in the guinea pig colon under standard conditions.

We also investigated the extent to which 4-DAMP mustard treatment antagonized oxotremorine-M-mediated contractions (table 2). The aziridinium ion of 4-DAMP mustard is an irreversible, muscarinic antagonist that alkylates the M_3 receptors selectively over the M_2 receptor (Thomas *et al.*, 1993). The isolated colon was incubated with the aziridinium ion of 4-DAMP mustard (40 nM) in the presence of AF-DX 116 (1 μM) for either 1 or 2 hr and washed extensively as described in "Materials and Methods". Incubation for 1 hr with 4-DAMP mustard caused a significant 27.9-fold ($P = 8.1 \times 10^{-5}$) increase in the EC_{50} value and no significant effect on the maximum contraction (fig. 2A). After the 2-hr 4-DAMP mustard treatment, the EC_{50} value for oxotremorine-M increased significantly by 20.2-fold ($P = 9 \times 10^{-7}$), and the maximal response decrease by 62.1% (fig. 2B and

TABLE 1

pK_B values for antagonism of oxotremorine-M-induced contraction in guinea pig colonic segments by AF-DX 116, pirenzipine and p-F-HHSiD and binding affinities (pK_D) for the same antagonists at the human (h) M_2 and M_3 receptors transfected into Chinese hamster ovary cells

Antagonist	Concentration	Concentration Ratio[a]	pK_B[b]	pK_D	
				hM_2[c]	hM_3[c]
	μM				
AF-DX 116	1	2.19	6.07 ± 0.10	7.27 ± 0.05	6.10 ± 0.06
Pirenzepine	1	5.2	6.62 ± 0.08	5.96 ± 0.05	6.59 ± 0.03
p-F-HHSiD	0.1	6.5	7.74 ± 0.09	6.09 ± 0.06	7.3 ± 0.04

[a] "Concentration ratio" denotes the EC_{50} value of oxotremorine-M in the presence of the antagonist divided by the EC_{50} value measured in its absence.
[b] Each pK_B is the mean ± S.E.M. of three experiments.
[c] Data from Esqueda *et al.,* 1996.

TABLE 2

Effects of AF-DX 116, 4-DAMP mustard treatment, pertussis toxin treatment and their combination on the contractile response to oxotremorine-M under standard conditions

Conditions	pEC_{50}[a]	
	Control	AF-DX 116 (1 μM)
Control	7.32 ± 0.13 (8)	7.22 ± 0.11[b] (4)
Pertussis toxin	6.99 ± 0.28 (6)	N.D.
4-DAMP mustard (1 hr)	5.97 ± 0.30[c] (4)	N.D.
4-DAMP mustard (2 hr)	6.02 ± 0.26 (8)	5.35 ± 0.08[d] (5)
4-DAMP mustard (2 hr) + pertussis toxin	5.07 ± 0.73 (6)	N.D.

[a] Each pEC_{50} value represents the mean ± S.E.M. of the experiments conducted. The number of experiments is given in parentheses.
[b] The control EC_{50} value was estimated from the pooled data from eight experiments. However, AF-DX 116 was investigated in only four of these experiments. The control EC_{50} value for these four experiments was 0.0273 μM (pEC_{50} = 7.56).
[c] The control EC_{50} value was estimated from the pooled data from eight experiments. However, contractions in 1-hr 4-DAMP mustard-treated colon were investigated in only four of these experiments. The control EC_{50} value for these four experiments was 0.0380 μM (pEC_{50} = 7.42).
[d] The control EC_{50} value was estimated from the pooled data from eight experiments. However, the effect of AF-DX 116 on contractions in 2-hr 4-DAMP mustard-treated colon was investigated in only five of these experiments. The control EC_{50} value for these five experiments was 1.04 μM (pEC_{50} = 5.98).

table 2). The effect of 4-DAMP mustard at 1 and 2 hr corresponded to receptor inactivation values of 94.6 and 96.7%, respectively as estimated by the method of Furchgott (see "Materials and Methods"), assuming that a single receptor type mediates the contractile response. This large inhibitory effect of 4-DAMP mustard is consistent with the postulate that the M_3 receptor mediates the contractile response under standard conditions.

Figure 3 shows the effects of the M_2 selective antagonist, AF-DX 116 (1 μM), on the contractions elicited by oxotremorine-M in the guinea pig colon under standard conditions before and after treatment with 4-DAMP mustard. AF-DX 116 caused a 2.19-fold increase in the EC_{50} value which yielded a calculated pK_B value of 6.08 (fig. 3 and table 2). The residual oxotremorine-M concentration-response curve that persisted after the 2-hr 4-DAMP mustard treatment was shifted to the right 4.3-fold in the presence of AF-DX 116 yielding a calculated pK_B value of 6.52. Using the binding affinities for AF-DX 116 that our laboratory previously estimated for the cloned human M_2 (pK_D = 7.27) and M_3 (pK_D = 6.10) receptors in HEPES buffered KRB (Esqueda *et al.*, 1996), we estimate that AF-DX 116 (1.0 μM) should cause 19.6- and 2.3- fold shifts in M_2 and M_3 mediated responses, respectively. Therefore, the effects of AF-DX 116 on the standard contractile response to oxotremorine-M in the guinea pig colon are consistent with those expected for antagonism of an M_3-mediated response as mentioned above in connection with the data described in table 1. However, following 4-DAMP mustard treatment, the pK_B value of AF-DX 116 (6.52) was a little greater than that expected for a pure M_3 response (pK_D = 6.10).

Contractile studies in 4-DAMP mustard-treated tissue in the presence of histamine and forskolin or isoproterenol. Although the M_3 receptor mediates contractions under standard conditions, the M_2 receptor has been shown to mediate contractions to oxotremorine-M in the ileum and trachea when measured after 4-DAMP mustard treatment and in the presence of histamine and a cAMP stimulating agent, like forskolin (Thomas *et al.*, 1993; Thomas and Ehlert, 1994, 1996). Under these latter conditions, it is likely that the M_2 receptor inhibits the cAMP-mediated, relaxant effects of forskolin and allows histamine to contract the smooth muscle (Thomas *et. al.*, 1993; Thomas and Ehlert, 1994, 1996). In other words, the M_2 receptor mediates a disinhibition of histamine-induced contractions. To investigate whether the M_2 receptor mediates contractile effects in the colon, we used a slightly modified version of the method described by Thomas *et al.* (1993). After 2 hr 4-DAMP mustard treatment (see "Materials and Methods"), colon segments were contracted with .30 μM histamine, then relaxed back to resting tension with either isoproterenol (0.60 μM) or forskolin (10 μM). The contraction elicited to cumulative addition of oxotremorine-M was measured while histamine and the cAMP stimulating agent remained in the bath. The results of these experiments are shown in figure 4. The dotted lines in this figure indicate the contractile response to histamine (upper line) and the resting level of contraction measured in the presence of histamine and either forskolin or isoproterenol (lower line). In experiments where forskolin was used to cause relaxation, the EC_{50} value and Hill coefficient of the oxotremorine-M concentration-response curve were 0.27 μM and 1.2, respectively (fig. 4A). AF-DX 116 (1.0 μM) caused a significant 8.0- to 13.2-fold (P = .004) increase in the EC_{50} value and an increase in the Hill coefficient to 1.84. The shift in the EC_{50} value corresponds to a pK_B value of 7.09 - 6.85 for AF-DX 116 (table 2). When isoproterenol was used to cause relaxation, the oxotremorine-M concentration-response curve had a EC_{50} value of 0.97 μM and a Hill coefficient of 1.22 (fig. 4B). AF-DX 116 caused a significant 3.0- to 5.8-fold (P = .05) increase in the EC_{50} value of oxotremorine-M and an increase in the Hill coefficient to 1.47, with a corresponding pK_B value of 6.30 to 6.68. In the presence of forskolin, the K_B and fold-shift values for AF-DX 116 were in close agreement with those predicted from the estimate of the K_D value of AF-DX 116 measured in binding experiments on the cloned M_2 receptor (pK_D = 7.27 and 19.6-fold; Esqueda *et al.*, 1996). In contrast, when isoproter-

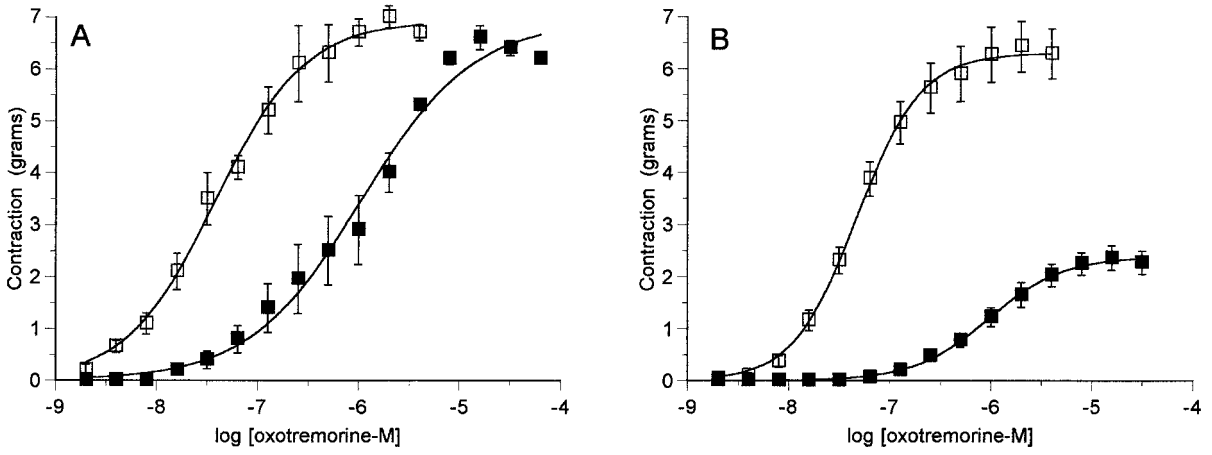

Fig. 2. Effects of 4-DAMP mustard treatment on the contractile response to increasing concentrations of oxotremorine-M under standard conditions. All contractile measurements were made in tissues that had been incubated with the aziridinium ion of 4-DAMP mustard (40 nM) for either 1 (A) or 2 (B) hr in the presence of AF-DX 116 (1 μM) at 37°C. Control (□) and 40 nM 4-DAMP mustard (■). Each data point represents the mean ± S.E.M. of four to eight experiments.

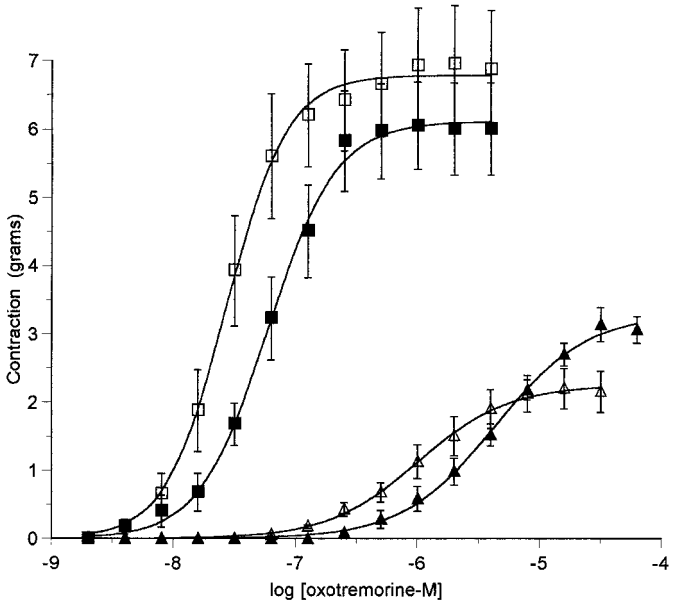

Fig. 3. Effects of AF-DX 116 (1 μM) on the contractile responses to oxotremorine-M in control and 2-hr 4-DAMP mustard-treated tissues. Contractile measurements were made in untreated (□,■) and 4-DAMP mustard-treated (△,▲) colon, in the absence (□,△) and presence (■,▲) of AF-DX 116 (1.0 μM). Each data point represents the mean ± S.E.M. of four experiments.

enol was used as the relaxant agent, the pK_B value (6.30–6.68) of AF-DX 116 at 1.0 μM was intermediate to those expected for either a purely M_2 (7.27) or M_3 (6.1) mediated response (Esqueda *et al.*, 1996).

Effect of pertussis toxin on the contractile response to oxotremorine-M under standard conditions. The M_2 receptor is known to elicit cellular responses by interacting with pertussis toxin-sensitive G proteins of the G_i family (Kurose and Ui, 1983; Sankary *et al.*, 1988; Zhang and Buxton, 1991). Pertussis toxin catalyzes the ADP-ribosylation of the *alpha* subunit of G_i and G_o, thereby preventing their coupling to M_2 receptors (Katada and Ui, 1982; Kurose *et al.*, 1983; Brown *et al.*, 1984). Consequently, we were interested in determining the pertussis toxin sensitivity of the contractile response of the colon under different experimental conditions to gain more insight into the muscarinic receptor subtypes that mediate contraction. Pertussis toxin treatment had no inhibitory effects on the contractile response to oxotremorine-M measured under standard conditions (fig. 5). In fact, a small potentiation in contraction by pertussis toxin was noted at high concentrations of oxotremorine-M. After 2-hr 4-DAMP mustard treatment, however, pertussis toxin caused a significant 8.8-fold ($P = 2 \times 10^{-4}$) increase in the EC_{50} value of oxotremorine-M under standard conditions. These results suggest that the M_2 receptor may contribute to the standard contractile response after most of the M_3 receptors have been inactivated. The results of these experiments are summarized in table 2.

Effects of pertussis toxin treatment on contractions measured after 4-DAMP mustard treatment and in the presence of histamine and either forskolin or isoproterenol. We also investigated the effects of pertussis toxin treatment on the contractile response to oxotremorine-M measured after 4-DAMP mustard treatment and in the presence of histamine and either forskolin or isoproterenol. In one experiment, pertussis toxin treatment caused a 17.4-fold increase in the EC_{50} value (fig. 6A and table 3) for those segments relaxed with forskolin. In three other experiments with forskolin, oxotremorine-M was unable to elicit contractions after pertussis toxin treatment (fig. 6A). Nevertheless, in the absence of forskolin, oxotremorine-M was able to elicit contractions in these tissues after pertussis toxin treatment as shown in figure 5. Moreover, pertussis toxin treatment did not affect histamine-induced contractions. Therefore, the lack of responsiveness of the tissue after 4-DAMP mustard treatment and in the presence of histamine and forskolin cannot be attributed to a pertussis toxin-induced depression in contractility. The variation in the effectiveness of pertussis toxin in the experiments described above (fig. 6A) is probably caused by variability in the absorption of pertussis toxin after intraperitoneal injection. In the experiments where isoproterenol was used to relax the colon segments after contraction with histamine, pertussis toxin caused a significant 32.5-fold ($P = .02$) increase in the EC_{50} value (fig. 6B). These results strongly indicate that the M_2 receptor mediates contractions under these conditions because the contractile response is pertussis toxin-sensitive. We investigated the ef-

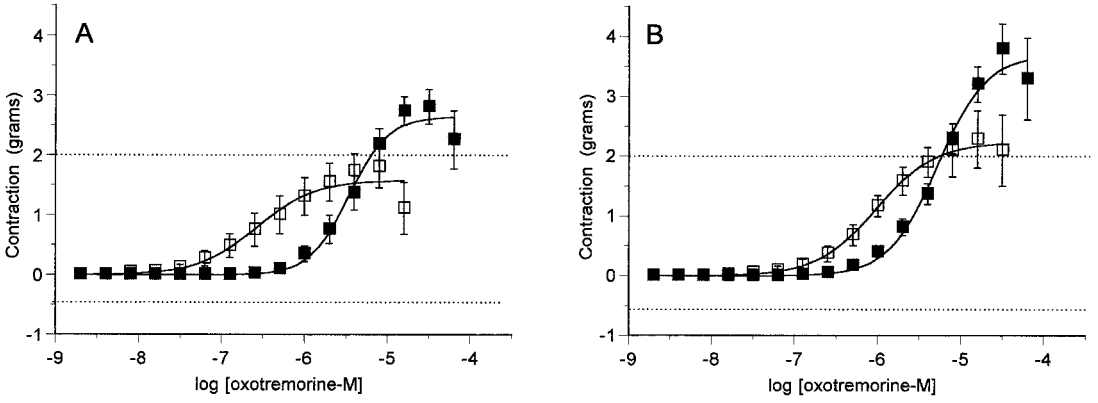

Fig. 4. Effects of AF-DX 116 on the contractile response to oxotremorine-M measured in the presence of histamine and either forskolin or isoproterenol after 4-DAMP mustard treatment. Colon segments were contracted with histamine (0.30 μM) and relaxed with forskolin (10 μM) (A) or isoproterenol (1 μM) (B) before oxotremorine-M-induced contractions were measured. Control (□) and 1 μM AF-DX 116 (■). Each data point represents the mean ± S.E.M. of seven experiments.

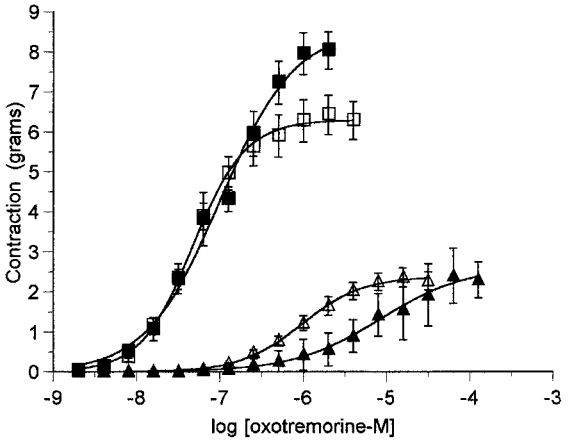

Fig. 5. Effects of pertussis toxin treatment on the contractile response to oxotremorine-M in control and 4-DAMP mustard-treated tissues. Contractile responses were measured in 2-hr 4-DAMP mustard-treated (△,▲) and untreated (□,■) colon from pertussis toxin-treated (■,▲) and untreated (□,△) guinea pigs. Each data point represents the mean ± S.E.M. of six experiments.

fects of AF-DX 116 on the residual contractile response that persisted after pertussis toxin treatment to determine what muscarinic receptor subtype was mediating the response. The EC_{50} value of oxotremorine-M was 15.1 μM (fig. 6C) when histamine-induced contractions were relaxed with isoproterenol. AF-DX 116 (1 μM) caused a 2.1-fold increase, which yielded a calculated pK_B value of 6.04 (table 3). These data indicate that the receptor mediating the contraction elicited by oxotremorine-M after pertussis toxin treatment is the M_3 because the pK_B value and fold increase in EC_{50} value were consistent with that expected for a purely M_3-mediated response (pK_D = 6.10 and 2.3-fold increase, respectively).

Discussion

In previous experiments with circular smooth muscle of canine colon, the muscarinic agonist carbachol inhibited forskolin-stimulated cAMP accumulation in muscle strips and isoproterenol-stimulated adenylate cyclase activity in broken cell preparations (Zhang and Buxton, 1991). In this investigation, the muscarinic agonist oxotremorine-M inhibited forskolin-, isoproterenol-, SKF 38393- and cicaprost-stimulated cAMP accumulation (fig. 1) in a manner consistent with a M_2-mediated response, as described in previous investigations with circular smooth muscle from canine colon and longitudinal muscle from the guinea pig ileum (Zhang and Buxton, 1991; Thomas *et al.*, 1993; Ostrom and Ehlert, 1997; Kurose and Ui, 1983). The fold increase in cAMP and the percent inhibition elicited in the presence of oxotremorine-M for forskolin, isoproterenol, SKF 38393 and cicaprost were similar to those observed in the guinea pig ileum (Ostrom and Ehlert, 1997).

In this investigation, the standard contractile response of the guinea pig colon to oxotremorine-M was unaffected by pertussis toxin treatment (fig. 5) and antagonized by pirenzipine, p-F-HHSiD and AF-DX 116 (table 1, fig. 3) in a manner consistent with an M_3 receptor-mediated event. This hypothesis is consistent with the well known role of the M_3 receptor in mediating contraction under standard conditions in a variety of other smooth muscles (see Ehlert *et al.*, 1997).

Previously, we showed that treatment with 4-DAMP mustard (40 nM) in the presence of AF-DX 116 (1.0 μM) for 1 hr caused a large inhibition of M_3-mediated responses including oxotremorine-M-stimulated phosphoinositide hydrolysis in the ileum and oxotremorine-M-stimulated contractions in the ileum and trachea. In contrast, the same treatment had no significant effect on oxotremorine-M-mediated inhibition of isoproterenol-stimulated cAMP accumulation in the ileum, an M_2-mediated response. With use of the observed rate constant for alkylation (0.00135 min^{-1}) at M_2 receptors that we previously measured for 4-DAMP mustard (40 nM) in the presence of AF-DX 116, we estimate that 4-DAMP mustard should only inactivate approximately 7.8% and 15% of the M_2 receptors after 1 and 2 hr of 4-DAMP mustard treatment, respectively. These levels of receptor inactivation should only shift an M_2-mediated concentration response curve to the right 1.1- to 1.2-fold. Therefore, the large inhibitory effects of 4-DAMP mustard shown in figure 2 and table 2 are also consistent with the postulate that the M_3 receptor mediates the standard contractile response to oxotremorine-M.

However, after 2-hr 4-DAMP mustard treatment, the standard contractile response to oxotremorine-M was pertussis toxin-sensitive (fig. 5). Under these conditions, pertussis toxin had a great effect on the remaining contraction elicited to oxotremorine-M, making it 8.8-fold less potent. Eglen *et al.* (1987) and others (Peralta *et al.,* 1988; Kurose *et al.*, 1983)

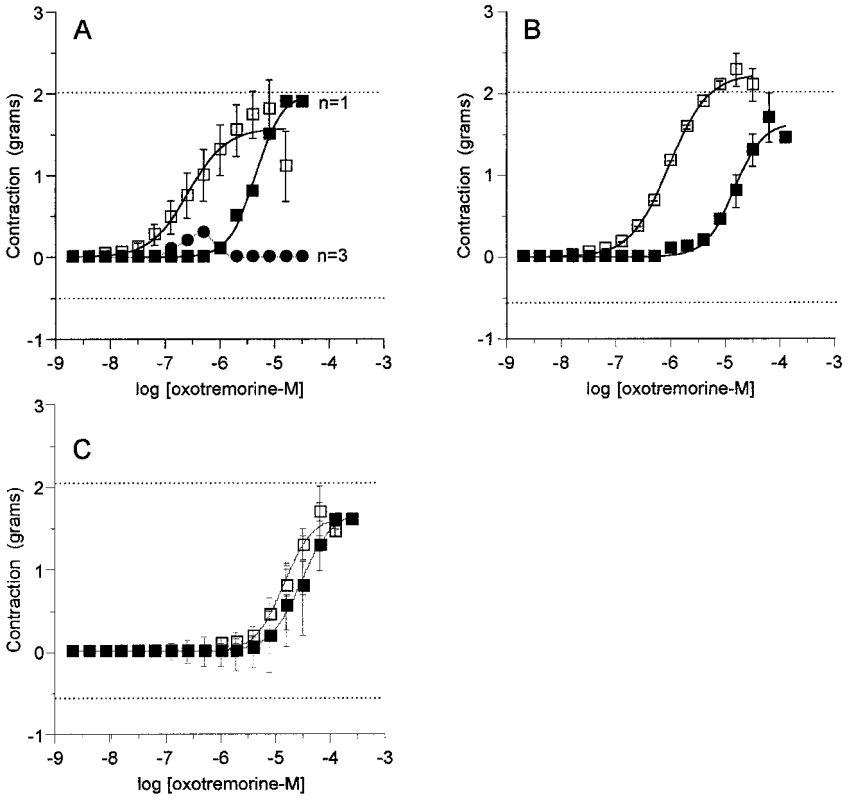

Fig. 6. Effects of pertussis toxin treatment and AF-DX 116 on the contractile response to oxotremorine-M measured in the presence of histamine and either forskolin or isoproterenol after 4-DAMP mustard treatment. All contractile responses were measured in colon treated with 4-DAMP mustard for 2-hr as described under "Material and Methods." (A) Contractile responses to oxotremorine-M were measured in the presence of histamine and forskolin in colon from control (□) and pertussis toxin-treated (■) guinea pigs. Pertussis toxin treatment completely blocked contractile response to oxotremorine-M under these conditions in three out of four experiments. The number of experiments are denoted by "n." (B) Contractile responses to oxotremorine-M were measured in the presence histamine and isoproterenol in a colon from control (□) and pertussis toxin-treated (■) guinea pigs. Each data point represents the mean ± S.E.M. of seven (no pertussis toxin treatment) to two (pertussis toxin treated) experiments. (C) Contractile responses to oxotremorine-M were measured in the presence of histamine and isoproterenol in colon from pertussis toxin-treated guinea pigs. Contractions were measured in the absence (□) and presence (■) of AF-DX 116. Each data point represents the mean ± S.E.M. of two experiments.

TABLE 3

Effects of AF-DX 116, pertussis toxin treatment and their combination on the contractile response to oxotremorine-M measured in 4-DAMP mustard-treated colon in the presence of histamine (0.30 μM) and either isoproterenol (0.60 μM) or forskolin (10 μM)

Conditions	pEC_{50}[a]	
	Control	AF-DX 116 (1 μM)
Histamine/isoproterenol		
Control (7)[b]	6.01 ± 0.54	5.27 ± 0.19
Pertussis toxin (2)	4.82 ± 0.06	4.50 ± 0.29
Histamine/forskolin		
Control (7)	6.57 ± 0.23	5.45 ± 0.16
Pertussis toxin[c] (1)	5.33	4.99

[a] Each pEC_{50} value is the mean ± S.E.M. of the experiments.
[b] Number of experiments is given in parentheses.
[c] In three other experiments, oxotremorine-M was unable to elict contraction in the presence of forskolin.

have shown that pertussis toxin uncouples M_2- and M_4-mediated responses without affecting M_1-, M_3- and M_5-mediated responses. Therefore, the M_2 receptor may be contributing to the standard contractile response of the guinea pig colon when most M_3 receptors have been inactivated by 4-DAMP mustard. Data obtained by use of AF-DX 116 to antagonize oxotremorine-M-induced contractions before and after 4-DAMP mustard treatment suggest that both the M_2 and the M_3 receptors contribute to the contractile response (fig. 3). The pK_B value (6.52) estimated after 4-DAMP mustard treatment did not correspond to a purely M_2- or M_3-mediated response, but rather, to an intermediate value, which suggests a role for both the M_2 and M_3 receptors. We have previously shown that antagonism of responses mediated by both the M_2 and M_3 receptors is complex and that the antagonistic profile can resemble an M_3 response even though there is a contribution of the M_2 receptor (Thomas and Ehlert, 1994; Ehlert *et al.*, 1997).

Bolton and Zholos (1997) have used subtype selective antagonists to demonstrate that the M_2 receptor couples to a nonselective cationic channel. Perhaps contractions mediated by this M_2 receptor-activated cation channel can be revealed after most of the M_3 receptors have been inactivated with 4-DAMP mustard.

Analysis of 4-DAMP mustard reaction kinetics also suggests that two receptors are mediating contraction in guinea pig colon after 2-hr treatment. Our laboratory has described the kinetics of alkylation of glandular M_3 muscarinic receptors as being consistent with a first-order decay model having an observed rate constant for alkylation (k_{obs}) of 0.0321 min^{-1} when AF-DX 116 (1.0 μM) is present and the concentration of 4-DAMP mustard is 40 nM. Accordingly, 4-DAMP mustard should alkylate 85.4% and 97.8% of the M_3 receptors after 1 and 2 hr of incubation, respectively. This degree of receptor inactivation was observed in pertussis toxin-treated tissue after a 2-hr treatment with 4-DAMP mustard (99.2%,

k_{obs} = 0.040 min^{-1}). However, in control tissue, the calculated level of receptor inactivation after 1 hr (94.6%) was not much different from that estimated at 2 hr (96.7%), even though there was a striking difference in the shapes of the two corresponding oxotremorine-M concentration-response curves (see fig. 2). Thus, the calculations seem inconsistent with our empirical observations; this suggests that the one-site, first-order decay model on which the calculations are based is inaccurate. Furthermore, the one-site, first-order decay model predicts that the estimate of the rate constant for alkylation should be the same for the two periods. However, the k_{obs} at 1 hr (0.049 min^{-1}) was approximately 73% larger than that estimated at 2 hr (0.0284 min^{-1}). Also, the method for calculating receptor inactivation (see "Material and Methods") provides an estimate of the dissociation constant (K_A) of oxotremorine-M for the receptor mediating the response. This estimate at 1-hr treatment (pK_A = 4.95) was more than 10-fold greater than that measured after 2-hr treatment (pK_A = 6.00). These inconsistencies suggest that the one-site model is inaccurate and that at least two receptors (*i.e.,* M_2 and M_3) showing differential sensitivity to 4-DAMP mustard contribute to the contractile response. A likely interpretation is that the 4-DAMP mustard-insensitive M_2 receptor rescues the contractile response after 2-hr 4-DAMP mustard treatment. By eliminating this M_2 response with pertussis toxin treatment, we should observe data consistent with a single M_3 receptor model. Accordingly, the estimates of pK_A of oxotremorine-M and the k_{obs} in pertussis toxin-treated tissue after 2-hr 4-DAMP mustard treatment (5.35 and .040 min^{-1}) were similar to those measured after 1-hr 4-DAMP mustard treatment (4.95 and .049 min^{-1}).

Our experiments on 4-DAMP mustard-treated colon show that oxotremorine-M elicits contraction through the M_2 receptor when histamine and either forskolin or isoproterenol are present (fig. 4). Presumably under these conditions, the M_2 receptor inhibits the cAMP-mediated relaxant effects of forskolin and isoproterenol, thereby allowing histamine to cause contraction. Similar results have been reported for the guinea pig ileum (Thomas *et al.*, 1993; Reddy *et al.*, 1995; Ostrom and Ehlert, 1997). The contractile response (disinhibition of contraction) elicited by oxotremorine-M in the presence of histamine and either forskolin or isoproterenol was antagonized by AF-DX 116 in a manner consistent with a M_2 receptor-mediated event. Also, after *in-vivo* pertussis toxin treatment, three of four colon segments precontracted with histamine (0.30 μM) and relaxed with forskolin (10 μM) did not contract when oxotremorine-M was added even though oxotremorine-M was able to elicit contraction in these tissues under standard conditions (fig. 5). In colon segments precontracted with histamine and relaxed with isoproterenol, pertussis toxin caused a 32.5-fold (fig. 6B) increase in EC_{50} for contractions elicited to oxotremorine-M. The pertussis toxin sensitivity of the contractile response strongly indicates the role of the M_2 receptor. AF-DX 116 (1 μM) shifted the remaining contractile response in pertussis toxin-treated tissue 2.1-fold (fig. 6C) when isoproterenol was used as the relaxant agent. These data suggest that the remaining contraction elicited to oxotremorine-M is mediated primarily by the M_3 receptor as evidenced by the extremely reduced potency of oxotremorine-M and the inability of AF-DX 116 to shift the remaining contraction more than 2.2-fold.

The guinea pig colon behaves like the ileum in the sense that the colonic M_2 receptor opposes the relaxant effects of both forskolin and isoproterenol, and the role of the M_2 receptor is easier to demonstrate when forskolin is used as the relaxant agent as compared with isoproterenol (see above; also Thomas *et al.*, 1993; Reddy *et al.*, 1995; Ostrom and Ehlert, 1997). We have previously discussed the dependence of the M_2 contractile response in the ileum on the nature of the relaxant agent (see Ostrom and Ehlert, 1997). However, in the trachea, the M_2 receptor only opposes the relaxant effects of forskolin (Ehlert and Thomas, 1996) but not isoproterenol (Watson *et al.*, 1995; Ostrom and Ehlert, unpublished observations).

In summary, our data demonstrate that the M_3 muscarinic receptor mediates the standard contractile response to oxotremorine-M in isolated guinea pig colon. Our data also suggest a role for the M_2 receptor in the standard contraction elicited to oxotremorine-M when the number of M_3 receptors is extremely reduced by 4-DAMP mustard. As observed in other smooth muscle preparations, the M_2 receptor in the guinea pig colon can mediate an indirect contraction by preventing the relaxant effects of forskolin and isoproterenol on histamine-induced contractions.

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