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Involvement of the M_2 Muscarinic Receptor in Contractions of the Guinea Pig Trachea, Guinea Pig Esophagus, and Rat Fundus

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ABSTRACT. The involvement of the M_2 muscarinic receptor in contractile responses of the guinea pig trachea, guinea pig esophagus, and rat fundus was investigated. In the standard assay, oxotremorine-M elicited contractions of the trachea with an EC_{50} value of approximately 73 nM. [[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one (AF-DX 116) at 1 and 10 μM antagonized these contractions by 2.1- and 9.0-fold increases in the EC_{50} value for oxotremorine-M. These effects are consistent with antagonism of an M_3-mediated contractile response. In subsequent experiments, the M_3 receptors were first inactivated selectively by incubation with *N*-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP mustard) (40 nM) for 1 hr in the presence of AF-DX 116 (1 μM) followed by extensive washing. In 4-DAMP mustard treated trachea, oxotremorine-M elicited contractions with an EC_{50} value of 0.31 μM in the presence of histamine (10 μM) and forskolin (4 μM). Under these conditions, AF-DX 116 at 1 and 10 μM antagonized contractions to oxotremorine-M by 8- and 59-fold increases in the EC_{50}, respectively, while *para*-fluorohexahydrosiladiphenidol (p-F-HHSiD) (0.1 μM) had no effect. These effects are consistent with a contraction being mediated by an M_2 receptor. In the guinea pig esophagus and rat fundus, AF-DX 116 and p-F-HHSiD blocked contractions measured under similar conditions with magnitudes intermediate between what would be expected from an M_2 and an M_3 receptor, suggesting that perhaps both subtypes contribute to the overall contractile response under these conditions. In addition, contractions of the guinea pig trachea measured in the presence of histamine and forskolin were pertussis toxin sensitive. These results indicate that, in the trachea, M_2 receptors can dominate the contractile response after a majority of the M_3 receptors have been inactivated, whereas in the guinea pig esophagus and rat fundus, M_2 receptors may contribute to, but do not play a dominant role in the overall response. BIOCHEM PHARMACOL 51;6:779–788, 1996.

KEY WORDS. muscle, smooth, contraction of trachea, M_2 muscarinic receptors; trachea, M_2 muscarinic receptors and contraction; fundus, M_2 muscarinic receptors and contraction; esophagus, M_2 muscarinic receptors and contraction; muscarinic receptors, M_2 and M_3 subtypes; pertussis toxin, inhibition of tracheal smooth muscle contractions

In smooth muscle containing tissues, activation of muscarinic receptors causes a stimulation of phospholipase C as well as inhibition of adenylate cyclase. The former response is mediated by the M_3 muscarinic receptor, which makes up approximately 20% of the total population of muscarinic receptors [1–3], whereas the latter response is mediated by the more abundant M_2 receptor, which accounts for roughly 80% of the muscarinic receptors [2–4]. Previous studies from our laboratory [5–7] as well as those of Reddy *et al.* [8] have demonstrated that contraction of the guinea pig ileum can be mediated by either the M_2 or the M_3 receptor, depending on the conditions of the assay. The M_3 receptor mediates contraction under standard assay conditions as discussed previously by a number of groups [1, 2, 9, 10]. Alternatively, when M_3 receptors are inactivated by treatment with 4-DAMP mustard,§ the M_2 receptor can elicit an indirect contraction of ileal smooth muscle by inhibiting the relaxant effect of isoproterenol or forskolin on histamine-induced contractions [5–7, 8]. Presumably, the mechanism for this indirect contraction involves an M_2-mediated inhibition of the cyclic AMP accumulation elicited by isoproterenol or forskolin. Like the ileum, numerous other smooth muscles contain a majority of M_2 receptors [11–19]; thus, the question remains whether M_2 receptors can also influence contraction in these other tissues.

An important tissue that has been the focus of research for many years is tracheal smooth muscle. In trachea from various

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§ *Abbreviations:* 4-DAMP mustard, *N*-(2-chloroethyl)-4-piperidinyl diphenylacetate; AF-DX 116, [[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; p-F-HHSiD, *para*-fluorohexahydrosiladiphenidol; KRB buffer, Krebs-Ringer bicarbonate buffer.

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species, it has long been noted that isoproterenol is less potent in relaxing tissues contracted with muscarinic agonists than other agonists such as histamine or leukotrienes [20–22]. This phenomenon could be explained by a muscarinic-induced inhibition of β-adrenergic receptor stimulation of adenylate cyclase resulting in a decreased relaxation [20, 23], although other mechanisms have been proposed (see Roffel *et al.* [24]). In more recent studies, it has been demonstrated that the M_2 selective antagonists AF-DX 116 and methoctramine, as well as pertussis toxin, can enhance isoproterenol-induced relaxation of canine and guinea pig trachea that have been contracted with muscarinic agonists [25, 26], indicating that M_2 receptor activation can prevent the relaxing effects of isoproterenol. It should be noted, however, that similar studies have not demonstrated a functional antagonism between the M_2 receptor and the β-adrenergic receptor in bovine trachea [24].

Studies investigating the functional role of muscarinic receptors in mediating whole tissue responses are beset by the lack of highly selective agonists and antagonists for receptor subtypes. To overcome this problem, we developed a protocol in guinea pig ileum where the M_3 receptors are irreversibly alkylated so that the remaining M_2 receptors can be activated preferentially with a non-selective muscarinic agonist [5]. Also, we have characterized the muscarinic response in these tissues using the standard pharmacological null method for competitive antagonism so that it was possible to estimate the dissociation constants of antagonists (i.e. K_B values) from their ability to interfere with the response [5, 7]. From these quantitative estimates of affinity, it was possible to assign a receptor subtype to the response with greater certainty. In the present report, we describe the results of a study in which the approach described above was used to investigate whether M_2 receptors elicit indirect contractions in the guinea pig trachea and esophagus by inhibiting the relaxant effect of forskolin on histamine-induced contractions. We also investigated whether M_2 receptors could inhibit the relaxant effect of isoproterenol on serotonin-induced contractions of the rat fundus. This group of tissues was selected in order to get some idea of how widespread the M_2 modulation of contraction might be. Our results indicate that, in the trachea, M_2 receptors can dominate the contractile response when cyclic AMP levels are elevated with forskolin and a majority of the M_3 receptors have been inactivated, whereas in the guinea pig esophagus and rat fundus, M_2 receptors may contribute to, but do not play a strong role in the overall response. Recently, Watson *et al.* [27] have used similar techniques to inactivate M_3 receptors in the guinea pig trachea and have shown a lack of involvement of the M_2 receptor in preventing the relaxant effect of isoproterenol on histamine-induced contractions. As discussed below, these results may suggest that the M_2 receptor is less effective at preventing the relaxant effects of isoproterenol, as compared with forskolin, on histamine-induced contractions in the guinea pig trachea.

MATERIALS AND METHODS

Contractile Responses

Male Hartley guinea pigs (300–350 g) or Sprague–Dawley rats (175–225 g) were asphyxiated with CO_2. The trachea, stomach, or esophagus was rapidly removed and placed in warm KRB buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 10 mM glucose) gassed with O_2/CO_2 (19:1). Tracheal tubes were cut free of adhering connective tissue, and the epithelium was removed by rubbing the inner surface with a cotton swab. The tubes were cut longitudinally on the ventral side to form a rectangular shape and then cut in a zigzag manner as described by Emmerson and Mackay [28]. The zigzag strips were suspended under a resting tension of 1.0 g. The longitudinal fundic strip was prepared from the stomach essentially as described by Coleman [29]. Strips (10 × 3 mm) were cut from the fundus along the longitudinal muscle and were suspended under 1.0 g of resting tension. The isolated esophagus muscularis mucosae was prepared as described by Kamikawa *et al.* [30]. The outer striated muscle coat was cut longitudinally and gently peeled away leaving an inner smooth muscle tube. The tube (approximately 15 mm), which included the muscular mucosae, was suspended under 0.5 g of resting tension. All tissues were mounted in a 50-mL 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. After a 30- to 60-min equilibrium period, three test doses of the muscarinic agonist, oxotremorine-M, were added to the bath to ensure reproducibility of the preparation. The tracheal strips equilibrated 3–4 hr before contractile responses were measured to avoid any time-dependent increases in contraction [31]. Concentration-dependent responses to oxotremorine-M were measured by adding increasing concentrations (0.3 log units) of oxotremorine-M in a cumulative manner and measuring the responses relative to resting tension. Indomethacin (1 μM) was included in all the experiments in which contractions of the isolated trachea were measured. Indomethacin is often included in *in vitro* tracheal tissue bath experiments for a variety of reasons including removal of the influence of endogenous prostaglandins [32, 33], dissipation of spontaneous tracheal tone [34], and maintenance of tracheal tone [35], although the explanation for many of these reasons is unclear. When present, AF-DX 116 and p-F-HHSiD were added 30 min prior to measuring contractions. 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. 4-DAMP mustard (10 μM) was preactivated to form the reactive aziridinium ion by incubation in 10 mM phosphate buffer (pH 7.4) for 30 min at 37° and used immediately.

Pertussis Toxin Treatment

Tracheal strips, with epithelium removed, were incubated in an enclosed organ chamber with a continuous flow of O_2/CO_2 (19:1) in Eagle's Minimum Essential Medium supplemented with streptomycin (100 μg/ml), penicillin (10,000 U/mL) and calf serum (5%), in the presence of HEPES (25 mM). Pertussis toxin was activated with 25 mM dithiothreitol for 30 min at

37°. Pertussis toxin (10 μg/mL) or an equal volume (0.5 mL) of saline (0.9%) was then added, and the strips were incubated for 18 hr at 35°.

Calculations

The EC_{50} values of oxotremorine-M for contraction were estimated by nonlinear regression analysis of the data according to a logistic equation, as described previously [2]. The K_B values for the selective antagonists were calculated using the following relationship:

$$K_B = \frac{[B]}{\text{concentration ratio} - 1} \quad (1)$$

where K_B denotes the dissociation constant of the antagonist for the receptor eliciting the response, [B] denotes the concentration of antagonist in the organ bath, and concentration ratio represents the EC_{50} value measured in the presence of antagonist divided by that measured in the absence of antagonist. To determine whether the estimate of the K_B value of an antagonist was different when measured at different concentrations of the antagonist, Student's *t*-test was used. The percent of receptors irreversibly bound by 4-DAMP mustard (% occupancy) was determined using the relationship described by Paton [36]:

$$\text{Occupancy} = \frac{\text{concentration ratio} - 1}{\text{concentration ratio}} \times 100 \quad (2)$$

Compounds

4-DAMP mustard was synthesized in our laboratory as described previously [37]. Compound p-F-HHSiD was provided by Dr. Gunter Lambrecht, University of Frankfurt, F.R.G., and Dr. Reinhold Tacke, University of Karlsruhe, F.R.G.; AF-DX 116 was provided by Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT.

RESULTS

Contractions Measured Under Standard Conditions

Previous studies have shown that the M_3 receptor mediates contractions of isolated smooth muscle tissues under standard conditions ("basal conditions," i.e. when no other heterologous agonists are present) [1, 2, 9, 10, 15, 38, 39]. To establish the lack of involvement of the M_2 receptor in contraction of the guinea pig trachea, esophagus, and rat fundus under standard conditions, we measured the potency with which the M_2-selective antagonist AF-DX 116 blocked contractions elicited by the highly efficacious muscarinic agonist oxotremorine-M. Oxotremorine-M caused concentration-dependent contractions of the guinea pig trachea with half-maximal contraction occurring at a concentration (EC_{50}) of 73 ± 15 nM. In this tissue, AF-DX 116 at concentrations of 1 and 10 μM shifted the concentration–effect curve to the right by 2.1- and 9.0-fold, respectively (Fig. 1). In guinea pig esophagus, AF-DX 116 at 1 and 10 μM antagonized oxotremorine-M-induced contractions by 3.2- and 13.5-fold increases in EC_{50}, respectively, and 1 μM AF-DX 116 caused a 2.1-fold increase in the EC_{50} for oxotremorine-M in the fundus. The K_B values for AF-DX 116 in each of these tissues were calculated using equation 1, defined in Materials and Methods, and are summarized in Table 1. These values correspond well with the reported affinity of AF-DX 116 for the M_3 receptor ($K_B \approx 1$ μM) [2, 40, 41], with the exception of the K_B value of 0.45 μM for the esophagus, which is approximately 2-fold lower. The two values for the esophagus (0.45 and 0.80 μM), however, were not significantly different from each other ($P > 0.05$), and previous studies have shown that the M_3 receptor medi-

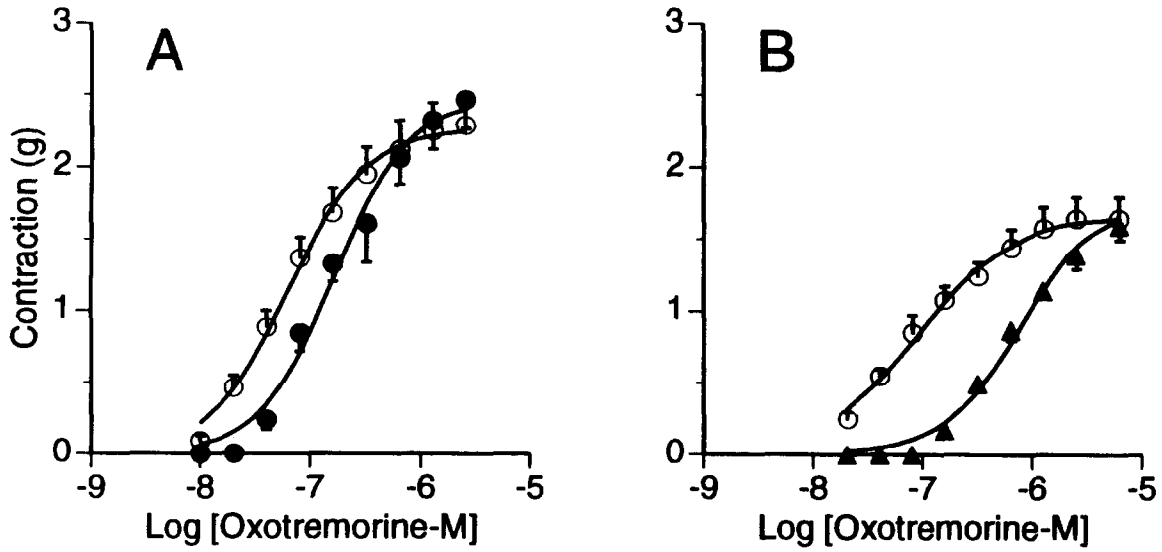

FIG. 1. Effects of AF-DX 116 on the contractile response to oxotremorine-M in the guinea pig trachea under standard conditions. (A) Contractile responses were measured in the absence (○) and presence (●) of 1 μM AF-DX 116. Contractile responses were measured in the absence (○) and presence (▲) of 10 μM AF-DX 116. Data points represent the means ± SEM of five experiments.

TABLE 1. Estimates of the dissociation constants (K_B) of AF-DX 116 for blocking oxotremorine-M-induced contractions measured under standard, basal conditions

AF-DX 116 concentration (μM)	Trachea		Fundus		Esophagus	
	Concentration ratio	K_B (μM)	Concentration ratio	K_B (μM)	Concentration ratio	K_B (μM)
1	2.1	0.90	2.1	0.90	3.2	0.45
10	9.0	1.25	ND*		13.5	0.80

* ND = not determined.

ates contraction of the esophagus under standard conditions [15, 30, 42]. The M_3 receptor, therefore, appeared to be mediating the contraction in all three tissues.

We have previously used the irreversible, M_1/M_3 selective antagonist 4-DAMP mustard to inactivate M_3 receptors selectively in the guinea pig ileum [5, 37]. To test the effectiveness of 4-DAMP mustard at inactivating M_3 receptors in the guinea pig trachea, contractile responses were measured under standing conditions before and after 4-DAMP mustard treatment. Tracheal strips were incubated with 4-DAMP mustard (40 nM) for 1 hr at 37° 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 inactivate 4-DAMP mustard. Incubation in the presence of AF-DX 116 has been shown previously to protect the M_2 receptors from alkylation by 4-DAMP mustard, thereby enabling a more selective alkylation of M_3 receptors by 4-DAMP mustard [37]. This technique, therefore, was used throughout the remainder of the experiments to inactivate the M_3 receptors selectively and is referred to as "4-DAMP mustard treatment." This treatment shifted the oxotremorine-M concentration–effect curve to the right 50-fold (see Fig. 2; EC_{50} control, 0.096 μM; EC_{50} after 4-DAMP mustard treatment, 4.8 μM). The magnitude of this shift corresponded to a 98% inactivation of the M_3 receptors (see equation 2 in Materials and Methods). These results suggest that 4-DAMP mustard treatment inactivated a large percentage of the M_3 receptors, and that contractile responses can still be mediated by the residual 2% of the receptors at greatly reduced potency. Although we did not characterize the nature of the response that persisted after 4-DAMP mustard treatment under standard conditions, it is likely that the M_3 receptor mediates the response for the following reasons. First, the EC_{50} value for the response is still less than the dissociation constant of oxotremorine-M for M_3 muscarinic receptors (5–10 μM; see Ringdahl [43]; Thomas *et al.* [5]). Second, in the guinea pig ileum, we have shown that the residual response to oxotremorine-M after 4-DAMP mustard treatment under standard conditions has a pharmacological profile consistent with an M_3 response [5]. Finally, in order to detect an M_2 response, it is necessary to carry out the contractile assay under special conditions (i.e. elevated levels of cyclic AMP; see Thomas *et al.* [5]; Ehlert and Thomas [7]).

Contractions Measured under Conditions of Elevated Cyclic AMP

To test the hypothesis that M_2 receptors can influence contraction in the guinea pig trachea by preventing the relaxing effects of adenylate cyclase stimulating agents, we first inactivated the M_3 receptors by 4-DAMP mustard treatment (see above). Next, we contracted the tracheal strips with histamine (10 μM), relaxed the tissues back to resting tension with the direct adenylate cyclase stimulating relaxant forskolin (4 μM), and then measured contractile responses to oxotremorine-M while these agents remained in the organ bath. In the remainder of the text, contractions measured under these conditions are referred to as "contractions measured after 4-DAMP mustard treatment and under conditions of elevated cyclic AMP." The contractile response to oxotremorine-M under these conditions cannot be attributed to a time-dependent decrease in the effectiveness of forskolin because we showed in preliminary studies that the relaxant effect of forskolin was constant for up to 30 min (data not shown). After 4-DAMP mustard treatment and under conditions of elevated cyclic AMP, oxotremorine-M elicited contractions of the guinea pig trachea with an average EC_{50} value of 0.31 μM. AF-DX 116 at concentrations of 1 and 10 μM shifted the concentration–effect curve to the right 8- and 59-fold, respectively (Fig. 3A and B).

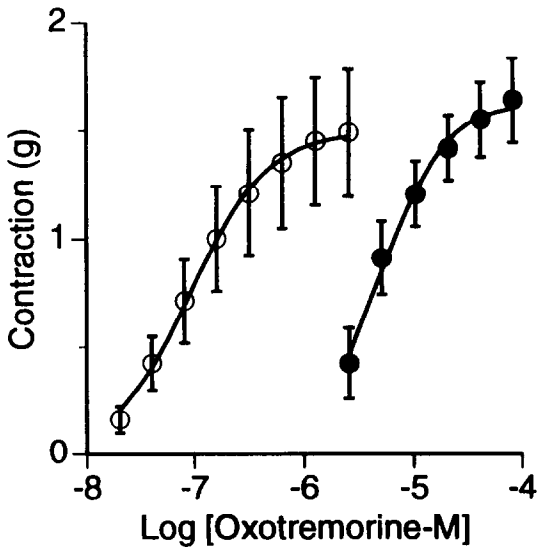

FIG. 2. Effect of 4-DAMP mustard treatment on the contractile response to oxotremorine-M in the guinea pig trachea under standard conditions. Contractile responses were measured in control (○) and 4-DAMP mustard treated (●) tissue. Tracheal strips were incubated with 4-DAMP mustard as described in Materials and Methods. Each point represents the mean ± SEM of four experiments.

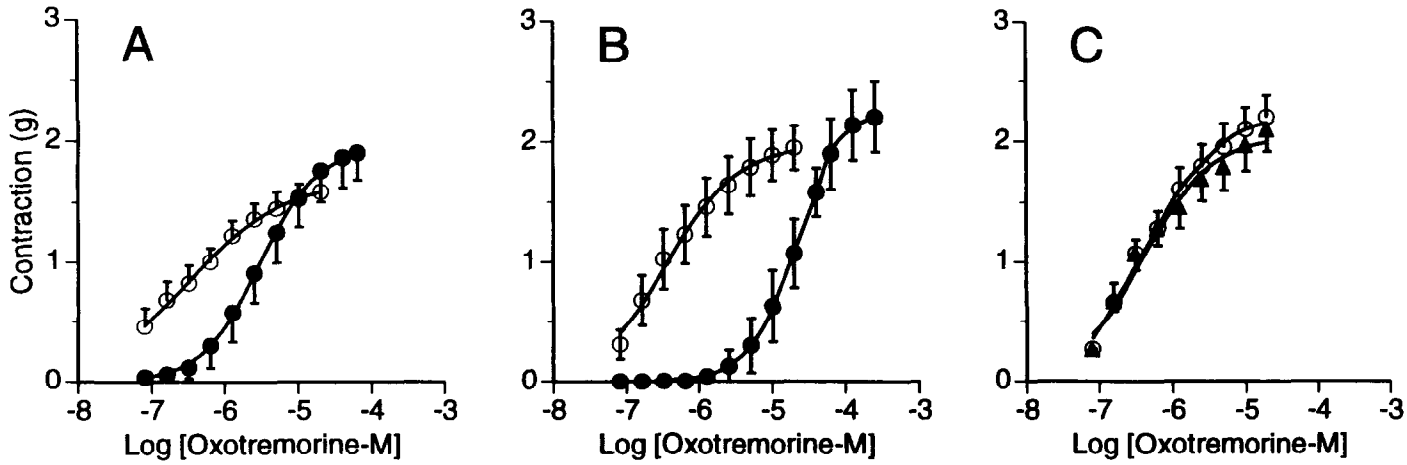

FIG. 3. Effects of AF-DX 116 (A and B) and p-F-HHSiD (C) on the contractile response to oxotremorine-M in the guinea pig trachea measured after 4-DAMP mustard treatment under conditions of elevated cyclic AMP. (A) Control (○) and 1 μM AF-DX 116 (●). (B) Control (○) and 10 μM AF-DX 116 (●). (C) Control (○) and 0.1 μM p-F-HHSiD (▲). In all experiments, tissues were treated with 4-DAMP mustard as described in Materials and Methods. Also, tissues were contracted with histamine (10 μM) and relaxed back to resting tension with forskolin (4 μM) before oxotremorine-M-induced contractions were measured. The histamine-induced contraction was approximately 70–80% of the maximal contraction elicited by oxotremorine-M. The data points represent the means ± SEM of five experiments.

The M_3 selective antagonist p-F-HHSiD (0.1 μM) had no effect on contraction measured under these conditions (Fig. 3C). The K_B values for AF-DX 116 are shown in Table 2 and correlate well with the dissociation constant of AF-DX 116 for the M_2 receptor determined by ligand binding (i.e. K_D = 0.1 μM) [2, 40, 41]. Presumably, oxotremorine-M is acting at M_2 receptors to inhibit adenylate cyclase, which prevents the relaxing effects of forskolin and allows histamine to contract the trachea. This mechanism can be referred to as an "M_2-mediated disinhibition of histamine-induced contractions." The effects of AF-DX 116 and p-F-HHSiD on the contractile responses to oxotremorine-M were also measured in the guinea pig esophagus and rat fundus under conditions similar to those used in the trachea except that, in the fundus, serotonin and isoproterenol were used in place of histamine and forskolin, respectively. The calculated K_B values for AF-DX 116 and p-F-HHSiD in these two tissues are summarized in Table 2. It can be seen that the K_B values for the esophagus and fundus are intermediate between those expected for M_2 and M_3 receptors.

Effects of Pertussis Toxin

To investigate the influence of the M_2 receptor on contraction in the guinea pig trachea further, the effect of pertussis toxin on the contractile response was examined. Pertussis toxin causes an uncoupling of G_i, from the M_2 receptor [44], thereby preventing M_2-mediated responses but not M_3-mediated effects [45, 46]. Tracheal strips were incubated with pertussis toxin (10 μg/mL) in organ culture for 18 hr before contractile responses to oxotremorine-M were measured. Control trachea were incubated in the same manner except for exposure to pertussis toxin. This lengthy incubation of control tissue reduced the potency of oxotremorine-M for eliciting contractions under standard conditions by one-half (i.e. 2-fold increase in EC_{50}), but had no effect on the maximal contractile

TABLE 2. Estimates of the Dissociation Constants (K_B) of Muscarinic Antagonists for Blocking Oxotremorine-M-Induced Contractions Measured in the Presence of Histamine (Trachea and Esophagus) or Serotonin (Fundus) and Forskolin (Trachea and Esophagus) or Isoproterenol (Fundus)*

	Trachea			Fundus			Esophagus			M_2‡	M_3‡§
Antagonist	Concn. (μM)	CR†	K_B (μM)	Concn. (μM)	CR	K_B (μM)	Concn. (μM)	CR	K_B (μM)	K_B (μM)	K_B (μM)
AF-DX 116	1	8	0.14	1	3.8	0.36	1	5.7	0.21	0.1	1.0
AF-DX 116	10	59	0.17	10	27	0.38	10	27	0.38		
p-F-HHSiD	0.1	1	–‖	0.5	2.8	0.28	0.1	2.5	0.07	1.0	0.01–0.07

* All tissues had been treated with 4-DAMP mustard before contractile response to oxotremorine-M was measured. Details of contractile measurements are given in Materials and Methods.
† "CR" denotes concentration-ratio.
‡ K_B values were taken from Candell *et al.* [2].
§ K_B values were taken from Eglen *et al.* [49].
‖ K_B values could not be determined due to lack of effect of antagonist.

response (2 to 2.5 g). Pertussis toxin treatment had no inhibitory effect on the contractions measured under standard conditions, but actually caused a slight increase in the maximal response to oxotremorine-M (Fig. 4A). We have no explanation for this increase. When measured after 4-DAMP mustard treatment and under conditions of elevated cyclic AMP, the contractile responses to oxotremorine-M were greatly attenuated by pertussis toxin treatment as manifested by a 76% reduction in the maximum response (Fig. 4B). These results are consistent with the postulate that the M_2 receptor elicits contraction under conditions of elevated cyclic AMP because previous studies have shown that pertussis toxin blocks M_2-mediated responses but not M_3-mediated responses [45, 46]. The effect of forskolin alone on the standard contractile responses of tissues that had not been treated with 4-DAMP mustard was also investigated to see if M_2 receptors could potentiate M_3-mediated contractions in addition to disinhibiting histamine-induced contractions as observed above. In control tissues, forskolin caused a 5-fold shift in the concentration–effect curve of oxotremorine-M (Fig. 5A), whereas in pertussis toxin treated tissues, forskolin had a 2-fold greater effect, namely, a 10-fold decrease in potency (Fig. 5B). These results suggest that, when cyclic AMP levels are elevated, M_2 receptors can act to potentiate M_3-mediated contractions.

DISCUSSION

Two different strategies have been used to uncover a contractile role for the M_2 receptor in preventing the relaxant effect of the β-adrenergic receptor in the trachea. First, Fernandes *et al.* [25] and Watson and Eglen [26] showed that M_2 selective antagonists [i.e. AF-DX 116 (0.1 to 1.0 μM) and methoctramine (0.3 μM)] enhanced the potency of isoproterenol for inhibiting the contractile response to a fixed concentration of a muscarinic agonist, whereas an M_3 selective antagonist (HHSiD) was ineffective. The simplest interpretation of these data is that, in the presence of isoproterenol, the contractile response to muscarinic agonists in the trachea is mediated through both the M_2 and M_3 receptor [24, 25]. The M_3 receptor causes a direct contraction through calcium mobilization, whereas the M_2 receptor causes an indirect contraction by inhibiting the increase in cyclic AMP elicited by isoproterenol [24, 25]. Thus, when M_2 receptors are competitively blocked, isoproterenol is more potent at inhibiting the contractile response of muscarinic agonists. It is important to note that this strategy tests for the ability of the M_2 receptor to potentiate or disinhibit M_3-mediated contractions. When this strategy was applied in bovine trachea, no evidence for a role of the M_2 receptor in contraction was found [26], suggesting species differences in the ability of the M_2 receptor to inhibit the relaxant effect of isoproterenol on M_3 contractile responses. However, we suggest caution in the interpretation of negative results from this type of experiment. It is possible that the potency of agonists for inhibiting cyclic AMP accumulation in the presence of isoproterenol is much greater than that for eliciting contraction through the M_3 receptor in bovine trachea. Thus, even in the presence of an M_2 selective antagonist (e.g. AF-DX 116), a muscarinic agonist could still cause an effective inhibition of adenylate cyclase over the same concentration range required to elicit an M_3 contractile response. This situation could yield results consistent with no effect of an M_2 selective antagonist on the relaxant potency of isoproterenol even though the M_2 receptor may still contribute to the contractile response. We have described this hypothesis in

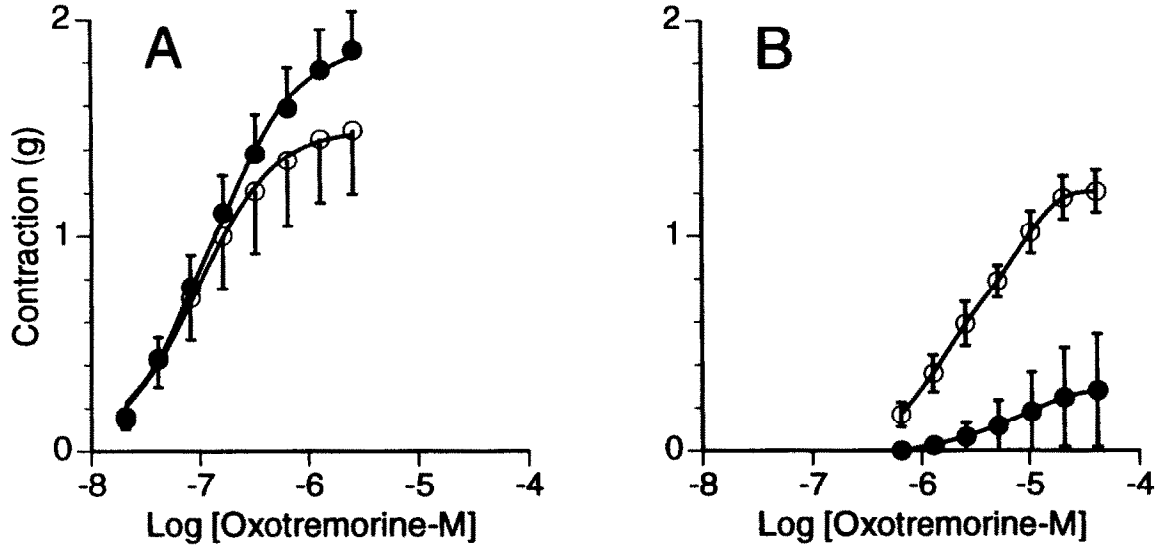

FIG. 4. Effects of pertussis toxin treatment on the contractile responses to oxotremorine-M measured under standard conditions (A) and after 4-DAMP mustard treatment under conditions of elevated cyclic AMP (B). Contractile responses were measured in control (○) and pertussis toxin treated (●) tissues. Tracheal strips were incubated with activated pertussis toxin (10 μg/mL) for 18 hr at 35°. (A) Standard conditions. (B) M_3 receptors were inactivated by treatment with 4-DAMP mustard as described in Materials and Methods. Contractile responses to oxotremorine-M were measured in the presence of histamine (10 μM) and forskolin (4 μM). Data points represent the means ± SEM of six experiments.

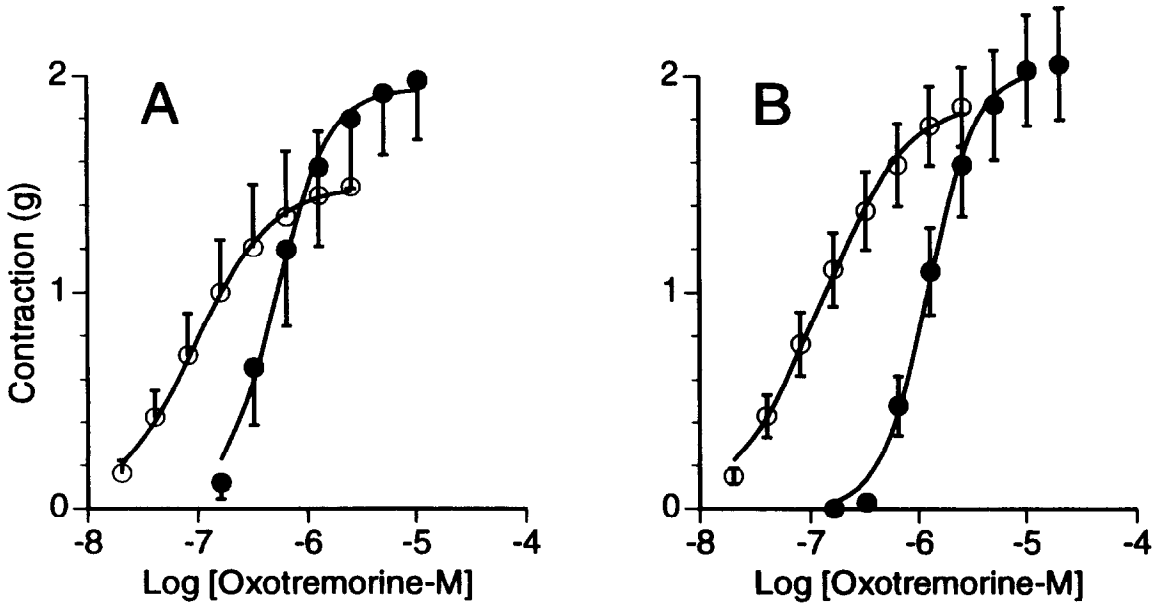

FIG. 5. Effects of forskolin on oxotremorine-M induced contractions under standard conditions in control (A) and pertussis toxin treated (B) trachea. (A) Contractile responses were measured in the absence (○) and presence (●) of forskolin (4 μM). (B) Contractile responses were measured in pertussis toxin treated trachea in the absence (○) and presence (●) of forskolin (4 μM). Tracheal strips were incubated with activated pertussis toxin (10 μg/mL) for 18 hr at 35°. Data points represent the means ± SEM of six experiments.

greater detail previously with regard to muscarinic responses in the guinea pig ileum (see Thomas and Ehlert [6]).

The second strategy that has been used to investigate the role of the M_2 receptor in the contractile response of the trachea is that used in the present study. With this method, the ability of a muscarinic agonist to elicit contraction in the presence of histamine and a cyclic AMP stimulating agent (e.g. forskolin or isoproterenol) is tested in tissue that has been treated previously with 4-DAMP mustard to inactivate M_3 muscarinic receptors. An advantage of this strategy is that it is possible to estimate the dissociation constants of antagonists for blocking the response (i.e. K_B values) and to compare these estimates with the binding affinities of the M_2 and M_3 receptors so that it is possible to determine with greater certainty which receptors contribute to the contractile response. A limitation of this technique is that it only measures the ability of the M_2 receptor to potentiate or disinhibit the contractile response of a heterologous receptor, like the H_1 histamine receptor, but not that of the M_3 muscarinic receptor. Using this strategy, Watson *et al.* [27] found no role for the M_2 receptor in inhibiting the relaxant effect of isoproterenol on histamine-induced contractions in the guinea pig trachea. However, in the present report, we found that the M_2 receptor was able to inhibit the relaxant effect of forskolin on histamine-induced contractions in the trachea. Thus, it appears as though the M_2 receptor is active at inhibiting the relaxant effect of forskolin, but not isoproterenol, on histamine-induced contractions of the guinea pig trachea. Previously, we reported a greater contribution of the M_2 receptor at inhibiting the relaxant effect of forskolin, as compared with isoproterenol, on histamine-induced contractions of the guinea pig ileum [5]. This conclusion is based on the greater potency of oxotremorine-M at eliciting contractions in 4-DAMP mustard-treated ilea in the presence of histamine and forskolin (EC_{50} = 0.059 μM) as compared with that measured in the presence of histamine and isoproterenol (EC_{50} = 0.46 μM)). Also, the K_B value of AF-DX 116 was in closer agreement to the dissociation constant of the M_2 receptor (K_D = 0.1 μM) when forskolin was used (K_B = 0.1 μM) as compared with isoproterenol (K_B = 0.17 μM). In addition, we have observed recently that forskolin causes a greater stimulation of cyclic AMP in the guinea pig ileum as compared with isoproterenol and that oxotremorine-M causes a greater inhibition of forskolin-stimulated cyclic AMP accumulation as compared with that elicited by isoproterenol (Ostrom RS and Ehlert FJ, unpublished observations). Thus, when forskolin is used to stimulate cyclic AMP, the M_2 receptor causes a greater inhibition of a greater response as compared with that observed with isoproterenol. This condition might explain why the M_2 receptor is more effective at preventing the relaxant effects of forskolin as compared with isoproterenol in the guinea pig ileum. It is conceivable that a similar situation exists in the trachea; however, additional work is needed to sort out this question.

A comparison of our present study on the role of the M_2 receptor in the guinea pig trachea with our previous work on the guinea pig ileum reveals some important differences. In the guinea pig ileum, treatment with 4-DAMP mustard (40 nM) and AF-DX 116 (1.0 μM) caused a 20-fold increase in the EC_{50} value of oxotremorine-M for eliciting contractions [5], whereas in the present report, the same treatment caused a 50-fold increase in the EC_{50} value of oxotremorine-M. The greater effect of 4-DAMP mustard in the trachea may be related to the smaller size of the trachea as compared with the ileum. We have noted previously that the alkylating ability of

4-DAMP mustard in brain homogenate is inhibited by relatively low tissue concentrations in a manner that cannot be explained by the depletion in the aziridinium ion concentration by receptor binding [37]. Thus, it appears that the aziridinium ion of 4-DAMP mustard is readily inactivated by tissue nucleophiles which are more abundant in a relatively large segment of ileum as compared with a rather small strip of trachea.

Another difference between our studies in the ileum and trachea is related to the EC_{50} values of oxotremorine-M for eliciting M_2 and M_3 muscarinic responses. Our results showed that in the guinea pig trachea, 4-DAMP mustard treatment inactivated 98% of the M_3 receptors; however, oxotremorine-M could still elicit a contraction under standard conditions via the M_3 receptor but with greatly reduced potency ($EC_{50} \approx 2.6$ μM). In contrast, when contractile responses were measured in 4-DAMP mustard treated trachea in the presence of forskolin, oxotremorine-M elicited contractions with approximately 10-fold greater potency ($EC_{50} \approx 0.31$ μM). Under these conditions, AF-DX 116 and p-F-HHSiD antagonized the contractile response with pK_B values consistent with those expected for an M_2 response (Fig. 3, Table 2). Nevertheless, the EC_{50} value of oxotremorine-M measured in 4-DAMP mustard treated trachea in the presence of forskolin was approximately 4-fold greater (i.e. less potent) than that observed for the standard M_3 mediated contraction ($EC_{50} = 73$ nM). In contrast, in the guinea pig ileum, the EC_{50} values of oxotremorine-M for eliciting standard contractions and those measured in the presence of histamine and forskolin after 4-DAMP mustard treatment were approximately the same. Thus, the M_2 receptor in the guinea pig ileum elicits a more potent contractile response in the presence of forskolin as compared with that observed here in the trachea. This less prominent role for the M_2 receptor in the trachea may contribute, in part, to the observations of Watson *et al.* [27] who observed no role for the M_2 receptor at inhibiting the relaxant effect of isoproterenol on histamine-induced contractions in the trachea. This situation also explains why it is essential to inactivate M_3 receptors in the trachea in order to observe an M_2 effect on contraction.

Our studies with pertussis toxin provide further support for the role of the M_2 receptor in the contractile response of the guinea pig trachea. Following 4-DAMP mustard treatment, the contractions measured in the presence of histamine and forskolin were pertussis toxin sensitive, whereas those measured under standard conditions were insensitive to pertussis toxin (Fig. 4). These results strongly indicate that the M_2 receptor elicits contraction under conditions of elevated cyclic AMP because previous studies have demonstrated that pertussis toxin prevents M_2-mediated responses, but has no effect on M_3-mediated responses [45, 46].

In the rat fundus and guinea pig esophagus, the involvement of the M_2 receptor in contraction appears to be less. When measured after 4-DAMP mustard treatment under conditions of elevated cyclic AMP, AF-DX 116 and p-F-HHSiD antagonized the contractile responses to oxotremorine-M with pK_B values that were intermediate between those expected for the individual M_2 and M_3 subtypes. This situation might be expected if both the M_2 and the M_3 receptor subtypes contribute to the overall contractile response under these conditions. An area of ambiguity in our experiments is related to the pK_B value of p-F-HHSiD. This antagonist has been suggested to be M_3-selective on the basis of its high pA_2 value for antagonizing M_3-mediated contractions of the guinea pig ileum (8.0) relative to its lower pA_2 values for antagonizing M_1-mediated inhibition of neuronal outflow of the rabbit vas deferens (6.7) and M_2-mediated negative inotropic responses in the guinea pig heart (6.0) [9, 39, 47]. It has been shown, however, that pA_2 values for p-F-HHSiD can vary with the agonist and the tissue [48, 49]. For example, using carbachol as the agonist in the standard contractile assay, the pA_2 value of p-F-HHSiD in the guinea pig trachea (7.13) was reported to be much lower than that observed in the guinea pig ileum (7.85), whereas the pA_2 value in the esophagus was significantly higher (8.22). In each of these tissues, M_3 receptors are thought to mediate the standard contractile response. Eglen *et al.* [48, 49] have suggested a few explanations for this phenomenon. Despite this variability in the pA_2 values of p-F-HHSiD for the M_3 receptor, p-F-HHSiD still appears to distinguish between the M_2 and the M_3 receptors. The complete lack of effect that we observed for p-F-HHSiD (0.1 μM) in the trachea supports the postulate that the M_2 receptor elicits contraction when cyclic AMP levels are elevated after 4-DAMP mustard treatment. Under similar conditions in the fundus, the calculated pK_B value of p-F-HHSiD is intermediate between those expected for M_2 and M_3 receptors, but closer to that of the M_2 receptor. Using the pA_2 value (8.22) reported by Eglen *et al.* [49] for the esophagus, one would predict that 0.1 μM p-F-HHSiD would cause a 17-fold shift of the concentration–effect curve in the esophagus if contractions were elicited by the M_3 receptor. However, in our experiments, 0.1 μM p-F-HHSiD only caused a 2.5-fold shift of the concentration–effect measured in the presence of histamine and forskolin, which would indicate that the M_3 receptor was not solely responsible for the contractile response, even though the calculated pK_B value (7.15) was closer to that expected for the M_3 receptor (8.22) as compared with the M_2 receptor (6.0) (Table 2).

The variation in the extent to which M_2 receptors participate in the contractile response in different tissues may be related to how well the M_2 receptor inhibits cyclic AMP levels. In the guinea pig ileum, M_2 receptors play a major role in contraction when cyclic AMP levels are increased, and we have shown that oxotremorine-M causes a complete inhibition (101%) of isoproterenol-stimulated cAMP accumulation in this tissue [6]. In membranes and whole cells of the canine trachea, muscarinic agonists cause a 38–43% decrease in isoproterenol-stimulated adenylate cyclase activity [50] and cAMP accumulation [4, 51], and a clear role for the M_2 receptor in contraction when cyclic AMP levels are increased has been demonstrated in this report. In contrast, oxotremorine-M only caused a 13.5% reduction in isoproterenol-stimulated cAMP accumulation in slices of the rat fundus (data not shown), which may account for the lack of an exclusive effect of the M_2 receptor on contraction in this tissue. To our knowl-

edge, the evidence for muscarinic agonist-induced inhibition of cAMP accumulation in the guinea pig esophagus is lacking; therefore, we are unable to correlate the size of the adenylate cyclase response in this tissue with the involvement of the M_2 receptor in contraction. Nevertheless, the available evidence suggests that M_2 receptors must be efficiently coupled to the inhibition of adenylate cyclase if they are to participate in the contractile response.

In conclusion, our results show that, after M_3 receptors have been inactivated, the M_2 receptor can play a dominant role in the contractile response of the guinea pig trachea in the presence of histamine when cyclic AMP levels are increased with forskolin. Other investigators have shown that the M_2 receptor is ineffective under similar conditions when isoproterenol is used as the relaxing agent instead of forskolin [27]. Our results also show that M_2 receptors can enhance M_3-mediated contractions dampened by forskolin in trachea that has not been treated with 4-DAMP mustard. Similarly, other investigators [24, 25] have observed the same phenomenon when isoproterenol is used as the relaxing agent. Thus, the role of the M_2 receptor in contraction depends on both the primary contractile receptor (e.g. M_3 muscarinic or H_1 histamine) and the cyclic AMP generating, relaxing agent (e.g. forskolin or isoproterenol). The involvement of the M_2 receptor in contractions of the guinea pig esophagus and rat fundus, measured after 4-DAMP mustard treatment and under conditions of elevated cyclic AMP, is less because it appears that both the M_2 and the M_3 receptor contribute to the overall contractile response.

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References

1. Roffel AF, Meurs H, Elzinga CRS and Zaagsma J, Characterization of the muscarinic receptor subtypes involved in phosphoinositide metabolism in bovine tracheal smooth muscle. *Br J Pharmacol* **99:** 293–296, 1990.
2. Candell LM, Yun SH, Tran LLP and Ehlert FJ, Differential coupling of subtypes of the muscarinic receptor to adenylate cyclase and phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum. *Mol Pharmacol* **38:** 689–697, 1990.
3. Michel AD and Whiting RD, Methoctramine reveals heterogeneity of M_2 muscarinic receptors in longitudinal ileal smooth muscle membranes. *Eur J Pharmacol* **45:** 305–311, 1988.
4. Yang CM, Characterization of muscarinic receptors in dog tracheal smooth muscle cells. *J Auton Pharmacol* **11:** 51–61, 1991.
5. Thomas EA, Baker SB and Ehlert FJ, A functional role for the M_2 muscarinic receptor in smooth muscle of guinea pig ileum. *Mol Pharmacol* **44:** 102–110, 1993.
6. Thomas EA and Ehlert FJ, Pertussis toxin blocks M_2 muscarinic receptor-mediated effects on contraction and cyclic AMP in the guinea pig ileum, but not M_3-mediated contractions and phosphoinositide hydrolysis. *J Pharmacol Exp Ther* **271:** 1042–1050, 1994.
7. Ehlert FJ and Thomas EA, Functional role of M_2 muscarinic receptors in the guinea pig ileum. *Life Sci* **56:** 965–971, 1995.
8. Reddy H, Watson N, Ford APDW and Eglen RM, Characterization of the interaction between muscarinic M_2 receptors and β-adrenoceptor subtypes in guinea-pig isolated ileum. *Br J Pharmacol* **114:** 49–56, 1995.
9. Lambrecht G, Feifel R, Moser U, Wagner-Roder M, Choo LK, Camus J, Tastenoy M, Waelbroek M, Strohmann C, Tacke R, Rodrigues de Miranda JF, Christophe J and Mustchler E, Pharmacology of hexahydro-difenidol, hexahydro-sila-difenidol, and related selective muscarinic antagonists. *Trends Pharmacol Sci* (Suppl) **10:** 60–64, 1989.
10. Noronha-blob L, Lowe V, Patton A, Canning B, Costello D and Kinnier WJ, Muscarinic receptors: Relationships among phosphoinositide breakdown, adenylate cyclase inhibition, *in vitro* detrusor muscle contractions and *in vivo* cystometrogram studies in guinea pig bladder. *J Pharmacol Exp Ther* **249:** 843–851, 1989.
11. Zhang L, Horowitz B and Buxton ILO, Muscarinic receptors in canine colonic circular smooth muscle. I. Coexistence of M_2 and M_3 subtypes. *Mol Pharmacol* **40:** 943–951, 1991.
12. Yang CM, Chou S and Sung T, Muscarinic receptor subtypes coupled to generation of different second messengers in isolated tracheal smooth muscle cells. *Br J Pharmacol* **104:** 613–618, 1991.
13. Del Tacca M, Danes R, Blandizzi C and Bernardini MC, Differential affinities of AF-DX 116, atropine and pirenzepine for muscarinic receptors in guinea pig gastric fundus, atria and urinary bladder: Might atropine distinguish among muscarinic receptor subtypes? *Pharmacology* **40:** 241–249, 1990.
14. Li Z, Ruan Y, Guo ZD, Cong H, Zhang KY and Takemura H, Functional and localization of high and low affinity binding sites to muscarinic receptors in longitudinal and circular smooth muscles of human stomach. *Res Commun Chem Pathol Pharmacol* **67:** 31–42, 1990.
15. Eglen RM and Whiting RL, Comparison of the muscarinic receptors of the guinea-pig oesophageal muscularis mucosae and trachea *in vitro*. *J Auton Pharmacol* **8:** 181–189, 1988.
16. Maeda A, Kubo T, Mishina M and Numa S, Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes. *FEBS Lett* **239:** 339–342, 1988.
17. Fryer AD and El-Fakahany EE, Identification of three muscarinic receptor subtypes in rat lung using binding studies with selective antagonists. *Life Sci* **47:** 611–618, 1990.
18. Madison JM, Jones CA, Tom-Moy M and Brown JK, Affinities of pirenzepine for muscarinic cholinergic receptors in membranes isolated from bovine tracheal mucosa and smooth muscle. *Am Rev Respir Dis* **135:** 719–724, 1987.
19. Roffel AF, in't Hout WG, de Zeeuw RA and Zaagsma J, The M_2 selective antagonist AF-DX 116 shows high affinity for muscarinic receptors in bovine tracheal membranes. *Naunyn Schmiedebergs Arch Pharmacol* **335:** 593–595, 1987.
20. Torphy TJ, Differential relaxant effects of isoproterenol on methacholine versus leukotriene D_4-induced contraction in the guinea-pig trachea. *Eur J Pharmacol* **102:** 549–553, 1984.
21. Russell JA, Differential inhibitory effect of isoproterenol on contractions of canine airways. *J Appl Physiol* **57:** 801–807, 1984.
22. Koenig SM, Mitchell RW, Kelly E, White SR, Leff AR and Popovich KJ, β-Adrenergic relaxation of dog trachealis: Contractile agonist-specific interaction. *J Appl Physiol* **67:** 181–185, 1989.
23. Gunst SJ, Stropp JQ and Flavahan NA, Muscarinic receptor reserve and β-adrenergic sensitivity in tracheal smooth muscle. *J Appl Physiol* **67:** 1294–1298, 1989.
24. Roffel AF, Meurs H, Elzinga CR and Zaagsma J, No evidence for a role of muscarinic M_2 receptors in functional antagonism in bovine trachea. *Br J Pharmacol* **115:** 665–671, 1995.
25. Fernandes LB, Fryer AD and Hirshman CA, M_2 muscarinic receptors inhibit isoproterenol-induced relaxation of canine airway smooth muscle. *J Pharmacol Exp Ther* **262:** 119–126, 1992.
26. Watson N and Eglen RM, Effects of muscarinic M_2 and M_3 receptor stimulation and antagonism on responses to isoprenaline of guinea-pig trachea *in vitro*. *Br J Pharmacol* **112:** 179–187, 1994.
27. Watson N, Reddy H and Eglen RM, Role of muscarinic M_2 and

M_3 receptors in guinea-pig trachea: Effects of receptor alkylation. *Eur J Pharmacol* **278:** 195–201, 1995.
28. Emmerson J and Mackay D, The zig-zag tracheal strip. *J Pharm Pharmacol* **31:** 798, 1979.
29. Coleman RA, Methods in prostanoid receptors classification. In: *Prostaglandins and Related Substances* (Eds. Benedetto E, McDonald-Gibson RG, Nigam S and Slater TF), pp. 267–301. IRL Press, Oxford, 1987.
30. Kamikawa Y, Uchida K and Shimo Y, Heterogeneity of muscarinic receptors in the guinea pig esophageal muscularis mucosae and ileal longitudinal muscle. *Gastroenterology* **88:** 706–716, 1985.
31. Watts SW and Cohen ML, Time-dependent improvement in guinea pig tracheal contractility: Modification by cyclooxygenase inhibitors. *J Pharmacol Exp Ther* **266:** 950–957, 1993.
32. Muccitelli R, Tucker SS, Hay DWP, Torphy TJ and Wasserman MA, Is the guinea pig isolated trachea a good *in vitro* model of human large and central airways? Comparison of leukotriene-, methacholine-, histamine- and antigen-induced contractions. *J Pharmacol Exp Ther* **243:** 467–473, 1987.
33. Undem BJ and Adams GK, An analysis of the functional interactions of selected contractile agonists in the guinea pig isolated trachea. *J Pharmacol Exp Ther* **246:** 47–53, 1988.
34. Small RC, Good DM, Dixon JS and Kennedy I, The effects of epithelium removal on the actions of cholinomimetic drugs in opened segments and perfused tubular preparations of guinea pig trachea. *Br J Pharmacol* **100:** 516–522, 1990.
35. Mansour S and Daniel EE, Maintenance of tone, role of arachidonate metabolites, and effects of sensitization in guinea pig trachea. *Can J Physiol Pharmacol* **64:** 1096–1103, 1986.
36. Paton WDM, A theory of drug action based on the rate of drug receptor combination. *Proc R Soc Lond B Biol Sci* **154:** 21–69, 1961.
37. Thomas EA, Hsu HH, Griffin MT, Hunter AL, Luong T and Ehlert FJ, Conversion of *N*-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP mustard) to an aziridinium ion and its interaction with muscarinic receptors in various tissues. *Mol Pharmacol* **41:** 718–726, 1992.
38. Monferini E, Giraldo E and Ladinsky H, Characterization of the muscarinic receptor in the rat urinary bladder. *Eur J Pharmacol* **147:** 453–458, 1988.
39. Lambrecht G, Feifel R, Wagner-Roser M, Strohmann C, Zilch H, Tacke R, Waelbroeck M, Christophe J, Boddecke H and Mutschler E, Affinity profiles of hexahydro-sila-difenidol analogues at muscarinic receptor subtypes. *Eur J Pharmacol* **168:** 71–78, 1989.
40. Pedder KE, Eveleigh P, Pouner D, Hulme E and Birdsall NJM, Modulation of the structure-binding relationships of antagonists for muscarinic acetylcholine receptor subtypes. *Br J Pharmacol* **103:** 1561–1567, 1991.
41. Kashihara K, Varga EV, Waite SL, Roeske WR and Yamamura HI, Cloning of the rat M3, M4 and M5 muscarinic acetylcholine receptor genes by the polymerase chain reaction (PCR) and the pharmacological characterization of the expressed genes. *Life Sci* **51:** 955–971, 1992.
42. Eglen RM, Harris GC, Cox H, Sullivan AO, Stefanich E and Whiting RL, Characterization of the interaction of the cervane alkaloid, imperialine, at muscarinic receptors *in vitro*. *Naunyn Schmiedebergs Arch Pharmacol* **346:** 144–151, 1992.
43. Ringdahl B, Structural requirements for muscarinic receptor occupation and receptor activation by oxotremorine analogs in the guinea-pig ileum. *J Pharmacol Exp Ther* **232:** 67–73, 1985.
44. Katada T and Ui M, Direct modification of the membrane adenylate cyclase system by islet activating protein due to ADP-ribosylation of a membrane protein. *J Biol Chem* **257:** 7210–7216, 1982.
45. Eglen RM, Huff MM, Montgomery WW and Whiting RL, Differential effects of pertussis toxin and lithium on muscarinic responses in the atria and ileum: Evidence for receptor heterogeneity. *Br J Pharmacol* **91:** 6–8, 1987.
46. Kurose H, Katada T, Amano T and Ui M, Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via α-adrenergic, cholinergic, and opiate receptors in neuroblastoma × glioma hybrid cells. *J Biol Chem* **258:** 4870–4875, 1983.
47. Lambrecht G, Feifel R, Forth B, Strohmann C, Tacke R and Mutschler E, *p*-Fluoro-hexahydro-sila-difenidol: The first $M_2\beta$-selective muscarinic antagonist. *Eur J Pharmacol* **152:** 193–194, 1988.
48. Eglen RM, Michel AD, Montgomery WW, Kunysz EA, Machado CA and Whiting RL, The interaction of *para*fluorohexahydrosiladiphenidol at muscarinic receptors *in vitro*. *Br J Pharmacol* **99:** 637–642, 1990.
49. Eglen RM, Cornett CM and Whiting RL, Interaction of p-F-HHSiD (*p*-fluro-hexahydrosila-difenidol) at muscarinic receptors in guinea-pig trachea. *Naunyn Schmiedebergs Arch Pharmacol* **342:** 394–399, 1990.
50. Jones CA, Madison JM, Tom-Moy M and Brown JK, Muscarinic cholinergic inhibition of adenylate cyclase in airway smooth muscle. *Am J Physiol* **253:** C97–C104, 1987.
51. Sankary RM, Jones CA, Madison JM and Brown JK, Muscarinic cholinergic inhibition of cyclic AMP accumulation in airway smooth muscle: Role of a pertussis toxin-sensitive protein. *Am Rev Respir Dis* **138:** 145–150, 1987.