M₂ Muscarinic Receptors Inhibit Forskolin- but not Isoproterenol-Mediated Relaxation in Bovine Tracheal Smooth Muscle

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Accepted for publication March 16, 1998 This paper is available online at http://www.jpet.org

ABSTRACT
The ability of the M₂ muscarinic receptor to inhibit the relaxant effects of forskolin and isoproterenol was investigated in bovine trachea. In most experiments, we measured contractile responses to oxotremorine-M in smooth muscle isolated from bovine trachea in which a majority of M₃ receptors were inactivated by treatment with N-(2-chloroethyl)-4-piperidinyl diphenylacetate. In the presence of histamine (20 μM), the histamine H₂ antagonist cimetidine (10 μM) and forskolin (4 μM), responses to oxotremorine-M were antagonized by [2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3b][1,4]benzodiazepine-6-one (1 μM) in a manner consistent with contractions mediated predominantly by M₃ receptors. When similar experiments were conducted in the presence of isoproterenol (0.1 μM) instead of forskolin, contractions were antagonized in a manner consistent with an M₂ receptor-mediated response. In similar experiments, we measured the relaxant potency of isoproterenol and forskolin against histamine-induced contractions in N-(2-chloroethyl)-4-piperidinyl diphenylacetate-treated trachea. By itself, oxotremorine-M (7.5 nM) had no contractile effect; however, it caused a substantial reduction in the relaxant potency of forskolin although having little effect on that of isoproterenol. These experiments establish that M₂ receptors inhibit the relaxant effects of forskolin, but not isoproterenol. In untreated tissues, the relaxant responses to isoproterenol and forskolin were 10.8- and 14.2-fold more potent, respectively, against histamine than against oxotremorine-M-induced contractions of equal magnitude. Similarly, the maximal stimulation of cAMP accumulation elicited by isoproterenol and forskolin was inhibited 58 and 62%, respectively, in the presence of oxotremorine-M (80 nM) compared to that measured in the presence of histamine (20 μM). Analysis of the data indicated that isoproterenol elicited relaxation at concentrations well beyond those that stimulated maximal levels of cAMP accumulation. Our results indicate that part of the relaxant response to isoproterenol is mediated through a non-cAMP-dependent mechanism, and that this mechanism is largely unopposed by the M₂ receptor.

Abbreviations: cAMP, adenosine 3’, 5’ cyclic monophosphate; 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; IBMX, isobutylmethylxanthine; KRB buffer, Krebs ringer bicarbonate buffer.

Received for publication October 29, 1997.

This work was supported by National Institutes of Health Grant NS 30882.

Muscarinic receptor populations in mammalian smooth muscle of the gastrointestinal tract and the airways are known to consist of approximately 80% M₂ and 20% M₃ receptors (Candell et al., 1990; Gies et al., 1989; Haddad et al., 1991; Maeda et al., 1988). M₃ receptors mediate direct contractions of smooth muscle and couple to the G₃ family of G proteins causing activation of phospholipase C-β (Candell et al., 1990; Roffel et al., 1990; Yang et al., 1991). The more predominant M₂ receptor is known to mediate inhibition of adenylyl cyclase through the pertussis toxin-sensitive G protein, Gᵣ, in airway smooth muscle and in cells transfected with the M₂ subtype (Kurose et al., 1983; Sankary et al., 1988). Conversely, beta adrenergic agonists stimulate adenylyl cyclase through the cholera toxin-sensitive G protein, Gₐ, and induce relaxation of smooth muscle via cAMP-dependent activation of protein kinase A. M₂ receptors have been shown to mediate contractions by inhibiting the relaxant effects of isoproterenol or the direct adenylyl cyclase activator, forskolin, in smooth muscle of the gastrointestinal tract (Reddy et al., 1995; Thomas et al., 1993). These studies used treatment of tissues with 4-DAMP mustard to inactivate a majority of the M₂ receptors selectively. Under this condition, responses elicited by a muscarinic agonist in the presence of histamine and either isoproterenol or forskolin were attributed to M₂ receptors through the use of selective antagonists. The contractile mechanism involved an M₂ receptor-mediated inhibition of the relaxant effect of isoproterenol or forskolin on histamine-induced contractions. Furthermore, the ability of muscarinic agonists to inhibit the relaxant effects of isoproterenol or forskolin has been correlated with M₂ receptor-mediated inhibition of stimulated cAMP accumulation (Ostrom and Ehlert, 1997).
The contractile role of the M₂ receptor is less clear in airway smooth muscle. Using the method of inactivating M₃ receptors with 4-DAMP mustard, Thomas and coworkers showed that the M₂ receptor inhibited the relaxant effect of forskolin on histamine-induced contractions of the guinea pig trachea (Thomas and Ehler, 1996). However, using a similar strategy, Watson et al. (1995) were unable to detect a role for the M₂ receptor when isoproterenol was used as the relaxant agent. One reason for this discrepancy may lie in the ability of forskolin to stimulate more cAMP than isoproterenol. Unfortunately, cAMP accumulation is difficult to measure in the small amount of smooth muscle of the guinea pig trachea, so this hypothesis remains untested. However, in equine trachea it was recently demonstrated that isoproterenol stimulated far less cAMP at equivalent levels of relaxation than did forskolin (Kume et al., 1994). These investigators also demonstrated that beta adrenergic receptors stimulate calcium-activated potassium channels through direct coupling to Gₛ. The M₂ receptor, however, appears to be capable of inhibiting this same channel directly via Gₛ irrespective of any effect on cAMP (Kume and Kotlikoff, 1991).

Our study was undertaken to investigate the contractile role of the M₂ receptor in bovine tracheal smooth muscle. The large size of the bovine trachea enabled us to measure both contraction and cAMP accumulation in a relatively homogeneous preparation of smooth muscle cells. We show that activation of M₂ receptors inhibits the increase in cAMP elicited by both forskolin and isoproterenol, and that this effect is due to a mechanism resistant to functional antagonism by the M₃ receptor.

Methods

cAMP accumulation. Whole trachea from steer were obtained at a local slaughterhouse (Shamrock Meats, Vernon, CA) and transported in ice-cold Krebs Ringer bicarbonate buffer (KRB buffer; 124 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 26 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.8 mM CaCl₂, 10 mM glucose) pregassed with O₂/CO₂ (19:1). A 2-inch length of the trachea was cut through the cartilaginous rings on the anterior side, and the mucosa carefully dissected to expose the underlying smooth muscle. The muscle was then removed and dissected free of all connective tissue. Large strips of muscle were chopped three times at 400 μm with a McIlwain tissue chopper, rotating the stage 60° between each pass. Slices were washed extensively and equilibrated at 37°C for 30 min. The tissue was incubated in 20 ml of KRB buffer with [³²]Hadenine (2.0 μM, 100 μCi for 40 min at 37°C to allow uptake and incorporation into endogenous ATP. Slices were washed three times to remove unincorporated [³²]Hadenine, and equilibrated for another 15 min. Aliquots (70 μl) of gently packed tissues slices were incubated for 10 min at 37°C in KRB buffer (0.7 ml) containing IBMX (0.5 mM), indomethacin (1 μM) and various agonists of interest. In some experiments, IBMX was omitted and the incubation time was reduced to 4 min. Reactions were stopped by the addition of 0.3 ml trichloroacetic acid (30% v/v). Approximately 2000 cpm of [³²]P]cAMP was added to each sample as an internal standard. The tubes were centrifuged at 2000 × g for 2 min, and the [³²]H]cAMP and [³²]H]ATP were separated from the supernatant fraction using the chromatography method of Salomon et al. (1974) and as described previously (Ostrom and Ehler, 1997).

Isolated trachealis muscle strips. Trachea were prepared as described above, except that small strips of smooth muscle (20 × 3 mm) were carefully cut as described by Hashjin et al. (1995) and mounted in an organ bath containing KRB buffer with indomethacin (1 μM) at 37°C gassed with O₂/CO₂ (19:1). In experiments where repeated concentration-response curves to oxotremorine-M were measured, the smooth muscle strips were incubated overnight at 37°C in modified Eagle’s media containing 100 μg/ml streptomycin, 10,000 U penicillin, 5% fetal bovine serum and 25 mM NaHEPES pH 7.4, and continually gassed with O₂/CO₂ (19:1). This length of incubation was necessary to eliminate spontaneous contractions and to increase reproducibility. Isometric contractions were measured with a force transducer and recorded on a polygraph (Grass Instruments, Quincy, MA). Data are expressed as the mass (grams) required to generate the measured force. Tracheal smooth muscle strips were equilibrated for 1 hr at a resting tension equivalent to a load of 1.0 g. Two test doses of the muscarinic agonist oxotremorine-M or histamine were added to the bath to ensure viability of the preparation. Between each test dose the strips were washed with fresh KRB buffer and incubated for 5 min. To calculate an EC₅₀ value for a compound, several concentrations, geometrically spaced every 0.33 log units, were added cumulatively to the bath and contractile responses were recorded. After data for an EC₅₀ value were obtained, the tissue was washed and incubated for 30 min before additional measurements were made. To obtain EC₅₀ values for relaxant agents such as isoproterenol and forskolin, tissues were first contracted with either histamine or oxotremorine-M and allowed to achieve a stable contraction. Then increasing concentrations of the relaxant agent were added, and the responses measured as a decrease in tension. In some experiments, tissues were incubated with the aziridinium ion of 4-DAMP mustard (40 nM) for a total of 2 hr in the presence of 1 μM AF-DX 116. After one hour, the tissue was washed and replenished with fresh 4-DAMP mustard and AF-DX 116. After 2 hr, tissues were washed extensively to remove AF-DX 116 and unreacted 4-DAMP mustard. When an EC₅₀ value for oxotremorine-M was measured in the presence of AF-DX 116, the antagonist was incubated with the tissue for 20 min before measurement of contractions.

Formation of the aziridinium ion of 4-DAMP mustard. When used, a solution of 4-DAMP mustard (100 μM) was first incubated in 10 mM phosphate buffer (pH 7.4) at 37°C for 30 min to allow formation of the reactive aziridinium ion (Thomas et al., 1992). Immediately after cyclization, the solution of the aziridinium ion was placed on ice and used immediately.

Data analysis. The EC₅₀ values of oxotremorine-M (concentration of oxotremorine-M required for half-maximal response) for contraction and inhibition of cAMP accumulation were estimated by nonlinear regression analysis of the data according to an increasing or decreasing logistic equation as described previously (Candell et al., 1990).

Compounds. 4-DAMP mustard was synthesized in our laboratory as described previously (Thomas et al., 1992). Radiolabeled chemicals were obtained from NEN Life Science Products (Boston, MA). AF-DX 116 was acquired from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). Triprolidine, cimetidine, thioperamide and oxotremorine-M were obtained from Research Biochemicals Incorporated (Natick, MA). Forskolin was obtained from Calbiochem (La Jolla, CA). All other drugs and chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Results

Contractile responses in 4-DAMP mustard-treated bovine tracheal smooth muscle. 4-DAMP mustard was used to inactivate M₃ receptors selectively so that responses through the M₂ receptor might be more easily detected. Tracheal smooth muscle strips were treated for 2 hr with 4-DAMP mustard (40 nM with 1 μM AF-DX 116) and washed repeatedly. This treatment inactivates most of the M₃ recep-
tors without affecting the M₂ receptors (Thomas et al., 1993). The contractile potency of oxotremorine-M was measured before and after 4-DAMP mustard treatment. This treatment reduced the potency of oxotremorine-M 11.5-fold, from an EC₅₀ value of 0.31 μM in untreated tissue to a value of 3.55 μM in treated tissue. This degree of shift of the contractile response to oxotremorine-M is consistent with inactivation of approximately 97% of the M₂ receptor population.

To investigate contractile effects of the M₂ receptor, we used a novel strategy previously developed in our laboratory for experiments in the guinea pig ileum (Thomas et al., 1993). Contractile responses to oxotremorine-M were measured in 4-DAMP mustard-treated ileum in the continued presence of histamine and one of the relaxant agents, isoproterenol or forskolin. When present together, histamine and the relaxant agent had no contractile effect. However, when these agents were present, ileal M₂ receptors mediated contractions to oxotremorine-M, presumably by inhibiting the relaxant effect of isoproterenol or forskolin on histamine-induced contraction. In the present studies, isolated tracheal smooth muscle was treated with 4-DAMP mustard before being contracted with histamine (10 μM). After a stable contraction was achieved, either isoproterenol (0.1 μM) or forskolin (4 μM) was added. When a stable relaxation was achieved, a cumulative concentration-response curve to oxotremorine-M was measured. Because a small proportion of the M₂ receptor population remains and can elicit contractile responses, this measurement was repeated in the presence of AF-DX 116 (1 μM) to characterize the subtype of the muscarinic receptor mediating the contraction. In tissues relaxed with isoproterenol, contractions to oxotremorine-M were antagonized 2.0-fold by AF-DX 116 (1 μM), whereas tissues relaxed with forskolin were antagonized 3.2-fold (fig. 1). These degrees of shift are consistent with M₂ receptor-mediated contractions, as the calculated pKᵦ values (6.00 and 6.34, respectively) are in close agreement with the binding affinity of AF-DX 116 for the cloned M₂ receptor (pKᵦ = 6.10) (Esqueda et al., 1996). In this latter study, the binding affinity of AF-DX 116 was measured in a modified KRB buffer similar to that used in our study.

The results shown in figure 1 provide no evidence for a contractile effect of the M₂ receptor. However, experiments described below indicate that under the experimental conditions of figure 1, histamine potentiates the cAMP accumulation elicited by forskolin and that this effect is blocked by the histamine H₂ antagonist, cimetidine. To eliminate the cAMP-stimulating effect of histamine, we repeated the experiments of figure 1 in the presence of cimetidine (10 μM). When isoproterenol was used as the relaxant agent under these new conditions, AF-DX 116 caused a small increase in maximal contraction and a 2.6-fold increase in the EC₅₀ value of oxotremorine-M; a shift that yields a calculated pKᵦ value of 5.78 to 6.20, a range still consistent with an M₂ receptor-mediated response (fig. 2A; table 1). However, when forskolin was used as the relaxant agent instead of isoproterenol, the potency of oxotremorine-M increased 7-fold in the control condition, and much higher levels of contraction were attained (fig. 2B; table 1). AF-DX 116 (1 μM) shifted the EC₅₀ value of oxotremorine-M 6.7-fold under this condition and reduced the maximal contraction by 24%. This shift by AF-DX 116 yields a calculated pKᵦ value of 6.76–7.16, a range that nearly includes the binding affinity of AF-DX 116 for cloned M₂ (pKᵦ = 7.27) but not M₃ (pKᵦ = 6.10) receptors (Esqueda et al., 1996).

**Inhibition of isoproterenol and forskolin relaxation in tissues treated with 4-DAMP mustard.** We used another approach to determine if M₂ receptors can inhibit the relaxant effects of isoproterenol and forskolin. The potency of isoproterenol and forskolin for relaxing a histamine-induced contraction was measured in tissues treated with 4-DAMP mustard then antagonized by a single, low concentration of oxotremorine-M (7.5 nM). This concentration of oxotremorine-M elicited large contractions in untreated tissues, but consistently elicited no response after treatment with 4-DAMP mustard. Any effect of oxotremorine-M under these conditions, can be attributed primarily to activation of M₂ receptors. Addition of oxotremorine-M caused a 1.8-fold decrease in isoproterenol relaxant potency, with the pEC₅₀ going from 9.68 to 9.43 (fig. 3A). However, forskolin relaxant potency was antagonized by oxotremorine-M 5-fold, with the pEC₅₀ going from 8.01 to 7.33 (fig. 3B). These results further indicate that the relaxant potency of forskolin, but not isoproterenol, is inhibited by M₂ receptor activation.

**Relaxant potency of isoproterenol and forskolin on muscarinic and histamine-induced contractions.** We investigated the ability of isoproterenol and forskolin to inhibit contractions elicited by either histamine (20 μM) or oxotremorine-M (80 nM) in strips of tracheal smooth muscle. Tension elicited by histamine and oxotremorine-M was of similar magnitude; namely 8.0 ± 2.7 and 7.45 ± 2.3 g, respectively (not significantly different, P = .502, paired t test). Cumulative addition of isoproterenol caused a complete in-

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**Fig. 1.** The effects of AF-DX 116 on the contractile response to oxotremorine-M in tissues treated with 4-DAMP mustard. Tracheal smooth muscle strips were contracted with histamine (10 μM) and relaxed with .1 μM isoproterenol (A) or 4 μM forskolin (B) before oxotremorine-M-induced contractions were measured. Control, ■ and 1 μM AF-DX 116, □. Dotted line denotes the level of precontraction elicited by histamine. In all experiments, tissues were treated with 4-DAMP mustard as described in “Methods.” Each point represents the mean ± S.E.M. of four experiments.
The inhibition of both histamine- and oxotremorine-M-induced contractions, although the potency of isoproterenol was 10.8-fold less against oxotremorine-M (fig. 4A; table 2). Over the concentration range investigated, forskolin caused a complete inhibition of histamine-induced contractions, but only a partial inhibition of oxotremorine-M-induced contractions. The concentration of forskolin causing 50% inhibition of contraction was 14.2-fold greater in the presence of oxotremorine-M as compared to that measured in the presence of histamine (fig. 4B; table 2).

Table 2 also contains estimates of the pEC$\text{_{50}}$s for isoproterenol and forskolin relaxation when contractions were elicited with threshold concentrations of histamine or oxotremorine-M. These lower concentrations of contractile agents elicited nearly equal levels of contraction as the higher concentrations used above (oxotremorine-M 8.14 ± 1.44 g, histamine 6.36 ± 0.98 g). This behavior was due to a greater maximum contractile response of the tissues used in these latter experiments (as compared to those used above) as well as a large threshold response characteristic of these strips of tracheal smooth muscle. Nonetheless, the potency of isoproterenol and forskolin increased between 50- and 200-fold, with the lower concentration of histamine and oxotremorine-M. Moreover, both relaxant agents still exhibited 10- to 20-fold greater relaxant potency against histamine- as compared to oxotremorine-M-induced contractions. In guinea pig trachea, the differential relaxant potency of isoproterenol is...
The presence of oxotremorine-M (pEC50-dependent manner in the presence of both agonists (fig. 5A). Isoproterenol increased cAMP accumulation in a concentration-whereas oxotremorine-M inhibited it (0.69 106 alone slightly enhanced cAMP accumulation (1.26 105). Both the H1 (triprolidine, 10 µM) selective histamine antagonists had no effect on cAMP accumulation while having virtually no effect on basal cAMP levels by itself.

Isoproterenol and forskolin stimulation of cAMP in the presence of histamine or oxotremorine-M. To investigate the role of cAMP in mediating relaxation, we measured cAMP levels in trachea under the conditions of the experiments just described in figure 3. For these experiments, the ability of isoproterenol and forskolin to stimulate cAMP accumulation in the presence of either histamine (20 µM) or oxotremorine-M (80 nM) was measured (fig. 5). Basal cAMP was 0.97 ± 0.13% when expressed as percent conversion of [3H]adenine labeled nucleotides into [3H]cAMP. Histamine alone slightly enhanced cAMP accumulation (1.26 ± 0.11%) whereas oxotremorine-M inhibited it (0.69 ± 0.08%). Isoproterenol increased cAMP accumulation in a concentration-dependent manner in the presence of both agonists (fig. 5A). However, its potency and maximal response were lower in the presence of oxotremorine-M (pEC50 = 7.90; Emax = 1.90%) as compared to that measured in the presence of histamine (pEC50 = 8.21; Emax = 4.53%). Forskolin-stimulated cAMP accumulation was also diminished when measured in the presence of oxotremorine-M as compared to histamine (fig. 5B). It is difficult to estimate the potency of these responses since no maximum was obtained with the concentrations of forskolin studied. However, over the range of concentrations used, the concentration-effect curve of forskolin shifted to the right approximately 80-fold in the presence of oxotremorine-M. We also noted that histamine itself caused a sizable potentiation of forskolin-stimulated cAMP accumulation while having virtually no effect on basal cAMP levels by itself.

To characterize this effect of histamine further, experiments were carried out using histamine receptor subtype-specific antagonists. Histamine (20 µM) caused a 2.1-fold increase in the cAMP accumulation elicited by forskolin (4 µM). Both the H3 (triprolidine, 10 µM) and H5 (thioperamide, 10 µM) selective histamine antagonists had no effect on this cAMP response to histamine. In contrast, the H2 selective antagonist (cimetidine, 10 µM) reduced the histamine potentiation of forskolin-stimulated cAMP to non-significant levels (by paired t test) over forskolin alone (data not shown).

Muscarinic inhibition of isoproterenol- and forskolin-stimulated cAMP accumulation. The highly efficacious muscarinic agonist oxotremorine-M inhibited cAMP accumulation in slices of bovine tracheal smooth muscle stimulated by either isoproterenol (0.1 µM) or forskolin (4 µM) in a concentration-dependent manner (fig. 6). Isoproterenol stimulated basal cAMP from 0.51 ± 0.07% conversion to 2.82 ± 0.31%, although forskolin stimulated basal cAMP to 2.70 ± 0.56%. The potency (pEC50) of oxotremorine-M against isoproterenol and forskolin-stimulated cAMP was 8.00 and 7.77, respectively. Of the amount of cAMP stimulated by isoproterenol over basal levels, oxotremorine-M inhibited only 48.9% (Emax). The maximum inhibition of forskolin-stimulated cAMP by oxotremorine-M was 81.7% (table 3). These proportions are very similar to that previously observed in guinea pig ileum, where oxotremorine-M maximally inhibited 38% of isoproterenol-stimulated cAMP accumulation and 70% of forskolin-stimulated cAMP accumulation (fig. 7) (Ostrom and Ehler, 1997).

Relationship between cAMP accumulation and relaxation for isoproterenol and forskolin. Figure 8 shows the relationship between cAMP production and relaxation for isoproterenol and forskolin in the presence of either histamine or oxotremorine-M. Conditions were kept identical for both measurements, except that in A and B, IBMX (0.5 mM) was present in the cAMP determinations. Figure 8A shows the relationship between cAMP and inhibition of histamine-induced contractions for isoproterenol and forskolin. It can be seen that for any level of relaxation, the amount of cAMP generated by isoproterenol is much less than that stimulated by forskolin, especially at high levels of relaxation (>60%). Similar results were obtained when oxotremorine-M was used as the contractile agent (B), although the discrepancy in the cAMP-relaxation relationship for isoproterenol and for

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**Table 2**

<table>
<thead>
<tr>
<th>Drug</th>
<th>High Concentrations*</th>
<th>Low Concentrations*</th>
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<tbody>
<tr>
<td></td>
<td>vs. Hist (pEC50)</td>
<td>vs. Oxo-M (pEC50)</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>8.01 ± 0.14</td>
<td>7.00 ± 0.20</td>
</tr>
<tr>
<td>Forskolin</td>
<td>6.21 ± 0.01</td>
<td>5.52 ± 0.24</td>
</tr>
</tbody>
</table>

* Tissues were contracted with 20 µM histamine or 80 nM oxotremorine-M (high concentrations), or 1.5 µM histamine and 7.5 nM oxotremorine-M (low concentrations).

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![Fig. 5](image-url) Concentration-response curves for stimulation of cAMP accumulation by isoproterenol (A) or forskolin (B) in the presence of 20 µM histamine (□) or 80 nM oxotremorine-M (○). Data are presented as the percent conversion of [3H]ATP to [3H]cAMP. Each point represents the mean ± S.E.M. of five experiments.
isomer on levels of relaxation of more than 40%. C shows the relationship between relaxation and cAMP accumulation in the presence of oxotremorine-M when IBMX was not included in the cAMP determinations. The potencies of isoproterenol and forskolin at stimulating cAMP accumu-

![Fig. 6](image)

**Fig. 6.** Oxotremorine-M inhibition of cAMP accumulation stimulated by 0.1 μM isoproterenol, (■) or 4 μM forskolin (□). Basal level of cAMP accumulation is represented by hatched bar and horizontal line. Data are presented as the percent conversion of [3H]ATP to [3H]cAMP. Each bar or point represents the mean ± S.E.M. of four experiments.

**Table 3**

Estimates of the pEC50 values and maximal inhibitory effects of oxotremorine-M inhibition on isoproterenol- and forskolin-stimulated cAMP accumulation

<table>
<thead>
<tr>
<th>Condition</th>
<th>Oxotremorine-M</th>
<th>pEC50 ± S.E.M.</th>
<th>Emax ± S.E.M.</th>
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<tbody>
<tr>
<td>Isoproterenol (0.1 μM)</td>
<td>8.00 ± 0.22</td>
<td>48.9%</td>
<td></td>
</tr>
<tr>
<td>Forskolin (4 μM)</td>
<td>7.77 ± 0.13</td>
<td>81.7%</td>
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*Calculated as the average and S.E.M. of the pEC50 from each individual experiment.

Maximal effect is the percent inhibition of stimulated cAMP after subtraction of basal.

kolin only occurred at levels of relaxation of more than 40%. C shows the relationship between relaxation and cAMP accumulation in the presence of oxotremorine-M when IBMX was not used in the cAMP determinations. The potencies of isoproterenol and forskolin at stimulating cAMP accumula-

**Discussion**

In our study, we used 4-DAMP mustard to inactivate a majority of the M₂ receptors selectively, inducing a large rightward shift of the direct contractile response elicited by this receptor subtype. We then precontracted the tissue with a heterologous contractile agent (histamine) and induced relaxation with a cAMP-stimulating agent (either isoproterenol or forskolin) before measuring contractile responses to oxotremorine-M. When present together, histamine and the relaxant agent have no contractile effect because the latter acts to inhibit the histamine-induced contraction. However, under these conditions, the M₂ receptor has been shown to mediate contraction because its inhibition of the cAMP-stimulating effect of the relaxant agent essentially releases the brake on the original histamine contraction. Subtype-selective muscarinic antagonists have confirmed that contractile responses elicited by muscarinic agonists in this way are mediated by the M₂ receptor of the guinea pig ileum and colon when either forskolin or isoproterenol are used as relaxant agents (Reddy *et al.*, 1995; Thomas *et al.*, 1993; Sawyer and Ehlert, 1998). In the guinea pig trachea, the M₂ receptor inhibits the relaxant effect of forskolin, but not isoproterenol, on histamine-induced contractions (Thomas and Ehlert, 1996; Watson *et al.*, 1995). Finally, in the rat esophageal muscularis mucosa, the M₂ receptor elicits contractions by inhibiting the relaxant effect of serotonin and isoproterenol on contractions elicited by the thromboxane receptor agonist, U46619 (Eglen *et al.*, 1996).

When we used this strategy on the bovine trachea in the present study, no evidence for involvement of M₂ receptors was obtained, no matter which relaxant agent was present (isoproterenol or forskolin). However, we noted that histamine potentiated forskolin-stimulated cAMP accumulation and that this effect was mediated by the H₂ receptor. Therefore, we added the H₂ antagonist, cimetidine (10 μM), to the experimental paradigm described above to reduce the large cAMP response of forskolin in the presence of histamine. In this new condition, the M₂ receptor mediated part of the contractile response in the presence of forskolin. We postulate that the M₂ receptor was unable to inhibit enough of the adenyl cyclase activity when histamine was allowed to potentiate forskolin-stimulated cAMP accumulation. When this potentiation was blocked by cimetidine, oxotremorine-M had sufficient efficacy to contribute to the contraction through the M₂ receptor. The increased potency of oxotremorine-M when cimetidine was included is evidence of the activation of a new receptor population. The addition of cimetidine did not alter the outcome of these experiments in the presence of isoproterenol, presumably because histamine did not potentiate cAMP stimulated by this agent.

There is a precedence for small numbers of H₂ receptors in smooth muscle coupled to stimulation of adenyl cyclase (Brink, 1987). We previously reported that dimaprit, an H₂ receptor-specific agonist, stimulated cAMP accumulation to moderate levels in guinea pig ileal smooth muscle (Ostrom and Ehlert, 1997). We have also observed similar effects in guinea pig colon (Sawyer GW and Ehlert FJ, unpublished)
Furthermore, it has been reported that certain isozymes of adenylyl cyclase (particularly type II) expressed in Sf9 cells exhibit marked potentiation of forskolin-stimulated adenylyl cyclase activity in the presence of exogenously added Gs (Sutkowski et al., 1994). Perhaps H2 receptor-mediated activation of Gs potentiates forskolin-stimulated adenylyl cyclase activity in bovine tracheal smooth muscle. It is further possible that \( \beta \gamma \) subunits liberated from stimulation of H2 receptors cause this potentiation of adenylyl cyclase (Thomas and Hoffman, 1996).

Two factors may account for the inability of the M2 receptor to inhibit isoproterenol-induced relaxation. First, isoproterenol appeared to mediate relaxation through a non-cAMP-dependent mechanism (see below). If the M2 receptor had no means of inhibiting this relaxant pathway then it would not play a role in cholinergic contractions in the presence of isoproterenol. Second, the cAMP accumulation that was stimulated by isoproterenol was more resistant to inhibition by a muscarinic agonist. Oxtremorine-M was capable of inhibiting 82% of cAMP stimulated by forskolin, but only 49% of that stimulated by isoproterenol. However, a similar situation exists in the guinea pig ileum where oxtremorine-M (1.0 \( \mu \)M) inhibits 70 and 38% of the cAMP accumulation elicited by forskolin and isoproterenol, respectively. This similarity between these tissues is illustrated in figure 7. In the guinea pig ileum, however, activation of M2 receptors inhibits the relaxant response of both forskolin and isoproterenol. Therefore, it is unlikely that the lack of a contractile role of the M2 receptor observed in our studies is due to its inability to inhibit isoproterenol-stimulated cAMP.

Figure 8 shows the relationship between cAMP levels and relaxation for both isoproterenol and forskolin when contraction is initiated with histamine (A) or oxtremorine-M (B). If the relaxant responses of both isoproterenol and forskolin are mediated solely through cAMP, then the relationship between cAMP and relaxation should be the same for both agents. However, the plots for isoproterenol indicate that relaxation continued after cAMP could be stimulated no further. In the presence of oxtremorine-M, in fact, the relaxant response elicited by isoproterenol increased from 35 to 97% although cAMP levels remained constant at approximately 1.4% conversion (B). The plot of relaxation vs. cAMP levels curves upward and attains a vertical slope throughout most of the plot. Thus, most of the relaxation elicited by isoproterenol is independent of cAMP when cAMP levels are inhibited by oxtremorine-M. In contrast, forskolin caused a gradual increase in both cAMP and relaxation, with the maximal relaxant response reaching a plateau at high levels of cAMP (fig. 8A). This behavior is consistent with a causal relationship between cAMP and relaxation. To eliminate the possibility that the presence of a phosphodiesterase inhibitor did not artificially create this relationship between cAMP and relaxation, we measured isoproterenol- and forskolin-stimulated cAMP accumulation in the presence of oxtremorine-M (80 nM) without IBMX present (table 4). The relationship between cAMP and relaxation is not altered under this new condition (fig. 8C).

These data can be rationalized by the model shown in figure 9. Accordingly, the beta adrenergic receptor activates

---

**TABLE 4**

<table>
<thead>
<tr>
<th>Drug</th>
<th>( \text{pEC}_{50} ) ± S.E.M.</th>
<th>( E_{\text{max}}^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol</td>
<td>7.75 ± 0.03</td>
<td>0.20%</td>
</tr>
<tr>
<td>Forskolin</td>
<td>4.67 ± 0.06</td>
<td>13.5%</td>
</tr>
</tbody>
</table>

\( ^a \) Maximal effect is expressed as percent conversion of cAMP for the average curve.

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**Fig. 8.** Comparison of cAMP accumulation and relaxant activity for isoproterenol (square symbols) and forskolin (round symbols) in the presence of either 20 \( \mu \)M histamine (A) or 80 nM oxtremorine-M (B). cAMP accumulation data in the presence of 80 nM oxtremorine-M was also obtained without using IBMX (C). Each data point represents the cAMP response (percent conversion with basal subtracted) and the relaxant response (percent relaxation of the developed contraction) of equivalent concentrations of isoproterenol or forskolin. Mean of three to five experiments.

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**Fig. 9.** Schematic diagram illustrating the effector mechanisms of isoproterenol and forskolin. See “Discussion” for a more complete description.
G, which in turn activates adenyl cyclase. However, we postulate that G, has an alternate more effective means of causing relaxation. This pathway is shown by the more prominent arrow. In contrast, forskolin acts downstream at the level of adenyl cyclase; consequently, it can only mediate relaxation through cAMP. This model provides an explanation for the ability of the M2 receptor to mediate an inhibition of forskolin-induced relaxation without affecting isoproterenol-induced relaxation. The model does not rule out the possibility that the beta adrenergic receptor can elicit relaxation through cAMP, but rather, it postulates that there is an alternate, more effective mechanism.

Torphy (1994) has previously suggested that the beta adrenergic receptor may mediate a cAMP-independent relaxation in the trachea and that the mechanism might involve a stimulation of Ca++ activated K+ channels (K Ca channels) via G, (see Kume et al., 1994; Kume et al., 1995). This mechanism could explain the lack of effect of the M2 receptor in antagonizing beta adrenergic receptor induced relaxation, if the M2 receptor has no means of inhibiting the Ca++ activated potassium conductance. However, Kume and coworkers have demonstrated that beta adrenergic activation of K Ca channels is inhibited by muscarinic receptor activation in a pertussis toxin-sensitive manner (Kume and Kotlikoff, 1991). These data demonstrate both a direct activation of the K Ca channel by G, and a direct inactivation by G, and suggest an additional mechanism for functional antagonism between beta adrenergic and M2 muscarinic receptors. However, if beta adrenergic activation of K Ca channels is the non-cAMP-dependent mechanism in question, then the M2 receptor should participate in inhibiting relaxation by isoproterenol. Because we did not observe this functional antagonism, it may be that the inhibition of K Ca channels by muscarinic agonists exhibits low potency, leaving a large portion of the beta adrenergic activation of this channel unopposed. An alternative explanation is that yet another, unknown pathway is mediating the non-cAMP-dependent relaxation of the beta adrenergic receptor.

The question arises as to why isoproterenol is less potent at inhibiting oxotremorine-M-induced contractions in the trachea as compared to those elicited by an equieffective concentration of histamine. In the guinea pig ileum, we have shown that M2 receptor-mediated inhibition of cAMP levels can account for this difference in relaxant potency (Ostrom and Ehlerdt, 1997). However, in bovine trachea, the relaxant response to isoproterenol appears to be largely independent of cAMP, and the M2 receptor does not oppose the relaxant effect of isoproterenol (see above). This situation directs attention to the M3 receptor in bovine tracheal smooth muscle. Indeed, when M3 receptors are inactivated by treatment with 4-DAMP mustard, isoproterenol relaxation of histamine contractions is almost unaffected by a low concentration of oxotremorine-M (fig. 3). Forskolin relaxation in these same conditions, however, is still significantly antagonized by oxotremorine-M. Therefore, M2 receptor activation is responsible for the observed differential potency of isoproterenol in relaxing histamine vs. muscarinic induced contractions, while M2 and, presumably, M3 receptors are both at work causing this differential potency of forskolin.

Rofil and coworkers (1995) have previously proposed that the relaxant response to isoproterenol in the trachea is more greatly inhibited by M3 receptor-mediated activation of PLC as compared to that activated by histamine. Although the maximum contractile and phosphoinositide responses to muscarinic agonists are greater than those of histamine, equieffective concentrations of these agonists have been shown to elicit the same degree of Ca++ mobilization (Hoiting et al., 1996). It is unclear why isoproterenol would exhibit differential relaxant effects in the presence of equieffective concentrations of histamine and a muscarinic agonist. Perhaps the muscarinic agonist elicits a greater contractile “signal” (e.g., phosphoinositides) than histamine even at equivalent levels of contraction, giving the relaxant agent more to overcome.

The ability of the M2 receptor to oppose the relaxant effect of forskolin has relevance to situations in which forskolin or highly selective phosphodiesterase inhibitors are used to induce bronchial relaxation. However, the inability of the M2 receptor to oppose the relaxation elicited by isoproterenol raises the question of the physiological function of bronchial M2 muscarinic receptors. Evidence for a contractile role of these receptors has been obtained in Basenji-greyhound dog (Emala et al., 1995). This strain exhibits impaired relaxation of bronchial smooth muscle and, interestingly, they express higher levels of tracheal M2 receptors as compared to mongrel dogs.

In summary, our studies indicate that the M2 receptor can functionally antagonize the relaxant actions of forskolin, but not isoproterenol in bovine tracheal smooth muscle. Oxotremorine-M is able, however, to inhibit the cAMP stimulated by both agents. We also find substantial evidence that isoproterenol mediates relaxation via mechanisms independent of cAMP and protein kinase A activation, possibly explaining the inability of the M2 receptor to inhibit its relaxant effects.

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