ABSTRACT

The ability of the M₂ muscarinic receptor to mediate an inhibition of the relaxant effects of forskolin and isoproterenol was investigated in guinea pig ileum and trachea. In some experiments, trachea was first treated with 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) mustard to inactivate M₃ receptors. The contractile response to oxotremorine-M was measured subsequently in the presence of both histamine (10 μM) and isoproterenol (10 nM). Under these conditions, [2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3b]-[1,4]benzodiazepine-6-one (AF-DX 116) antagonized the contractile response to oxotremorine-M in a manner consistent with an M₂ mechanism. However, when the same experiment was repeated using forskolin (4 μM) instead of isoproterenol, the response to oxotremorine-M exhibited greater potency and was antagonized by AF-DX 116 in a manner consistent with an M₃ mechanism. We also measured the effects of pertussis toxin treatment on the ability of isoproterenol to inhibit the contraction elicited by a single concentration of either histamine (0.3 μM) or oxotremorine-M (40 nM) in both the ileum and trachea. Pertussis toxin treatment had no significant effect on the potency of isoproterenol for inhibiting histamine-induced contractions in the ileum and trachea. In contrast, pertussis toxin treatment enhanced the relaxant potency of isoproterenol against oxotremorine-M-induced contractions in the ileum but not in the trachea. Also, pertussis toxin treatment enhanced the relaxant potency of forskolin against oxotremorine-M-induced contractions in the ileum and trachea. We investigated the relaxant potency of isoproterenol when very low, equi-effective (i.e., 20–34% of maximal response) concentrations of either histamine or oxotremorine-M were used to elicit contraction. Under these conditions, isoproterenol exhibited greater relaxant potency against histamine in the ileum but exhibited similar relaxant potencies against histamine and oxotremorine-M in the trachea. Following 4-DAMP mustard treatment, a low concentration of oxotremorine-M (10 nM) had no contractile effect in either the ileum or trachea. Nevertheless, in 4-DAMP mustard-treated tissue, oxotremorine-M (10 nM) reduced the relaxant potency of isoproterenol against histamine-induced contractions in the ileum, but not in the trachea. We conclude that in the trachea the M₂ receptor mediates an inhibition of the relaxant effects of forskolin, but not isoproterenol, and the decreased relaxant potency of isoproterenol against contractions elicited by a muscarinic agonist relative to histamine is not due to activation of M₃ receptors but rather to the greater contractile stimulus mediated by the M₃ receptor compared with the H₁ histamine receptor.

The smooth muscle of the airways and gastrointestinal tract abundantly expresses M₂ and M₃ muscarinic receptors in a ratio of approximately four to one (Maeda et al., 1988; Gies et al., 1989; Candell et al., 1990; Haddad et al., 1991). Several investigators have shown that it is the M₂ subtype that mediates contraction when isolated strips of airway and gastrointestinal smooth muscle are exposed to muscarinic agonists (Candell et al., 1990; Roffel et al., 1990; Yang et al., 1991). This mechanism can be explained by the coupling of the M₂ receptor to G proteins of the Gq family, which stimulate phosphoinositide hydrolysis and calcium mobilization (Peralta et al., 1988; Candell et al., 1990). The lack of involvement of the M₂ receptor in contraction can be explained by the coupling of this receptor to the Gi family of G proteins which, for the most part, does not cause a direct mobilization of calcium. The M₂ receptor has been shown to mediate an inhibition of adenyl cyclase when transfected into cells (Peralta et al., 1988). In tracheal and ileal smooth muscle, native M₂ receptors mediate an inhibition of adenyl cyclase activity in broken cell preparations (Sankary et al., 1988; Candell et al., 1990), and in intact cell preparations, the M₂ receptor mediates an inhibition of the cAMP accumulation elicited by forskolin and the beta adrenergic agonist, isoproterenol (Griffin and Ehlert, 1992; Ostrom and Ehlert, 1997; Ostrom and Ehlert, 1998). Because these latter agents cause relaxation of smooth muscle, it was suggested that the M₂ receptor may mediate an inhibition of the relaxant effect of cAMP stimulating agents, thereby facilitating the contractile response mediated by another contractile receptor, like the M₃ (Candell et al., 1990).

ABBREVIATIONS: 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine; AF-DX 116, [2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3b]-[1,4]benzodiazepine-6-one; IBMX, isobutylmethylxanthine; KRB, Krebs-Ringer bicarbonate buffer.
Such a role for the M2 receptor was first rigorously tested in a novel experimental paradigm designed to isolate the M2 facilitatory mechanism from the direct contractile mechanism of the M3 receptor (Thomas et al., 1993). In the first phase of this paradigm (Treatment Phase), smooth muscle is incubated with 4-diphenylacetoxycarbonyl-3-methylpiperidine (4-DAMP) mustard to inactivate M3 receptors selectively. In the second phase (Test Phase), smooth muscle is exposed to histamine followed by a cAMP-stimulating relaxant agent, like isoproterenol. The contractile effects of a muscarinic agonist are measured in the continued presence of histamine and isoproterenol. In combination, histamine and isoproterenol have no net contractile effect because their actions oppose one another. Under these conditions, activation of M2 receptors causes contraction, presumably by inhibiting the relaxant effect of isoproterenol on histamine-induced contractions. Subtype-selective competitive muscarinic antagonists have been used during the Test Phase to verify the M2 nature of the contractile response. Several investigators have used this approach in gastrointestinal smooth muscle to demonstrate that M2 receptors mediate an inhibition of the relaxant effects of both isoproterenol and forskolin on histamine-induced contractions (Thomas et al., 1993; Thomas and Ehler, 1994; Reddy et al., 1995). In the trachea of cows (Ostrom and Ehler, 1998) and guinea pigs (Thomas and Ehler, 1996), M2 receptors mediate an inhibition of the relaxant effects of forskolin on histamine-induced contractions; however, M2 receptors are unable to mediate an inhibition of the relaxant effect of isoproterenol on histamine-induced contractions (Watson et al., 1995; Ostrom and Ehler, 1998). Nevertheless, M2 receptors mediate an inhibition of the increase in cAMP elicited by isoproterenol in bovine trachea (Ostrom and Ehler, 1998). We have recently demonstrated the lack of a functional relationship between isoproterenol-induced relaxation and cAMP levels in bovine trachea, indicating that at least part of the relaxant mechanism for isoproterenol involves a non-cAMP mechanism (Ostrom and Ehler, 1998). This situation can explain the lack of a role of the M2 receptor in opposing isoproterenol-induced relaxation in the trachea.

The idea that the M2 receptor is unable to mediate an inhibition of the relaxant action of isoproterenol in bovine and guinea pig trachea appears to conflict with other empirical observations implicating such a function for the M2 receptor. For example, it has long been known that the relaxant potency of beta adrenergic agonists in airway smooth muscle is markedly decreased when measured against muscarinic agonist-induced contraction compared with that elicited by other contractile agents (e.g., histamine) (Russell, 1984; Van Amsterdam et al., 1989; Fernandes et al., 1992). The simultaneous interaction of a muscarinic agonist with both M2 and M3 receptors could account for the resistance of the muscarinic response to isoproterenol. This hypothesis is supported by the observation of Mitchell et al. (1993) that pertussis toxin treatment increases the relaxant potency of isoproterenol against acetylcholine-induced contractions in the canine trachea. Pertussis toxin treatment is known to uncouple M2 muscarinic receptor signaling mechanisms but not those of the M3 receptor.

In the present report, we used a variety of experimental conditions in the guinea pig trachea to investigate the ability of the M2 receptor to mediate an inhibition of the relaxant response to isoproterenol to clarify previous conflicting reports in this area of research. We show that activation of M2 receptors inhibits the relaxant effects of forskolin but not isoproterenol in guinea pig trachea under several conditions. Nevertheless, under the same conditions, M2 receptors in the guinea pig ileum are capable of mediating an inhibition of the relaxant effects of both isoproterenol and forskolin. Lastly, we demonstrate that the decreased relaxant potency of isoproterenol against muscarinic agonist-induced contractions in the trachea compared with histamine-induced contractions is due primarily to activation of M3 receptors.

Materials and Methods

Isolated Ileum. Animals were euthanized by asphyxiation with CO2 and the whole ileum was rapidly removed. The most distal 10 cm of ileum was discarded, and 2- to 3-cm ileal segments were cut, flushed with Krebs-Ringer bicarbonate (KRB) buffer to remove ileal contents and mounted longitudinally in an organ bath containing KRB buffer at 37°C gassed with O2/CO2 (19:1). Isometric contractions of the tissue were measured with a force transducer and recorded on a polygraph. All contractile responses are expressed as the mass (g) required to generate the recorded force. The ileum was allowed to equilibrate for 1 h at a resting tension equivalent to a load of 0.5 g. Three test doses of histamine or the muscarinic agonist, oxotremorine-M, were added to the bath to ensure reproducibility of the preparation. Ileal segments that did not achieve >60% of the maximum from the test doses were discarded. Between each test dose, the ileum was washed with fresh KRB buffer and incubated for 5 min. To calculate an EC50 value for a compound, 6 to 10 concentrations, spaced geometrically every 0.33 log units, were added cumulatively to the bath, and contractile responses were recorded. After a cumulative concentration-response curve was measured, the ileum was washed and incubated for 30 min before additional recordings were made. In some experiments, tissues were incubated with the aziridinium ion of 4-DAMP mustard (40 nM) for 1 h in the presence of [2-{[diethylamino)methyl]-1-piperidinyl}[acetyl]-5,11-dihydro-6H-pyrido[2,3-b]-[1,4]benzodiazepine-6-one (AF-DX 116) (1 μM). By itself, 4-DAMP mustard is moderately selective for M3 receptors over M2; however, this selectivity is enhanced by protection of the M3 receptor with AF-DX 116 (Thomas et al., 1992, 1993). Tissues were washed extensively to remove AF-DX 116 and untreated 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 ileum for 20 min before measurement of contractions.

Isolated Trachea. Animals were euthanized as described above, and the trachea was exposed by blunt dissection. Approximately 2 to 3 cm of trachea was dissected, and all adhering connective tissue was removed. The tube was then cut longitudinally on the ventral side, and the inner surface was rubbed with a cotton swab to remove the epithelium. Two zig-zag strips were prepared from each trachea by the method of Emmerson and Mackay (1979). The strips were mounted in an organ bath containing KRB buffer at 37°C gassed with O2/CO2 (19:1). Isometric contractions were measured with a force transducer and recorded on a polygraph. Trachea was equilibrated for 1 h at a resting tension equivalent to a load of 1.0 g. Test doses and cumulative dose-response curves were measured in the manner described above. In some experiments, tissues were incubated with 4-DAMP mustard as described above.

Treatment of Tissues with Pertussis Toxin. Pertussis toxin treatment of ilea was carried out in vivo by injecting animals i.p. with 100 μg/kg of pertussis toxin or an equivalent volume of saline (control). Animals were then euthanized 72 h later and the ilea dissected and prepared as described above. Tracheal smooth muscle is not effectively treated by pertussis toxin following i.p. injection of reasonable doses of the toxin. Therefore, trachea were treated in vitro by incubating the dissected and prepared tissue for 18 h in...
modified Eagle’s media containing penicillin/streptomycin, 5% fetal bovine serum, and 30 mM Na/HEPES buffer, pH 7.4, and either 5 μg/ml pertussis toxin that had been incubated with dithiothreitol (20 mM) for 30 min at 37°C, or an equivalent aliquot of dithiothreitol (20 mM) (control).

Formation of Aziridinium Ion of 4-DAMP Mustard. 4-DAMP mustard undergoes two sequential reactions in aqueous solution at neutral pH. The first of these is the cyclization to its reactive aziridinium ion, and the second is the hydrolysis of the aziridinium ion to the stable alcohol product. In all experiments in which it was used, 4-DAMP mustard (10 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). After cyclization, the solution was placed on ice and used immediately.

Data Analysis. The pEC50 values (negative log of the concentration required for half-maximal response) obtained for isoproterenol, forskolin, and oxotremorine-M in contractile assays were estimated by nonlinear regression analysis of the data according to an increasing or decreasing logistic equation as described previously (Candell et al., 1990). Observed pKb values were calculated using the following equation for competitive inhibition (Arunlakashana and Schild, 1959):

$$pK_b = -\log\left(\frac{B}{B^{\text{shift}} - 1}\right)$$

where B denotes the concentration of the antagonist used, and shift denotes the EC50 value of oxotremorine-M measured in the presence of the antagonist divided by that measured in its absence. In some instances, the maximal response to oxotremorine-M increased moderately (7–15%) in the presence of the competitive antagonist, AF-DX 116. In such instances, we also calculated the AF-DX 116-induced shift from the EC50 value of the control oxotremorine-M concentration-response curve and an equi-effective concentration of oxotremorine-M in the presence of AF-DX 116 on the basis of grams of tension. The two estimates of shift were then used to calculate two Kb values according to the above equation. These two Kb values are reported as a range in Table 1.

Compounds. Radiolabeled chemicals were obtained from NEN Life Science Products (Boston, MA). Oxotremorine-M was obtained from Research Biochemicals Inc. (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

Effects of AF-DX 116 on Contractile Response to Oxotremorine-M in 4-DAMP Mustard-Treated Trachea. To determine whether the M2 receptor mediates an inhibition of the relaxant response to isoproterenol, we used a strategy previously developed in our laboratory (see Thomas et al., 1993). First, we treated isolated tracheal strips for 1 h with 4-DAMP mustard (40 nM, with 1 μM AF-DX 116), which inactivates most of the M3 receptors while leaving M2 receptors largely unaffected (Thomas et al., 1993). Contractile responses to oxotremorine-M were then measured in the continuous presence of histamine (10 μM) and isoproterenol (0.1 μM), which together had no net contractile effect. Responses to oxotremorine-M elicited under these conditions in guinea pig ileum are mediated by M2 receptors, presumably due to this receptor inhibiting the relaxant effect of isoproterenol on the histamine-induced contraction. Responses to oxotremorine-M were then repeated in the presence of AF-DX 116 (1 μM) to characterize the muscarinic receptor subtype mediating the contraction. AF-DX 116 shifted the contractile-response curve of oxotremorine-M 1.4- to 1.6-fold to the right (Fig. 1 and Table 1). This degree of shift yields a calculated pKb range of 5.6 to 5.7, near the binding affinity of AF-DX 116 for the cloned M3 subtype (pKbD = 6.10), but not the M2 subtype (pKbD = 7.27) (Esqueda et al., 1996). These data indicate that the M2 receptor is unable to inhibit the relaxant effects of isoproterenol in the trachea, a finding that is in agreement with that previously published by Reddy et al. (1995).

For comparative purposes, we also included previously published data from our laboratory (Thomas and Ehlerl, 1996), in which forskolin (4 μM) was used as the relaxant agent instead of isoproterenol (Table 1). Under this condition, AF-DX 116 shifted the contractile-response curve to oxotremorine-M 5.0- to 9.0-fold to the right, yielding calculated pKb values from 6.60 to 6.90. This range of pKb values is in agreement with the binding affinity of AF-DX 116 in cells transfected with the M2 subtype, indicating that the M2 receptor mediates contraction in the trachea under these conditions.

Effects of Isoproterenol and Forskolin on Contractile Responses to Oxotremorine-M in Pertussis Toxin-Treated Trachea and Ileum. To assess the contractile role of the M2 receptor with a different approach, we used pertussis toxin treatment to ADP-ribosylate, the G protein involved in the M3 receptor signaling (Gq) (Kurose et al., 1983), and measured the ability of the relaxant agents isoproterenol and forskolin to antagonize the contractile responses to oxotremorine-M. If the M2 receptor inhibits the relaxant effects of a given agent, then pertussis toxin treatment would be expected to enhance the ability of that agent to antagonize oxotremorine-M-mediated contractions. A single concentration of isoproterenol or forskolin was used to antagonize oxotremorine-M-mediated contractions in both untreated and treated tissues.

### Table 1

<table>
<thead>
<tr>
<th>Relaxant Agent</th>
<th>Oxotremorine-M</th>
<th>Oxotremorine-M + AF-DX 116 (1.0 μM)</th>
<th>Shifta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol (0.1 μM)b</td>
<td>pEC50 = 5.72 ± 0.15</td>
<td>Emax = 1.51 ± 0.25</td>
<td>pEC50 = 5.52 ± 0.17</td>
</tr>
<tr>
<td>Forskolin (4.0 μM)c</td>
<td>pEC50 = 6.48 ± 0.21</td>
<td>Emax = 1.69 ± 0.28</td>
<td>pEC50 = 5.53 ± 0.22</td>
</tr>
</tbody>
</table>

a Denotes EC50 value of oxotremorine-M measured in the presence of indicated relaxant agent (i.e., isoproterenol or forskolin) divided by that measured in the absence of relaxant agent (i.e., control EC50 value).

b Data are from Fig. 1.

c Data are from Thomas and Ehlerl.

d Significantly different from 1.0, P < .05.
Effects of isoproterenol and forskolin on EC$_{50}$ value of oxotremorine-M (Oxo-M) for eliciting contractions in untreated and pertussis toxin-treated guinea pig trachea and ileum

<table>
<thead>
<tr>
<th>Tissue and Relaxant Agent</th>
<th>Untreated Tissue</th>
<th>Pertussis Toxin-Treated Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxo-M</td>
<td>Shift$^b$</td>
</tr>
<tr>
<td></td>
<td>pEC$_{50}$</td>
<td>fold</td>
</tr>
<tr>
<td>Trachea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.86 ± 0.10</td>
<td>6.66 ± 0.15</td>
</tr>
<tr>
<td>Isoproterenol (0.1 μM)</td>
<td>5.79 ± 0.09</td>
<td>11.5$^c$</td>
</tr>
<tr>
<td>Forskolin (4.0 μM)</td>
<td>6.55 ± 0.05</td>
<td>2.0$^d$</td>
</tr>
<tr>
<td>Ileum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.61 ± 0.05</td>
<td>7.42 ± 0.07</td>
</tr>
<tr>
<td>Isoproterenol (1.0 μM)</td>
<td>7.56 ± 0.09</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$^a$ Data are from Fig. 2. Mean pEC$_{50}$ values ± S.E.M. are shown for oxotremorine-M.

$^b$ Denotes EC$_{50}$ value of oxotremorine-M (Oxo-M) measured in the presence of indicated relaxant agent (i.e., isoproterenol or forskolin) divided by that measured in the absence of relaxant agent (control EC$_{50}$ value).

$^c$ Data are from Thomas et al. (1994).

$^d$ Significantly different from 1.0, P < .05.

$^e$ Significantly different from 1.0, P < .01.

$^f$ Significantly different from shift measured in untreated tissue, P < .01.

Effects of Pertussis Toxin on Relaxant Responses to Isoproterenol in Isolated Ileum and Trachea. To investigate the apparent heterogeneity of responses elicited by the M$_2$ receptor further, we measured the relaxant potency of isoproterenol in guinea pig ileum and trachea contracted with a single concentration of either histamine or oxotremorine-M. These measurements were repeated in tissues treated with pertussis toxin. Ileal segments were contracted with either histamine (0.3 μM) or oxotremorine-M (40 nM). These agents produced roughly equal contractions, with tension averaging 2.44 ± 0.24 g with histamine and 2.78 ± 0.19 g with oxotremorine-M (not significantly different, P = 0.62 by paired t test). Isoproterenol was 2.3-fold more potent at inhibiting histamine-induced contractions compared with those induced by oxotremorine-M (Fig. 3A). Moreover, isoproterenol caused a maximal 54% inhibition of the oxotremorine-M-induced contractions compared with a 93% inhibition of those induced by histamine. In contrast, isoproterenol was equi-effective at inhibiting histamine- and oxotremorine-M-induced contractions in ilea isolated from guinea pigs that had been treated 72 h before with pertussis toxin (100 μg/kg, i.p.) (Fig. 3B).

Tracheal segments were also contracted with either histamine (10 μM) or oxotremorine-M (0.8 μM). Tension averaged 1.45 ± 0.15 g with histamine and 1.79 ± 0.16 g with ox-
Histamine at stimulating phosphoinositide hydrolysis in airway smooth muscle (Hoiting et al., 1996). Therefore, it is likely that isoproterenol must overcome a greater contractile signal when a maximally effective concentration of oxotremorine-M is used to elicit contractions, compared to when a high concentration of histamine is used. This situation could account for a differential relaxant effect of isoproterenol similar to that observed in Fig. 3, A and C. Therefore, to eliminate such effects, we measured relaxation against contractions elicited by low concentrations of histamine or oxotremorine-M. Presumably, under these conditions, differences in the contractile effects of histamine and oxotremorine-M are minimized.

When used at concentrations of 0.3 and 0.1 μM in the guinea pig ileum, histamine elicited contractions of 2.37 ± 0.41 and 0.57 ± 0.05 g over resting tension, respectively, corresponding to responses of approximately 85 and 20% of the maximal response to histamine. The pEC<sub>50</sub> values of isoproterenol for inhibiting contractions elicited by the high and low concentrations of histamine were 8.03 and 8.59, respectively (Table 4). At concentrations of 40 and 10 nM, oxotremorine-M contracted the ileum to levels averaging 2.43 ± 0.25 and 0.82 ± 0.08 g over resting tension, respectively, corresponding to responses of approximately 76 and 26% of the maximal response to oxotremorine-M. Isoproterenol did not completely overcome the contraction elicited by 40 nM oxotremorine-M; the maximal inhibition of contraction was 71%. The pEC<sub>50</sub> value of this relaxant response was 7.63. In contrast, isoproterenol caused complete relaxation of contractions elicited by the low concentration of oxotremorine-M, with the pEC<sub>50</sub> value being 8.31 (Table 4).

Similar experiments were conducted in guinea pig trachea. Contractions elicited by histamine at 10 and 0.6 μM averaged 1.43 ± 0.25 and 0.50 ± 0.05 g over resting tension, respectively, corresponding to responses of 99 and 34% of the maximal response to histamine. The pEC<sub>50</sub> value of isoproterenol for inhibiting contractions elicited by 10 μM histamine was 8.39, whereas that for inhibiting contractions elicited by 0.6 μM histamine was 8.60 (Table 4). Contractions elicited by oxotremorine-M at 0.8 μM and 10 nM averaged 1.89 ± 0.42 and 0.50 ± 0.05 g over resting tension, respectively, corresponding to responses of 95 and 25% of the maximal response to oxotremorine-M. The pEC<sub>50</sub> values of isoproterenol for inhibiting these contractions were 7.79 and 8.48, respectively (Table 4).

**Effect of 4-DAMP Mustard Treatment on Intrinsic Differential Relaxant Potency of Isoproterenol.** We define the intrinsic differential relaxant potency as the difference in the potency of isoproterenol at inhibiting contractions

**TABLE 3**

Relaxant potency (pEC<sub>50</sub>) of isoproterenol and forskolin measured against histamine- and oxotremorine-M-induced contractions in untreated and pertussis toxin-treated trachea*  

<table>
<thead>
<tr>
<th>Relaxant Agent</th>
<th>Untreated Tissue</th>
<th>Pertussis Toxin-Treated Tissue</th>
<th>Shift&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt; vs. Histamine</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt; vs. Oxo-M</td>
<td>Fold</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>8.46 ± 0.07</td>
<td>7.80 ± 0.06</td>
<td>4.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Forskolin</td>
<td>7.46 ± 0.27</td>
<td>6.14 ± 0.09</td>
<td>20.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Data for isoproterenol are from Fig. 3, C and D. Mean pEC<sub>50</sub> values ± S.E.M. are shown for isoproterenol and forskolin. Relaxant potency was measured using histamine (0.3 μM) and oxotremorine-M (Oxo-M; 40 nM) as contractile agents.

<sup>b</sup> Denotes EC<sub>50</sub> value of relaxant agent measured against oxotremorine-M divided by that measured against histamine.

<sup>c</sup> Significantly different from 1.0, P < .05.

<sup>d</sup> Significantly different from shift measured in untreated trachea, P < .02.
elicited by low concentrations of either histamine or oxotremorine-M. The numerical data from the experiments in Table 4 (where low concentrations of the contractile agents were used) are shown graphically in Fig. 4. In the guinea pig ileum, isoproterenol was 1.9-fold more potent at inhibiting contractions elicited by a low concentration of histamine (0.1 μM) than contractions elicited by a low concentration of oxotremorine-M (10 nM) (Fig. 4A). In the guinea pig trachea, however, isoproterenol was only 1.3-fold more potent at inhibiting contractions elicited by a low concentration of histamine (0.6 μM) than contractions elicited by a low concentration of oxotremorine-M (10 nM) (Fig. 4B). These data illustrate that the intrinsic differential relaxant potency of isoproterenol is smaller in the trachea than in the ileum.

To determine the muscarinic receptor subtype mediating this difference in relaxant potency, we carried out an experiment similar to that described above in tissues treated with 4-DAMP mustard. Tissues were treated for 1 h with 4-DAMP mustard (40 nM) in combination with AP-DX 116 (1 μM) as described in Materials and Methods. This treatment completely eliminated the contractile response to the low concentration of oxotremorine-M (10 nM) in both the ileum and trachea. Tissues were contracted with either histamine or histamine in combination with the low concentration of oxotremorine-M. Oxotremorine-M had no effect on the contractile response to histamine. In the guinea pig ileum, isoproterenol was 4.1-fold more potent at inhibiting contractions elicited by histamine compared with those elicited by histamine in the presence of oxotremorine-M (Fig. 5A). In the trachea, however, isoproterenol was equi-potent at inhibiting contractions elicited by histamine alone or in combination with oxotremorine-M (Fig. 5B). These data indicate that oxotremorine-M is unable to inhibit the relaxant potency of isoproterenol in trachea once M3 receptors have been inactivated, but is able to reduce isoproterenol potency in ileum through the remaining M2 receptors.

### Discussion

Our laboratory previously developed a novel strategy for the detection of contractile responses mediated by the M2 receptor. The method involves first treating smooth muscle with 4-DAMP mustard to inactivate M3 receptors selectively. The contractile activity of a muscarinic agonist is measured subsequently in the presence of histamine and a cAMP-stimulating, relaxant agent, such as forskolin or isoproterenol. The combination of histamine and the relaxant agent has no net contractile effect because the latter inhibits the histamine-induced contraction. However, activation of M2 receptors by the muscarinic agonist can inhibit the cAMP-mediated relaxant effect and allow histamine to contract the muscle. The M2 nature of the contractile response can be confirmed with subtype-selective muscarinic antagonists. Using this approach, Thomas and Ehler (1996) demonstrated that M2 receptors mediate contraction of the guinea pig trachea by inhibiting the relaxant effects of forskolin, whereas Watson et al. (1995) showed that M2 receptors are

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**TABLE 4**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Contractile Agents, Low Concentrations&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Contractile Agents, High Concentrations&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt; vs. Histamine</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt; vs. Oxo-M</td>
</tr>
<tr>
<td>Ileum</td>
<td>8.59 ± 0.16</td>
<td>8.31 ± 0.15</td>
</tr>
<tr>
<td>Trachea</td>
<td>8.60 ± 0.14</td>
<td>8.48 ± 0.13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration of contractile agents were as follows: histamine, ileum, 0.1 μM; histamine, trachea, 0.6 μM; oxotremorine-M, ileum, 10 nM; oxotremorine-M, trachea, 10 nM.

<sup>b</sup> Concentration of contractile agents were as follows: histamine, ileum, 0.3 μM; histamine, trachea, 10 μM; oxotremorine-M, ileum, 40 nM; oxotremorine-M, trachea, 0.8 μM.

<sup>c</sup> Denotes pEC<sub>50</sub> value of isoproterenol measured against oxotremorine-M divided by that measured against histamine.

<sup>d</sup> Significantly different from 1.0, P < .05.

<sup>e</sup> Significantly different from 1.0, P < .01.
unable to mediate contractions by inhibiting the relaxant effects of isoproterenol in the same tissue. The purpose of the present study was to investigate this apparent discrepancy in the trachea and to compare the contractile role of M₂ receptors in the guinea pig ileum and trachea under identical conditions.

Using the strategy described above, we now show that the M₂ receptor is unable to oppose the relaxant effect of isoproterenol in the guinea pig trachea. These observations are in agreement with the findings by Watson et al. (1995). However, the experiments previously conducted in our laboratory show that the M₂ receptor is capable of inhibiting the relaxant effects of forskolin in the trachea (Thomas and Ehlert, 1996). Therefore, we confirm that the contractile role of the M₂ receptor in guinea pig trachea is dependent upon the relaxant agent present.

It is interesting that the M₂ receptor is unable to inhibit the relaxant effects of isoproterenol because there is long-standing evidence that isoproterenol is more potent at relaxing histamine-induced contractions compared with contractions elicited by muscarinic agonists (Van Amsterdam et al., 1989; Roffel et al., 1993; Watson and Eglen, 1994). These observations have been regarded as an index of the functional antagonism between muscarinic and beta adrenergic receptors. Most interpretations attribute this differential relaxant potency of isoproterenol to M₂ receptor-mediated inhibition of cAMP levels stimulated by isoproterenol. We previously showed that such a mechanism exists in guinea pig ileum (Ostrom and Ehlert, 1997).

To assess the role of the M₂ receptor in opposing the relaxant effects of isoproterenol, some investigators have measured the extent to which M₂-selective muscarinic antagonists affect the potency of isoproterenol for inhibiting contractions elicited by a muscarinic agonist. We previously outlined some pitfalls of this strategy (Thomas and Ehlert, 1994; Ehlert et al., 1997), and not surprisingly, investigators using this approach have reported conflicting results. In light of this disparity, we investigated similar experimental conditions but chose to use pertussis toxin instead of a muscarinic antagonist to infer a role for M₂ receptors. Pertussis toxin treatment ADP ribosylates G₁ and G₃ proteins, preventing their activation, and thereby uncoupling M₂ receptors from their signaling mechanism. As a comparison, we also conducted these experiments in guinea pig ileum, because M₂ receptors have been shown to inhibit the relaxant effects of isoproterenol in this tissue (Thomas et al., 1993; Ostrom and Ehlert, 1997). In the guinea pig ileum, pertussis toxin treatment enhanced the relaxant effects of isoproterenol so that there was no difference in its ability to inhibit histamine- or oxotremorine-M-induced contractions. In contrast, activation of M₂ receptors in bovine tracheal smooth muscle, much like in guinea pig trachea, does not oppose the relaxant effects of isoproterenol even though this activation does inhibit isoproterenol-stimulated cAMP levels in bovine trachea (Ostrom and Ehlert, 1998). This situation can be explained if beta adrenergic receptors mediate relaxation by a mechanism independent of cAMP and unopposed by M₂ receptor activation. Therefore, the observed differential relaxant potency of isoproterenol in the guinea pig trachea must be due to M₂ receptor activation, as previously suggested by Roffel and coworkers (Roffel et al., 1993). Both the contraction and the phosphoinositide response elicited by near-maximal concentrations of muscarinic agonists are greater than the corresponding responses to high concentrations of histamine (Van Amsterdam et al., 1989), although both agents generate the same degree of Ca²⁺ mobilization at equal levels of contraction (Hoit- ing et al., 1996). It may be possible that muscarinic agonists elicit a greater contractile “signal” (e.g., phosphoinositide hydrolysis) than histamine, even at equivalent levels of contraction, giving the relaxant agent more to overcome when the tissue is contracted with a muscarinic agonist.

We addressed this issue by measuring relaxation curves with various concentrations of isoproterenol after eliciting very small contractions (~0.5 g) with either histamine or oxotremorine-M. At low levels of activation of muscarinic or histaminergic receptors the contractile “signal” elicited by each agent should be more equivalent. Under these conditions, the differential relaxant potency of isoproterenol was very small in the guinea pig trachea, but still sizable in the ileum. The near absence of a differential relaxant effect of isoproterenol in the trachea under these conditions is consistent with the inability of the M₂ receptor to oppose isoproterenol-induced relaxation. To explore these conditions further, we inactivated M₂ receptors selectively with 4-DAMP mustard and measured the ability of oxotremorine-M to affect the relaxant potency of isoproterenol against histamine-induced contractions. Under these conditions, oxotremorine-M acts only through the M₂ receptor and is unable to elicit contraction by itself. We found that activation of M₂ receptors had no effect on the relaxant potency of isoproterenol (see Figs. 2 and 3). The results of the experiments described in the preceding paragraph appear to conflict with those of Mitchell et al. (1993) who found that pertussis toxin treatment enhanced the relaxant potency of isoproterenol against acetylcholine-induced contractions in canine trachea. Perhaps these results can be explained by species differences. So far, there is consistent evidence indicating that activation of the M₂ receptor does not oppose isoproterenol-induced relaxation in bovine (Ostrom and Ehlert, 1998) and guinea pig trachea (Watson et al., 1995 and this report), yet the work of Mitchell et al. (1993) suggests such a mechanism in canine trachea. The inability of M₂ receptors to mediate inhibition of the relaxant effect of isoproterenol in the guinea pig trachea raises the question as to why isoproterenol is less potent at inhibiting oxotremorine-M-induced contractions compared with those elicited by an equi-effective concentration of histamine (Fig. 4C). In the guinea pig ileum, we showed that M₂ receptor-mediated inhibition of cAMP levels accounts for this difference in isoproterenol relaxant potency (Ostrom and Eh- lert, 1997). In contrast, activation of M₂ receptors in bovine tracheal smooth muscle, much like in guinea pig trachea, does not oppose the relaxant effects of isoproterenol even though this activation does inhibit isoproterenol-stimulated cAMP levels in bovine trachea (Ostrom and Ehlert, 1998). This situation can be explained if beta adrenergic receptors mediate relaxation by a mechanism independent of cAMP and unopposed by M₂ receptor activation. Therefore, the observed differential relaxant potency of isoproterenol in the guinea pig trachea must be due to M₂ receptor activation, as previously suggested by Roffel and coworkers (Roffel et al., 1993). Both the contraction and the phosphoinositide response elicited by near-maximal concentrations of muscarinic agonists are greater than the corresponding responses to high concentrations of histamine (Van Amsterdam et al., 1989), although both agents generate the same degree of Ca²⁺ mobilization at equivalent levels of contraction (Hoit- ing et al., 1996). It may be possible that muscarinic agonists elicit a greater contractile “signal” (e.g., phosphoinositide hydrolysis) than histamine, even at equivalent levels of contraction, giving the relaxant agent more to overcome when the tissue is contracted with a muscarinic agonist.
enol against histamine-induced contractions in the trachea, but greatly reduced the potency of isoproterenol in the ileum.

Much of the confusing information regarding the functional role of M2 muscarinic receptors in airway smooth muscle is clarified in the present study. Our findings indicate that M2 receptors can inhibit the relaxant effects of forskolin but not isoproterenol in guinea pig trachea. It is possible that in guinea pig trachea, much like in bovine trachea, beta adrenergic receptors elicit relaxation in part via a non-cAMP-dependent mechanism that cannot be opposed by the M2 receptor. The reduced relaxant potency of isoproterenol against contractions elicited by a muscarinic agonist, compared with those elicited by histamine, is due to the strong contractile stimulus generated by M3 receptors in the trachea. Our novel methods with 4-DAMP mustard should have use for investigating the role of M2 receptors in other tissues.

References


Send reprint requests to: Frederick J. Ehler, Department of Pharmacology, College of Medicine, University of California, Irvine, Irvine, CA. fehler@uci.edu