Comparison of Functional Antagonism Between Isoproterenol and M2 Muscarinic Receptors in Guinea Pig Ileum and Trachea

RENNOLDS S. OSTROM and FREDERICK J. EHLERT

Department of Pharmacology, College of Medicine, University of California, Irvine, Irvine, California

Accepted for publication October 2, 1998

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 receptor. We also measured the effects of pertussis toxin treatment on the ability of isoproterenol to inhibit the contractile response 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 M3 subtype which 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 M3 receptor to G proteins of the G(q) 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 G(i) 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 M₂facilitatory 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-diphenylacetoxy-N-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 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 measured 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 aziridine ion of 4-DAMP mustard (40 nM) for 1 h in the presence of [2-{diethylamino(methyl)-1-piperidinyl}[acetyl]-5,11-di-hydro-6H-pyrido[2,3b]-[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 M₂ receptor with AF-DX 116 (Thomas et al., 1992, 1993). 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 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

Vol. 288
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 neutralized to pH 7.4 at 37°C for 30 min to allow formation of the reactive aziridinium ion that was then used immediately.

**Data Analysis.** The pEC<sub>50</sub> 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).

<table>
<thead>
<tr>
<th>Shift&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Shift&lt;sup&gt;b&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Shift&lt;sup&gt;c&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Shift&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol (0.1 μM)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.48 ± 0.21</td>
<td>1.69 ± 0.28</td>
<td>6.53 ± 0.22</td>
<td>1.96 ± 0.22</td>
<td>5.80 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>Forskolin (4.0 μM)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.72 ± 0.15</td>
<td>1.51 ± 0.25</td>
<td>5.52 ± 0.17</td>
<td>1.80 ± 0.21</td>
<td>5.00 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Shift&lt;sup&gt;e&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Shift&lt;sup&gt;f&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Shift&lt;sup&gt;g&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Shift&lt;sup&gt;h&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.21 1.69</td>
<td>0.15 1.51</td>
<td>0.21 1.4–1.6</td>
<td>0.25 5.52</td>
<td>0.17 1.60</td>
<td>0.21 1.4–1.6</td>
<td></td>
</tr>
</tbody>
</table>

**Results**

**Effects of AF-DX 116 on Contractile Response to Oxotremorine-M in 4-DAMP Mustard-Treated Trachea.** To determine whether the M<sub>2</sub> 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 M<sub>3</sub> receptors while leaving M<sub>2</sub> 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 M<sub>3</sub> 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 pK<sub>B</sub> range of 5.6 to 5.7, near the binding affinity of AF-DX 116 for the cloned M<sub>3</sub> subtype (pK<sub>B</sub> = 6.10), but not the M<sub>2</sub> subtype (pK<sub>B</sub> = 7.27) (Esqueda et al., 1996). These data indicate that the M<sub>2</sub> 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 Ehler, 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 pK<sub>B</sub> values from 6.60 to 6.90. This range of pK<sub>B</sub> values is in agreement with the binding affinity of AF-DX 116 in cells transfected with the M<sub>2</sub> subtype, indicating that the M<sub>2</sub> 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 M<sub>2</sub> receptor with a different approach, we used pertussis toxin treatment to ADP-ribosylate, the G protein involved in M<sub>2</sub> receptor signaling (G<sub>i</sub>) (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 M<sub>2</sub> 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 pertussis toxin-treated trachea and ileum. Under these conditions, AF-DX 116 (1 μM) antagonized the contractile response to oxotremorine-M in both treated tissues.

**TABLE 1**

<table>
<thead>
<tr>
<th>Shift&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Shift&lt;sup&gt;b&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Shift&lt;sup&gt;c&lt;/sup&gt;</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Shift&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol (0.1 μM)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.48 ± 0.21</td>
<td>1.69 ± 0.28</td>
<td>5.53 ± 0.22</td>
<td>1.96 ± 0.22</td>
<td>5.80 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>Forskolin (4.0 μM)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.72 ± 0.15</td>
<td>1.51 ± 0.25</td>
<td>5.52 ± 0.17</td>
<td>1.80 ± 0.21</td>
<td>5.00 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Denotes pEC<sub>50</sub> values 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 EC<sub>50</sub> value).
<sup>b</sup> Data are from Fig. 1.
<sup>c</sup> Data are from Thomas and Ehler.
<sup>d</sup> Significantly different from 1.0, P < .05.
and pertussis toxin-treated isolated trachea. Isoproterenol (0.1 μM) decreased the potency of oxotremorine-M, causing an 11.5-fold increase in the EC₅₀ value of oxotremorine-M for contraction in untreated trachea (Fig. 2A and Table 2). Similar results were observed in pertussis toxin-treated trachea; isoproterenol caused a 10.7-fold increase in the oxotremorine-M EC₅₀ value (Fig. 2B and Table 2). Forskolin (4 μM) induced a 2-fold rightward shift of the oxotremorine-M concentration-response curve (Fig. 2C and Table 2). However in pertussis toxin-treated trachea, the antagonism by forskolin was significantly greater (p < .01, paired t test), representing a 4.9-fold decrease in the contractile potency of oxotremorine-M (Fig. 2D and Table 2). These data suggest that M₂ receptors act to prevent the relaxant effects of forskolin but not isoproterenol in the guinea pig trachea.

**Effects of Pertussis Toxin on Relaxant Responses to Isoproterenol in Isolated Ileum and Trachea.** To investigate the apparent heterogeneity of responses elicited by the M₂ 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 a single concentration of either histamine or oxotremorine-M (40 nM).

**TABLE 2**

Effects of isoproterenol and forskolin on EC₅₀ 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>Shiftᵇ</td>
</tr>
<tr>
<td></td>
<td>pEC₅₀ fold</td>
<td></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>11.5ᵇ</td>
</tr>
<tr>
<td>Forskolin (4.0 μM)</td>
<td>6.55 ± 0.05</td>
<td>2.0ᶜ</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 pEC₅₀ values ± S.E.M. are shown for oxotremorine-M.
ᵇ Denotes EC₅₀ 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 EC₅₀ 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.

---

**Fig. 1.** Effects of AF-DX 116 on contractile response to oxotremorine-M measured after 4-DAMP mustard treatment and in the presence of histamine and isoproterenol. Trachea were contracted with histamine (10 μM) and relaxed with isoproterenol (0.1 μM) before oxotremorine-M-induced contractions were measured. Control, (■) and AF-DX 116 (1 μM), (□). Each point represents mean ± S.E.M. of five to six experiments.

**Fig. 2.** Effects of isoproterenol (A and B) and forskolin (C and D) on oxotremorine-M-induced contraction in control (A and C) and pertussis toxin-treated (B and D) trachea. Contractile responses to oxotremorine-M were measured in the absence (■) and presence (□) of relaxant agents, isoproterenol and forskolin. Each point represents mean ± S.E.M. of four to six experiments.
otaxine-M (not significantly different, $p = 0.44$ by paired $t$ test). Isoproterenol elicited relaxation with 4.6-fold greater potency against histamine-induced contractions compared with those induced by oxotremorine-M in untreated trachea (Fig. 3C and Table 3). Pertussis toxin treatment was without effect on the differential effects of isoproterenol. The relaxant potency of isoproterenol in pertussis toxin-treated trachea was 4.5-fold greater against histamine-induced contractions compared with those induced by oxotremorine-M (Fig. 3D and Table 3). Forskolin was 20.9-fold more potent at inhibiting histamine-induced contractions compared with those induced by oxotremorine-M. Pertussis toxin treatment significantly reduced the differential relaxant effects of forskolin in the trachea ($p < .02$, paired $t$ test), with forskolin being only 12.6-fold more potent against histamine compared with oxotremorine-M (Table 3).

### Relaxant Response to Isoproterenol Against High and Low Concentrations of Histamine and Oxotremorine-M

Muscarinic agonists are far more efficacious than 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 mM in the guinea pig ileum, histamine elicited contractions of $2.37 \pm 0.41$ and $0.57 \pm 0.05$ g over resting tension, respectively, corresponding to responses of approximately 85 and 20% of the maximal response to histamine. The pEC$_{50}$ 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 $2.43 \pm 0.25$ and $0.82 \pm 0.08$ 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$_{50}$ 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$_{50}$ value being 8.31 (Table 4).

Similar experiments were conducted in guinea pig trachea. Contractions elicited by histamine at 10 and 0.6 mM averaged $1.43 \pm 0.25$ and $0.50 \pm 0.05$ g over resting tension, respectively, corresponding to responses of 99 and 34% of the maximal response to histamine. The pEC$_{50}$ value of isoproterenol for inhibiting contractions elicited by 10 mM histamine was 8.39, whereas that for inhibiting contractions elicited by 0.6 mM histamine was 8.60 (Table 4). Contractions elicited by oxotremorine-M at 0.8 mM and 10 mM averaged $1.89 \pm 0.42$ and $0.50 \pm 0.05$ g over resting tension, respectively, corresponding to responses of 95 and 25% of the maximal response to oxotremorine-M. The pEC$_{50}$ 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

<table>
<thead>
<tr>
<th>Relaxant Agent</th>
<th>Untreated Tissue</th>
<th>Pertussis Toxin-Treated Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>relaxant agent</td>
<td>relaxant agent</td>
</tr>
<tr>
<td></td>
<td>$pEC_{50}$ vs. histamine</td>
<td>$pEC_{50}$ vs. Ox-M</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>$8.46 \pm 0.07$</td>
<td>$7.80 \pm 0.06$</td>
</tr>
<tr>
<td>Forskolin</td>
<td>$7.46 \pm 0.27$</td>
<td>$6.14 \pm 0.09$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relaxant Agent</th>
<th>Untreated Tissue</th>
<th>Pertussis Toxin-Treated Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>relaxant agent</td>
<td>relaxant agent</td>
</tr>
<tr>
<td></td>
<td>$pEC_{50}$ vs. histamine</td>
<td>$pEC_{50}$ vs. Ox-M</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>$8.49 \pm 0.11$</td>
<td>$7.84 \pm 0.04$</td>
</tr>
<tr>
<td>Forskolin</td>
<td>$7.61 \pm 0.20$</td>
<td>$6.51 \pm 0.08$</td>
</tr>
</tbody>
</table>

$^a$ Data for isoproterenol are from Fig. 3, C and D. Mean $pEC_{50}$ values $\pm$ S.E.M. are shown for isoproterenol and forskolin. Relaxant potency was measured using histamine (0.3 mM) and oxotremorine-M (Oxo-M; 40 nM) as contractile agents.

$^b$ Denotes EC$_{50}$ value of relaxant agent measured against oxotremorine-M divided by that measured against histamine.

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

$^d$ Significantly different from shift measured in untreated trachea, $P < .02$. 

---

**Fig. 3.** Effect of pertussis toxin on relaxant potency of isoproterenol in guinea pig ileum and trachea. Ileum (A and B) were contracted with either 0.3 mM histamine (●) or 40 nM oxotremorine-M (□). Trachea (C and D) were contracted with either 10 μM histamine (●) or 0.8 μM oxotremorine-M (□). Contractions were measured in control (A and C) and pertussis toxin-treated (B and D) tissues. Each point represents mean $\pm$ S.E.M. of four to five experiments.
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 equipotent 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 Ehlert (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
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 longstanding 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 Gq 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, in the trachea, the differential relaxant effects of isoproterenol against histamine– and oxotremorine-M-induced contractions were unaffected by pertussis toxin treatment. The ability of a single concentration of isoproterenol to antagonize muscarinic contractile responses was similarly unaffected by pertussis toxin in trachea but enhanced in ileum. Our protocol for treatment with pertussis toxin is effective because forskolin’s ability to antagonize muscarinic contractions in trachea was enhanced by this treatment. These experiments further demonstrate that tracheal M2 receptors can mediate an inhibition of the relaxant effects of forskolin, but not isoproterenol, whereas ileal M2 receptors are able to mediate an inhibition of the relaxant effects 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 Ehlert, 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 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.
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 M₃ muscarinic receptors in airway smooth muscle is clarified in the present study. Our findings indicate that M₃ 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 M₃ receptors in other tissues.

References


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