Comparison of the Antimuscarinic Action of \( p \)-Fluoro-hexahydrosiladifenidol in Ileal and Tracheal Smooth Muscle

Frederick J. Ehlert, Jake Ching-Hsuan Hsu, Kevin Leung, Alex G. Lee, Darakhshanda Shehnaz, and Michael T. Griffin

Department of Pharmacology, College of Medicine, University of California, Irvine, Irvine, California (F.J.E., J.C.-H.H., K.L., A.G.L.); Banting and Best Department of Medical Research, University of Toronto, Ontario, Canada (D.S.); and Department of Physical Sciences, Chapman University, Orange, California (M.T.G.)

Received July 28, 2004; accepted September 14, 2004

ABSTRACT

We investigated the ability of the muscarinic antagonist \( p \)-fluoro-hexahydrosiladifenidol to inhibit muscarinic agonist-induced contractions and phosphoinositide hydrolysis in the guinea pig ileum and trachea. This antagonist displayed higher potency at blocking oxotremorine-M-induced contractions of the ileum compared with those of the trachea. When estimated using a simple model for competitive antagonism, the observed dissociation constant of \( p \)-fluoro-hexahydrosiladifenidol exhibited approximately 12-fold higher potency in the ileum compared with the trachea. We also investigated the ability of \( p \)-fluoro-hexahydrosiladifenidol to affect the inhibition of contraction caused by the known competitive muscarinic antagonist atropine. Using resultant analysis to analyze this interaction, we found that the true dissociation constant of \( p \)-fluoro-hexahydrosiladifenidol for competitively antagonizing oxotremorine-M-induced contractions in the ileum exhibited significantly lower potency than when calculated assuming a simple competitive model. In contrast, resultant analysis showed little difference between the true and observed potentials of \( p \)-fluoro-hexahydrosiladifenidol for antagonizing oxotremorine-M-induced contractions in the trachea. Using a simple competitive model, we found little difference in the observed dissociation constant of \( p \)-fluoro-hexahydrosiladifenidol for antagonizing oxotremorine-M-induced phosphoinositide hydrolysis in guinea pig ileum and bovine trachea. We also noted that \( p \)-fluoro-hexahydrosiladifenidol (0.3–1.0 \( \mu \)M) moderately inhibited histamine-induced contractions of ileum but not of trachea. Our results suggest that \( p \)-fluoro-hexahydrosiladifenidol does not discriminate markedly between \( M_3 \) muscarinic receptors in the ileum and trachea and that it may possess a more potent, nonmuscarinic inhibitory effect on contraction in the ileum.

Although acetylcholine has an important physiological role in smooth muscle, its contractile action may be counterproductive in some instances and contribute to the symptoms of chronic obstructive pulmonary disease (Barnes, 2003), irritable bowel syndrome (Talley, 2003), and urinary incontinence (de Groat and Yoshimura, 2001), which explains why muscarinic antagonists are useful therapeutic agents for these conditions. A limitation in their use, however, is their lack of selectivity. An antagonist used to treat urinary incontinence, for example, might also interfere with muscarinic receptors in the salivary glands to cause dry mouth. Consequently, muscarinic antagonists with a tissue-selective action have tremendous therapeutic potential. Some muscarinic antagonists with this type of selectivity have been described, including tolterodine (Nilvebrant et al., 1997), darifenacin (Wallis and Napier, 1999), zamifenacin (Watson et al., 1995), and \( p \)-fluoro-hexahydrosiladifenidol (\( p \)-FHHSiD) (Eglen et al., 1990). The latter two agents exhibit tissue selectivity in experiments on isolated smooth muscle, indicating that the selectivity cannot be attributed to the route of administration or distribution of the drug in the intact animal. The compound \( p \)-FHHSiD exhibits a preference for antagonizing the contractile effects of muscarinic agonists in the isolated ileum compared with the trachea (Eglen et al., 1990), whereas zamifenacin is more potent at blocking contraction in the ileum and trachea compared with the urinary bladder (Watson et al., 1995). The mechanism for this selectivity is unknown. The antimuscarinic properties of \( p \)-FHHSiD were first described by Lambrecht et al. (1988, 1989) who found that the compound was 67-fold more potent at blocking \( M_3 \) Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.104.074898.

ABBREVIATIONS: \( p \)-FHHSiD, \( p \)-fluoro-hexahydrosiladifenidol; KRB, Krebs-Ringer bicarbonate; 4-DAMP, \( N,N \)-dimethyl-4-piperidinyl diphenylacetate; CHO, Chinese hamster ovary.
receptor-mediated contractions of the ileum compared with M₂ receptor-mediated inhibition of contraction in electrically paced atria.

In this study, we investigated the mechanism for the ileal-selective antimuscarinic action of p-FHHSiD. As reported by Eglen et al. (1990), we found that p-FHHSiD exhibited greater potency at blocking muscarinic agonist-induced contractions of the ileum compared with those of the trachea. Resultant analysis showed that only part of its inhibitory effect in the ileum could be attributed to competitive antagonism, whereas all of its effect in the trachea was competitive. There was little difference in the competitive component of p-FHHSiD for inhibiting contractions in the ileum and trachea. We also observed no difference in the potency of p-FHHSiD for antagonizing muscarinic agonist-induced phosphoinositide hydrolysis in bovine trachea and guinea pig ileum. Our results suggest that p-FHHSiD does not discriminate markedly between M₃ muscarinic receptors eliciting contraction and phosphoinositide hydrolysis in ileum and trachea but that it may exhibit a more potent, nonmuscarinic receptor-mediated inhibitory effect in the ileum.

Materials and Methods

Contractile Assays. Male guinea pigs (Hartley, 300–500 g) were euthanized with CO₂, and the trachea was removed and cleaned of adherent tissue. Segments of the ileum (2.5 cm) were removed in a rostral direction, beginning at a point approximately 10 cm from the caecum. The trachea was cut open longitudinally on its ventral surface, and transverse sections were cut, each containing three to four cartilaginous rings. Silk thread was attached to the cartilage at the ends of the tracheal strips, and the tissue was mounted in an organ bath containing 50 ml of Krebs-Ringer bicarbonate (KRB) buffer (124 mM NaCl, 1.2 mM KCl, 2.3 mM MgSO₄, 1.2 mM KH₂PO₄, 20 mM NaHCO₃, and 10 mM glucose) gassed with O₂/CO₂ (19:1). Indomethacin (1 µM) was present in the KRB buffer. The tracheal strips were connected to isometric force transducers, and contractile responses were recorded using a PowerLab system (ADInstruments, Grand Junction, CO) or Polygraph (Grass Instruments, Quincy, MA). Ileal segments were mounted longitudinally in a similar manner. Tracheal strips were allowed to equilibrate for 2 h before the start of experiments, whereas ilea were equilibrated for at least 1 h. Three test doses of the muscarinic agonist oxotremorine-M (40 nM) were applied to the tissues at the beginning of the experiments. After each test dose, the tissue was washed and allowed to rest for approximately 10 min. Concentration-response curves for the muscarinic agonist oxotremorine-M were measured in a cumulative manner. Only the stable plateau phase of the contraction was used in the calculation of data. During the collection of the data for concentration-response curves, the response to oxotremorine-M was measured for 20 to 30 s in the ileum and for 6 min in the trachea. When present, antagonists were allowed to equilibrate with the tissue for 1 h before the measurement of the concentration-response curve. Two control concentration-response curves to oxotremorine-M were measured in each tissue at the start of the experiment, with a 30-min rest period between each curve. The control EC₅₀ and Eₘₐₓ values of oxotremorine-M were estimated from these two curves by fitting the data simultaneously to a logistic equation as described below under Calculations.

Phosphoinositide Hydrolysis. Whole trachea from steer were obtained from a slaughterhouse (Shamrock Meats, Vernon, CA) and transported to the laboratory on ice. Slices of tracheal smooth muscle were prepared and placed in KRB buffer as described previously (Ostrom and Ehler, 1998). The longitudinal muscle of the guinea pig ileum was isolated as described by Paton and Vizi (1969). The tissue was cross-chopped at 90° and 350 µm using a MacIlwain tissue chopper. The slices were washed three times in warm (37°C) KRB buffer gassed with O₂/CO₂ (19:1) and allowed to equilibrate at 37°C. Phosphoinositide hydrolysis was measured using a technique similar to that described previously (Thomas et al., 1993). Our method incorporates the [³H]inositol labeling and ion exchange method of Berridge et al. (1982) and the perchloric acid extraction method of Kendall and Hill (1990). Tissue slices were incubated with [³H]inositol (200 µCi) for 90 min in a final volume of 10 ml of KRB buffer. Aliquots (50 µl) of sedimented tissue slices were incubated with the muscarinic agonist oxotremorine-M in a final volume of 0.3 ml containing KRB buffer. The incubations were carried out in plastic tubes gassed with O₂/CO₂ (19:1) and capped. The slices were first incubated in the presence of oxotremorine-M or oxotremorine-M and p-FHHSiD for 30 min to allow these drugs to reach equilibrium with muscarinic receptors in the presence of each other. Control experiments showed little increase in [³H]inositolphosphates in the absence of LiCl during this time. After this equilibration phase, an aliquot (3 µl) was added to achieve a final concentration of 10 µM, and the incubation was allowed to proceed for another 30 min. The incubations were stopped by the addition of an aliquot (200 µl) of perchloric acid (5% w/v), and the tubes were placed on ice for approximately 15 min. An aliquot (approximately 200 µl) containing KOH (0.525 M) and Tris base (10 mM) was added to precipitate the perchlorate, and the tubes were allowed to stand on ice for 15 min, but no longer. The tubes were centrifuged at approximately 3000g for 10 min, and most of the supernatant (0.6 ml) was removed and transferred to a tube containing 2.4 ml of 50 mM Tris/HCl, pH 7.4. The entire 3-ml extract was applied to an ion exchange column consisting of 1 ml of Dowex AG1 × 8 (100–200 mesh). The column was washed four times with 5 ml of water each time. [³H]inositolphosphates were eluted from the column and collected directly into scintillation vials with 2.5 ml of 1 M ammonium formate and 0.1 M formic acid. The data were expressed as [³H]inositolphosphates (cpm).

Calculations. The EC₅₀ and Eₘₐₓ (maximal response) values of oxotremorine-M were estimated by fitting an increasing logistic equation to the concentration-response curves for contraction or phosphoinositide hydrolysis as described previously (Candell et al., 1990). Two different methods were used to estimate the dissociation constant (Kᵦ) of p-FHHSiD, based on its ability to antagonize oxotremorine-M-induced responses. The first method rests on the assumption that the entire effect of p-FHHSiD is caused by competitive antagonism of the agonistic effect of oxotremorine-M at muscarinic receptors. In this instance, the Kᵦ value is calculated according to the method of Schild (Arunlakshana and Schild, 1959):

\[ Kᵦ = \frac{B}{(CR - 1)} \]  

In this equation, concentration ratio (CR) denotes the EC₅₀ value of oxotremorine-M measured in the presence of the antagonist divided by that measured in its absence, and B denotes the concentration of antagonist. The second method for estimating the Kᵦ of p-FHHSiD introduces the possibility that part of the effect of p-FHHSiD may be mediated through a nonmuscarinic receptor mechanism, as well as through competitive antagonism of the receptor. In this instance, the Kᵦ value for the competitive component of the effect of p-FHHSiD is calculated according to the method of Black et al. (1986). The method involves measuring the competitive antagonism caused by a known competitive muscarinic antagonist in the absence and presence of p-FHHSiD. In our experiments, we used atropine as the standard competitive antagonist, and the Kᵦ value was calculated using the following equation:

\[ Kᵦ = \frac{B(CR^* - 1)}{1 + C/Kᵦ - CR^*} \]
In this equation, \( CR^* \) denotes the EC\(_{50} \) value of oxotremorine-M measured in the presence of the atropine and p-FHHSiD divided by that measured in the presence of p-FHHSiD only, \( B \) denotes the concentration of p-FHHSiD, \( C \) denotes the concentration of atropine, and \( K_C \) denotes the dissociation constant of atropine estimated in separate experiments using the simple competitive method (eq. 1). In some experiments, \( N,N \)-dimethyl-4-piperidinyl diphenylacetate (4-DAMP) was used in place of p-FHHSiD.

In some experiments, the ability of p-FHHSiD to inhibit the contractile action of histamine was investigated, and a decrease in both \( E_{\text{max}} \) and potency (increase in EC\(_{50} \)) was noted in the ileum. In this circumstance, we tested whether the effect of p-FHHSiD could be described empirically as a reduction in agonist efficacy using a procedure described previously (Ostrom and Ehlert, 1997). We refer to decrease in agonist efficacy as a decrease in observed coupling efficiency. We make no conclusions regarding agonist efficacy; we simply use the technique as an empirical method to convert the inhibitory effects on the two disparate parameters, EC\(_{50} \) and \( E_{\text{max}} \), into common currency.

**Reagents.** Reagents were obtained from the following sources: oxotremorine-M and p-FHHSiD (Sigma/RBI, Natick, MA); atropine and indomethacin (Sigma-Aldrich, St Louis, MO), and \([3H]\)inositol (PerkinElmer Life and Analytical Sciences, Boston, MA). 4-DAMP was synthesized as described by Barlow et al. (1976).

**Results**

**Contractile Assays.** We investigated the ability of p-FHHSiD to antagonize the contractile action of the highly efficacious muscarinic agonist oxotremorine-M (Fig. 1). In the ileum, oxotremorine-M elicited potent contractile effects, characterized by a pEC\(_{50} \) value of 7.75 \( \pm \) 0.023 and an \( E_{\text{max}} \) of 5.18 \( \pm \) 0.32 g (Fig. 1a). At a concentration of 0.03 \( \mu \)M, p-FHHSiD shifted the concentration-response curve of oxotremorine-M to the right 3.1-fold. In the presence of a higher concentration of p-FHHSiD (0.1 \( \mu \)M), the shift in the concentration-response curve was 12.8-fold. A small increase in \( E_{\text{max}} \) was also noted in the presence of p-FHHSiD; however, control experiments showed that the \( E_{\text{max}} \) value of oxotremorine-M slowly increased in time, whereas there was no change in EC\(_{50} \). The dissociation constant (\( K_B \)) of p-FHHSiD for antagonizing the contractions elicited to oxotremorine-M was calculated from the shift in the concentration-response curve assuming a competitive model (eq. 1, and these values are listed in Table 1). There was little difference in the estimates of \( pK_B \) at the two different concentrations of p-FHHSiD; the average value was 6.89 \( \pm \) 0.056 in the trachea. Thus, it can be seen that the observed potency of p-FHHSiD in the ileum (\( pK_B = 7.95 \)) is 11.5-fold greater than that measured in the trachea (\( pK_B = 6.89 \)). This difference was highly significant (\( P = 8.1 \times 10^{-7} \)).

The estimation of the \( K_B \) value of p-FHHSiD in eq. 1 is based on the assumption that p-FHHSiD acts solely through competitive antagonism of the muscarinic receptor mediating contraction. To determine whether an additional nonreceptor-mediated inhibitory effect of p-FHHSiD might skew the estimate of its \( K_B \) value, we used resultant analysis to estimate the true \( K_B \) value of p-FHHSiD. With this method, it is possible to distinguish between a competitive effect of an antagonist and a possible nonreceptor-mediated effect on the stimulus-response function (e.g., contractile mechanism). The method involves comparing the extent of antagonism caused by a known competitive inhibitor, like atropine, in the presence of the test compound p-FHHSiD with that observed in its absence. Resultant analysis of the inhibitory effect of p-FHHSiD is shown in Fig. 2.

In the absence of p-FHHSiD, atropine (10 nM) shifted the concentration-response curve of oxotremorine-M to the right about 5.9-fold in the ileum (Fig. 2a). Similar effects were observed in the trachea (Fig. 2b). The estimates of the \( pK_B \) values of atropine in the ileum and trachea were 8.70 \( \pm \) 0.044 and 9.22 \( \pm \) 0.039, respectively. The ability of atropine to shift the concentration-response of oxotremorine-M curve was also measured in the presence of two concentrations of p-FHHSiD. In the ileum, the atropine-induced increases in the EC\(_{50} \) value of oxotremorine-M at low (0.03 \( \mu \)M) and high (0.1 \( \mu \)M) concentrations of p-FHHSiD were 4.1- and 2.6-fold, respectively (Fig. 2c). Using eq. 2, it is possible to estimate the true \( K_B \) value of p-FHHSiD based on its ability to interfere with the competitive antagonism caused by atropine. These esti-

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**Fig. 1. Effects of p-FHHSiD on the contractile response to oxotremorine-M in the guinea pig ileum (a) and trachea (b). a, contractile response to oxotremorine-M was measured in the ileum in the absence (○) and presence of p-FHHSiD at concentrations of 0.03 \( \mu \)M (△) and 0.1 \( \mu \)M (■). b, contractile response to oxotremorine-M was measured in the trachea in the absence (○) and presence of p-FHHSiD at concentrations of 0.5 \( \mu \)M (△) and 1.0 \( \mu \)M (■). The data represent the mean contractile measurements \( \pm \) S.E.M. from four to five experiments.**
The dissociation constants of p-FHHSiD estimated in functional assays on the ileum and trachea

The mean estimates ± S.E.M. are shown and were calculated from the data presented in Figs. 1, 2, and 4.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Ileum</th>
<th>Trachea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed $pK_B^{a}$</td>
<td>Resultant $pK_B^{b}$</td>
</tr>
<tr>
<td>Contraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low p-FHHSiD</td>
<td>7.84 ± 0.045$^a$</td>
<td>7.17 ± 0.20</td>
</tr>
<tr>
<td>High p-FHHSiD</td>
<td>8.07 ± 0.041$^a$</td>
<td>7.25 ± 0.11</td>
</tr>
<tr>
<td>Phosphoinositide hydrolysis</td>
<td>7.08 ± 0.046</td>
<td>7.06 ± 0.12</td>
</tr>
<tr>
<td>$p$-FHHSiD (1 μM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Negative logarithm of the observed $K_B$ value calculated according to eq. 1.
$^b$ Negative logarithm of the observed $K_B$ value calculated according to eq. 2.

The concentrations of $p$-FHHSiD in the ileum and trachea were 0.03 and 0.3 μM, respectively.

Significantly different from the corresponding resultant $pK_B$ in the ileum, $P = 0.011$.

Significantly different from the corresponding resultant $pK_B$ in the ileum, $P = 0.029$.

Fig. 2. Effects of $p$-FHHSiD and atropine on the contractile response to oxotremorine-M in the guinea pig ileum (a–c) and trachea (d–f). a, contractile response to oxotremorine-M was measured in the ileum in the absence (○) and presence of atropine (10 nM) (●). b, contractile response to oxotremorine-M was measured in the ileum in the presence of $p$-FHHSiD (0.03 μM) (□) and $p$-FHHSiD (0.03 μM) plus atropine (10 nM) (▲). c, contractile response to oxotremorine-M was measured in the ileum in the presence of $p$-FHHSiD (0.1 μM) (□) and $p$-FHHSiD (0.1 μM) plus atropine (10 nM) (▲). d, contractile response to oxotremorine-M was measured in the trachea in the absence (○) and presence of atropine (10 nM) (●). e, contractile response to oxotremorine-M was measured in the trachea in the presence of $p$-FHHSiD (0.3 μM) (□) and $p$-FHHSiD (0.3 μM) plus atropine (10 nM) (▲). f, contractile response to oxotremorine-M was measured in the ileum in the presence of $p$-FHHSiD (1.0 μM) (□) and $p$-FHHSiD (1.0 μM) plus atropine (10 nM) (▲). The data represent the mean contractile measurements ± S.E.M. from four to five experiments.

The mean estimates ± S.E.M. are shown and were calculated from the data presented in Figs. 1, 2, and 4.

The dissociation constants of $p$-FHHSiD estimated in functional assays on the ileum and trachea.

The mean estimates ± S.E.M. are shown and were calculated from the data presented in Figs. 1, 2, and 4.

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the trachea ($pK_B = 6.76$), the difference in potency was only 2.8-fold and not quite significant ($P = 0.067$) in contrast to the larger, highly significant 11.5-fold difference between the observed $K_B$ values in the two tissues. This similarity in resultant $pK_B$ values between the two tissues indicates that the true potency of $p$-FHHSiD for competitively antagonizing muscarinic receptors elicitng contraction in the ileum and trachea is similar, but perhaps not identical. Also, the data suggest that the majority of the differential blocking effect of $p$-FHHSiD in ileum and trachea (i.e., 11.5-fold difference in observed $pK_B$ values) is due to a mechanism not directly related to competitive antagonism of muscarinic receptors.

To validate our approach in the ileum, we used resultant analysis to analyze the interaction between atropine and another known competitive antagonist, 4-DAMP (Fig. 3). We expected that in this situation, there should be little or no difference between the simple competitive estimate of the $pK_B$ of 4-DAMP and that measured by resultant analysis. At concentrations of 3 and 10 nM, 4-DAMP shifted the oxotremorine-M concentration-response curve to the right 5.5- and 14-fold, respectively (Fig. 3a), yielding simple $pK_B$ values estimated assuming a competitive model (eq. 1) of 9.17 and 9.09 (Table 2). In these experiments, atropine (10 nM) shifted the oxotremorine-M concentration-response curve to the right 12-fold (Fig. 3b), which yields a $pK_B$ estimate of 9.04. In the presence of atropine (10 nM), the shifts in the oxotremorine-M concentration-response curve caused by 4-DAMP at 3 and 10 nM were 3.6- and 1.9-fold, respectively (Fig. 3, c and d). Resultant analysis yielded true $pK_B$ values of 9.04 and 9.15, respectively, for the effects of the two concentrations of 4-DAMP used in the presence of atropine. The $pK_B$ values of 4-DAMP calculated using the simple equation for competitive inhibition (eq. 1) are similar to those for resultant analysis (eq. 2). These results are consistent with the idea that both 4-DAMP and atropine inhibit the contractile effects of oxotremorine-M solely through a competitive mechanism.

As suggested above, the discrepancy between the observed and resultant $pK_B$ values of $p$-FHHSiD in the ileum may be explained by an inhibitory mechanism distinct from antagonism of muscarinic receptors. To explore this hypothesis, we measured the effects of $p$-FHHSiD on the contractile response to a nonmuscarinic contractile agent (i.e., histamine).

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**Table 2**

Dissociation constants of 4-DAMP and atropine estimated in contractile assays on the ileum

<table>
<thead>
<tr>
<th>Assay</th>
<th>Observed $pK_B^a$</th>
<th>Resultant $pK_B^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine (10 nM)</td>
<td>9.04 ± 0.071</td>
<td>NA</td>
</tr>
<tr>
<td>4-DAMP (3 nM)</td>
<td>9.17 ± 0.12</td>
<td>9.04 ± 0.16</td>
</tr>
<tr>
<td>4-DAMP (10 nM)</td>
<td>9.09 ± 0.14</td>
<td>9.15 ± 0.28</td>
</tr>
</tbody>
</table>

NA, not applicable.

^a Negative logarithm of the observed $K_B$ value calculated according to eq. 1.

^b Negative logarithm of the observed $K_B$ value calculated according to eq. 2.

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**Fig. 3.** Effects of 4-DAMP and atropine on the contractile response to oxotremorine-M in the guinea pig ileum. a, contractile response to oxotremorine-M was measured in the ileum in the absence (○) and presence of 4-DAMP at concentrations of 3.0 nM (△) and 10 nM (□). b, contractile response to oxotremorine-M was measured in the absence (○) and presence of atropine (10 nM) (●). c, contractile response to oxotremorine-M was measured in the presence of 4-DAMP (3.0 nM) (△) and 4-DAMP (3.0 nM) plus atropine (10 nM) (▲). d, contractile response to oxotremorine-M was measured in the presence of 4-DAMP (10 nM) (○) and 4-DAMP (10 nM) plus atropine (10 nM) (■). The data represent the mean contractile measurements ± S.E.M. from four experiments.
in the ileum and trachea. When tested in the ileum, p-FHHSiD (0.3 μM) caused a 1.5-fold increase in the EC₅₀ value of histamine and 6% decrease in the maximal response (Fig. 4a). At a concentration of 1 μM, p-FHHSiD caused a 1.9-fold increase in the EC₅₀ value of histamine and 34% decrease in the maximal response. These decreases in the contractile action of histamine at 0.3 μM and 1.0 μM p-FHHSiD are equivalent to those caused by 39 and 70% decreases, respectively, in the observed coupling efficiency of histamine receptors in the ileum. This decrease in the activity of histamine was significant at both the low (0.3 μM; *P* = 0.027) and high (1.0 μM; *P* = 0.044) concentrations of p-FHHSiD. In contrast, the same concentrations of p-FHHSiD had no significant effect on contractions elicited to histamine in the trachea (Fig. 4b). The results indicate that, in the concentration range of 0.3 to 1.0 μM, p-FHHSiD inhibits the contractile effects of histamine in the ileum, but not in the trachea.

The relatively small, 2.8-fold difference between the resultant pKᵦ values of p-FHHSiD in ileum and trachea suggests a small difference in the potency of p-FHHSiD for antagonizing muscarinic receptors eliciting contraction in the two tissues. Such a difference might be attributed to a greater contribution of the M₂ muscarinic receptor to contraction in the trachea compared with the ileum, because p-FHHSiD exhibits approximately 16-fold higher binding affinity for M₃ receptors compared with M₂ (Esquela et al., 1996). Consequently, we wondered whether another muscarinic antagonist with comparable selectivity for M₃ receptors over M₂ might exhibit a similar preference for the ileum relative to trachea. To test this idea, we measured the competitive antagonism of oxotremorine-M-induced contractions by 4-DAMP in ileum and trachea. 4-DAMP exhibits 10-fold higher binding affinity for human M₃ muscarinic receptors (pKᵦ = 8.81) expressed in CHO cells compared with human M₂ receptors (pKᵦ = 7.87) (Griffin et al., 2004). At a concentration of 10 nM, 4-DAMP caused similar shifts (10.2- and 15.9-fold) in the oxotremorine-M concentration-response curve in ileum and trachea, respectively. These results yielded pKᵦ values of 8.96 ± 0.12 and 9.17 ± 0.05 in ileum (n = 4) and trachea (n = 3), respectively. There was no significant difference between the pKᵦ values of 4-DAMP in ileum and trachea (P = 0.16). The pKᵦ values estimated for 4-DAMP from the data just described as well as those in Fig. 3 are similar to the binding affinity of 4-DAMP estimated at human M₃ muscarinic receptors expressed in CHO cells (pKᵦ = 8.81), but not with that estimated at human M₂ muscarinic receptors (pKᵦ = 7.87) (Griffin et al., 2004). In these latter assays, binding was measured in a modified KRB buffer similar to that used in the present study.

**Phosphoinositide Hydrolysis.** Since muscarinic agonist-induced contractions represent a complicated response involving several steps and perhaps more than one muscarinic receptor subtype, we were interested in examining the inhibitory effects of p-FHHSiD on a simpler response more closely coupled to the M₃ muscarinic receptor in the ileum and trachea. Using this approach, it seemed more likely that the inhibitory effect of p-FHHSiD would be confined to competitive antagonism of a single receptor subtype, i.e., the M₃. Consequently, we measured the ability of p-FHHSiD to inhibit oxotremorine-M-stimulated phosphoinositide hydrolysis in slices of the bovine trachea and longitudinal muscle of the guinea pig ileum. Since the tracheal smooth muscle from the guinea pig is too small to provide an adequate source of tissue, we used the cow as a source of trachea for these experiments. As shown in Fig. 5, oxotremorine-M caused a concentration-dependent increase in phosphoinositide hydrolysis in both ileum and trachea with the maximal effect being 36,000 ± 3000 and 35,000 ± 9400 cpm, respectively, when expressed as cpm of [³H]inositolphosphates. The pEC₅₀ values of oxotremorine-M in these two tissues were 5.18 ± 0.11 and 5.96 ± 0.15, respectively. At a concentration of 1 μM, p-FHHSiD shifted the concentration-response curve of oxotremorine-M to the right 13.0- and 12.6-fold in ileum and trachea, respectively. The pKᵦ values of p-FHHSiD were estimated from these shifts, and these values are listed in Table 1. The pKᵦ estimates in the ileum 7.08 ± 0.046 and trachea 7.06 ± 0.12 are the same.

**Discussion**

Our results on the antimuscarinic effects of p-FHHSiD in the guinea pig ileum and trachea are consistent with those of Eglen et al. (1990), who reported that this antagonist exhibits approximately 10-fold higher potency at blocking the ability of several muscarinic agonists to elicit contraction of the ileum compared with the trachea. When calculated using a simple competitive model, we estimated that the observed pKᵦ values of p-FHHSiD in the ileum and trachea were approximately 7.95 and 6.87, respectively, corresponding to a 12-fold difference in antagonist potency.

Several possibilities have been suggested for the mechanism of the tissue selective effect of p-FHHSiD, including...
Thus, these data show that \( p\)-FHHSiD exhibits approximately 16-fold higher affinity for \( M_3 \) muscarinic receptors over \( M_2 \) (Esqueda et al., 1996), it might seem that a greater contribution of the \( M_2 \) receptor to contraction in the trachea could explain the ideal selectivity of \( p\)-FHHSiD relative to trachea. However, we found that another muscarinic antagonist (4-DAMP) with 10-fold higher affinity for \( M_3 \) receptors over \( M_2 \) lacked ideal selectivity, which provides no support for the latter hypothesis. Studies on ileum and trachea from muscarinic receptor knockout mice have shown a large loss of muscarinic contractile function in \( M_3 \) knockout mice, a small loss of function in \( M_2 \) knockout mice, and a complete loss of function in \( M_2/M_3 \) double knockout mice (Matsui et al., 2000, 2002; Stengel et al., 2000, 2002). Nevertheless, the relatively small \( M_3 \) receptor-mediated contractions observed in \( M_3 \) receptor knockout mice are greater in trachea (Stengel et al., 2002) compared with ileum (Matsui et al., 2000), suggesting a greater role for the \( M_3 \) receptor in the trachea. In guinea pig, \( M_3 \) muscarinic receptors have been shown to mediate a high potency inhibition of the relaxant effects of agents that increase cAMP (Thomas et al., 1993) and a low potency enhancement in \( M_3 \) receptor-mediated contractions (Sawyer and Ehlert, 1999). However, these latter mechanisms are ultimately contingent upon \( Ca^{2+} \) mobilization by another receptor, like the \( M_3 \). It has been shown that the competitive inhibition of this type of receptor interaction has a tendency to resemble the pharmacological profile of the directly acting receptor (i.e., \( M_3 \)) and not that of the conditionally acting receptor (i.e., \( M_2 \)) (Ehlert et al., 1999; Sawyer and Ehlert, 1999; Ehlert, 2003). Thus, the ideal selectivity of \( p\)-FHHSiD relative to trachea cannot be explained by the differential contribution of the \( M_3 \) receptor to contraction.

Perhaps the strongest data to suggest an alternative mechanism for the tissue-selective effects is shown in Fig. 6, which shows a histogram of the binding affinities of \( p\)-FHHSiD at human recombinant \( M_2 \) and \( M_3 \) receptors plotted together with the \( pK_B \) values estimated by different methods in functional assays on the guinea pig ileum and guinea pig and bovine trachea. The binding affinities were measured in a prior study (Esqueda et al., 1996) using a modified KRB buffer nearly identical to that used for the contractile assays of this study. It can be seen that \( p\)-FHHSiD has approximately 16-fold higher binding affinity for \( M_3 \) receptors (\( pK_B = 7.30 \)) compared with \( M_2 \) (\( pK_B = 6.09 \)). Moreover, its binding affinities (\( pK_B \) values) for \( M_1 \) (7.08), \( M_4 \) (7.08), and \( M_5 \) (6.26) receptors are in between those shown for \( M_2 \) and \( M_3 \) receptors in Fig. 6 (Esqueda et al., 1996). Surprisingly, its observed \( pK_B \) value for antagonizing oxotremorine-M-mediated contractions in the ileum exceeds its binding affinity for the \( M_3 \) receptor as well as any known muscarinic subtype by at least 4.5-fold. In contrast, its observed \( pK_B \) value for blocking contractions in the trachea is nearly the same as its binding affinity at the \( M_3 \) receptor. If the tissue selective action of \( p\)-FHHSiD were caused by a differential contribution of the \( M_3 \) receptor to contraction, then one would expect the observed \( pK_B \) values to vary across tissues within a range bounded by the high affinity \( M_3 \) \( pK_B \) and the low affinity \( M_2 \) \( pK_B \). However, as just described, the observed \( pK_B \) in the ileum exceeds this range, which strongly suggests an alternative, nonmuscarinic receptor mechanism. In contrast, the resultant \( pK_B \) values of \( p\)-FHHSiD in the ileum and trachea are similar to its binding affinity at the \( M_3 \) receptor, which is
consistent with the well known direct role that the M3 receptor plays in contraction in these tissues. Moreover, the pK_B values of p-FHHSiD for antagonizing oxotremorine-M-mediated phosphoinositide hydrolysis are also in agreement with the binding affinity observed at M3 receptors. Collectively, the data show that p-FHHSiD does not discriminate markedly between M3 muscarinic receptors in the ileum and trachea.

Although our data show little difference between the binding affinity of p-FHHSiD for recombinant M3 receptors and its resultant pK_B values in ileum and trachea, we did observe that the resultant pK_B value of p-FHHSiD in the ileum was 2.8-fold greater than that observed in the trachea, although the difference was not quite significant (P = 0.067). This modest difference in resultant pK_B values cannot entirely explain the relatively large, ileal selectivity of p-FHHSiD relative to trachea.

Resultant analysis is an ingenious method for estimating the dissociation constant of an antagonist in situations where the antagonist has at least two actions: competitive antagonism at the receptor and some other nonreceptor-mediated effect. Our observation that the observed pK_B value of p-FHHSiD in the ileum (7.95) exceeds its corresponding resultant pK_B value (7.21) by a 5.5-fold difference in potency suggests that p-FHHSiD may possess an alternative, inhibitory mechanism on muscarinic agonist-induced contractions of the ileum distinct from competitive antagonism of M3 receptors. Accordingly, we noted that p-FHHSiD caused a moderate inhibition of histamine-induced contractions of the ileum in the concentration range of 0.3 to 1.0 μM, while having no effect on histamine-induced contractions of the trachea. This inhibitory effect on the ileum was substantial at 1 μM, equivalent to that caused by a 70% reduction in the intrinsic efficacy of histamine. However, we were unable to demonstrate an inhibitory effect on histamine-induced contractions of the ileum in the low concentration range (0.03–0.1 μM) where an ileal-selective, antimuscarinic action of p-FHHSiD is apparent.

In considering the actions of p-FHHSiD, it is important to note that the compound contains a chiral silicon atom. Homologous compounds with a similar chiral center, but lacking the Si-O bond, often exhibit a large difference in the antimuscarinic effects of the enantiomers (Tacke et al., 1995, 2001). Thus, it is possible that the different effects of p-FHHSiD (i.e., competitive antagonism and nonmuscarinic receptor mediated inhibition) observed in this study are mediated by different enantiomers. This question is difficult to address because enantiomers of silanol similar to p-FHHSiD racemize quickly in aqueous solution because of the highly polar Si-O bond (Tacke et al., 1987).

A variety of interesting antimuscarinic agents have been developed and analyzed for their muscarinic receptor binding properties and inhibitory effects in functional assays. Some of these agents have been shown to exhibit tissue selectivity in contractile assays on smooth muscle (e.g., zamifenacin; Watson et al., 1995). Our results illustrate the power of resultant analysis in determining the true muscarinic receptor selectivity of an antagonist and suggest caution in assuming that the apparent tissue selectivity of a muscarinic antagonist implies tissue differences in muscarinic receptors. Our results might also suggest the existence of a high affinity (pK_D = 8.0) nonmuscarinic binding site for p-FHHSiD that may nonetheless be a useful target for the development of tissue selective smooth relaxant agents that inhibit cholinergic induced contractions.

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Address correspondence to: Dr. Frederick J. Ehlert, Department of Phar- 
macology, University of California, Irvine, Irvine, CA 92697-4625. E-mail: fjehlert@uci.edu