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Comparison of the Antimuscarinic Action of *p*-Fluorohexahydrosiladifenidol in Ileal and Tracheal Smooth Muscle

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Abbreviations: 4-DAMP, N,N-dimethyl-4-piperidinyl diphenylacetate; EC_{50}, concentration of agonist eliciting half-maximal response; E_{max}, maximal response; NMS, N-methylscopolamine; *p*-FHHSiD, *p*-fluorohexahydrosiladifenidol

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ABSTRACT

We investigated the ability of the muscarinic antagonist, *p*-fluorohexahydrosiladifenidol, 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 as compared to those of the trachea. When estimated using a simple model for competitive antagonism, the observed dissociation constant of *p*–fluorohexahydrosiladifenidol exhibited approximately 12-fold higher potency in the ileum as compared to the trachea. We also investigated the ability of *p*-fluorohexahydrosiladifenidol 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*-fluorohexahydrosiladifenidol 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 potencies of *p*–fluorohexahydrosiladifenidol 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*–fluorohexahydrosiladifenidol for antagonizing oxotremorine-M-induced phosphoinositide hydrolysis in guinea pig ileum and bovine trachea. We also noted that p–fluorohexahydrosiladifenidol (0.3 to 1.0 μM) moderately inhibited histamine-induced contractions of ileum but not of trachea. Our results suggest that p–fluorohexahydrosiladifenidol does not discriminate markedly between M_3 muscarinic receptors in the ileum and trachea and that it may posses a more potent, non-muscarinic inhibitory effect on contraction in the ileum.

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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*-fluorohexahydrosiladifenidol (*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 as compared to the trachea (Eglen et al., 1990), whereas zamifenacin is more potent at blocking contraction in the ileum and trachea as compared to 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 and coworkers (1988; 1989) who found that the compound was 67-fold more potent at blocking M_3 receptor-mediated contractions of the ileum as compared to M_2 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 as compared to 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 M_3 muscarinic receptors eliciting contraction and phosphoinositide hydrolysis in ileum and trachea, but that it may exhibit a more potent, non-muscarinic receptor-mediated inhibitory effect in the ileum.

MATERIALS AND METHODS

Contractile assays: Male guinea pigs (Hartely, 300 – 500 g) were euthanized with CO_2 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 ceacum. 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 $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM $NaHCO_3$ and 10 mM glucose) gassed with O_2/CO_2 (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 fashion. Tracheal strips were allowed to equilibrate for two hr before the start of experiments, whereas ilea were equilibrated for at least one hr. 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 fashion. 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 – 30 sec in the ileum and for six min in the trachea. When present, antagonists were allowed to equilibrate with the tissue for 1 hr 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 EC_{50} and E_{max} 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 lab on ice. Slices of tracheal smooth muscle were prepared and placed in KRB buffer as described previously (Ostrom and Ehlert, 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 MacIlwaine tissue chopper. The slices were washed three times in warm (37°C) KRB buffer gassed with O_2/CO_2 (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 [^{3}H]inositol labeling and ion exchange method of Berridge (1982) and the perchloric acid extraction method of Kendal and Hill (1990). Tissue slices were incubated with [^{3}H]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 O_2/CO_2 (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 [^{3}H]inositolphosphates in the absence of LiCl during this time. Following this equilibration phase, an aliquot of LiCl (3µl) was added to achieve a final concentration of 10 mM, 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 3000 x g 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 AG1X8 (100 – 200 mesh). The column was washed four times with 5 ml of water each time. [^{3}H]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 [^{3}H]inositolphosphates (cpm).

Calculations: The EC_{50} and E_{max} (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 (K_B) 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 K_B value is calculated according to the method of Schild (Arunlakshana and Schild, 1959):

$$K_B = \frac{B}{(CR-1)} \qquad 1$$

In this equation, *CR* (concentration ratio) denotes the EC_{50} 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 K_B of *p*-FHHSiD introduces the possibility that part of the effect of *p*-FHHSiD may be mediated through a non-muscarinic receptor mechanism, as well as through competitive antagonism of the receptor. In this instance, the K_B value for the competitive component of the effect of *p*-FHHSiD is calculated according to the method of Black and coworkers (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 K_B value was calculated using the following equation:

$$K_B = \frac{B(CR^* - 1)}{1 + C/K_C - CR^*} \qquad 2$$

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 (equation 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_{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 (see Ostrom and Ehlert (Ostrom and Ehlert, 1997)). We refer to the apparent 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_{max}, into common currency.

Reagents: Reagents were obtained from the following sources: oxotremorine-M and *p*-FHHSiD, Sigma/RBI (Natick, MA); atropine, indomethacin and NMS, Sigma Chemical Company (St Louis, MO) and [^{3}H]inositol, Dupont NEN (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 (see Figure 1). In the ileum, oxotremorine-M elicited potent contractile effects, characterized by a pEC_{50} value of 7.75 ± 0.023 and an E_{max} of 5.18 ± 0.32 g (see Figure 1*a*). At a concentration of 0.03 μM, *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 μM), the shift in the concentration-response curve was 12.8-fold. A small increase in E_{max} was also noted in the presence of *p*-FHHSiD; however, control experiments showed that the E_{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 (equation 1), and these values are listed in Table 1 as observed pK_B values. There was a small, but nonetheless significant (P = 0.013), 1.7-fold difference in the estimates of K_B at the two different concentrations of *p*-FHHSiD; the average pK_B value in the ileum was 7.95 ± 0.033.

In the trachea, the pEC_{50} and E_{max} values of oxotremorine-M were 7.21 ± 0.029 and 3.9 ± 0.46 g, respectively (see Figure 1*b*). At a concentration of 0.3 μM, *p*-FHHSiD shifted the concentration-response curve of oxotremorine-M to the right about 3.3-fold. At a higher concentration of 1.0 μM, *p*-FHHSiD shifted the concentration-response curve of oxotremorine-M about 8.8-fold. As observed in the ileum, the E_{max} value of oxotremorine-M increased when measured in the presence of *p*-FHHSiD; however, control experiments showed that this effect could be attributed to time and not to *p*-FHHSiD. The dissociation constant (K_B) of *p*-FHHSiD

for antagonizing the contractions elicited to oxotremorine-M in the trachea was calculated from the shift in the concentration response curve assuming a competitive model (equation 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 ± 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 x 10^{-7}).

The estimation of the K_B value of *p*-FHHSiD by equation 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 non-receptor-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 non-receptor-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 Figure 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 (Figure 2*a*). Similar effects were observed in the trachea (see Figure 2*b*). The estimates of the pK_B values of atropine in the ileum and trachea were 8.70 ± 0.044 and 9.22 ± 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 μM) and high (0.1 μM) concentrations of *p*–FHHSiD were 4.1- and 2.6-fold respectively (see Figure 2*c*). Using equation 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 estimates of the resultant pK_B values of *p*-FHHSiD were 7.17 ± 0.20 and 7.25 ± 0.11 when calculated at concentrations of 0.03 and 0.1 μM, respectively, in the presence of atropine (10 nM) in the ileum. These resultant pK_B values are listed in Table 1. It can be seen that there is little difference between these estimates made at the two concentrations of *p*-FHHSiD in the ileum, and the average value was estimated at 7.21 ± 0.14. Similar experiments were run in the trachea (see Figure 2*d*), except that higher concentrations of *p*-FHHSiD were used. In the trachea, the atropine-induced increases in the EC_{50} value of oxotremorine–M at low (0.3 μM) and high (1.0 μM) concentrations of *p*–FHHSiD were 9.4- and 4.3-fold, respectively. There was little difference between the corresponding resultant pK_B values of *p*-FHHSiD in the trachea, and these were calculated to be 6.71 ± 0.21 and 6.82 ± 0.076, respectively, with the average estimate being 6.76 ± 0.14. Table 1 summarizes resultant analysis. It can be seen that, in the ileum, the resultant pK_B values of *p*-FHHSiD are significantly smaller (lower potency) than the corresponding observed pK_B values estimated using the simple competitive model. In contrast, there is little difference between the observed and resultant pK_B values of *p*–FHHSiD in the trachea. Although the average estimate of the resultant pK_B value of *p*–FHHSiD in the ileum (pK_B = 7.21) was greater than that observed in 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 eliciting 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 (see Figure 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 (see Figure 3*a*), yielding simple pK_B values estimated assuming a competitive model (equation 1) of 9.17 and 9.09 (see Table 2). In these experiments atropine (10 nM) shifted the oxotremorine-M concentration-response curve to the right 12-fold (see figure 3*b*), 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 cause by 4-DAMP at 3 and 10 nM were 3.6- and 1.9-fold, respectively (see figure 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 (equation 1) are similar to those for resultant analysis (equation 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's 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 non-muscarinic contractile agent (i.e., histamine) in the ileum and trachea. When tested in the ileum, *p*-FHHSiD (0.3 μM) caused a 1.5-fold increase in the EC_{50} value of histamine and 6% decrease in the maximal response (see Figure 4*a*). At a concentration of 1 μM, *p*-FHHSiD caused a 1.9-fold increase in the EC_{50} 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 (see Figure 4*b*). The results indicate that, in the concentration range of 0.3 – 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 pK_B 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 M_2 muscarinic receptor to contraction in the trachea as compared to the ileum, because p-FHHSiD exhibits approximately 16-fold higher binding affinity for M_3 receptors as compared to M_2 (Esqueda et al., 1996). Consequently, we wondered if another muscarinic antagonist with comparable selectivity for M_3 receptors over M_2 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 ten-fold higher binding affinity for human M_3 muscarinic receptors (pK_D = 8.81) expressed in CHO cells as compared to human M_2 receptors (pK_D = 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 pK_B 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 pK_B values of 4-DAMP in ileum and trachea (P = 0.16). The pK_B values estimated for 4-DAMP from the data just described as well as those in Figure 3 are similar to the binding affinity of 4-DAMP estimated at human M_3 muscarinic receptors expressed in CHO cells (pK_D = 8.81), but not with that estimated at human M_2 muscarinic receptors (pK_D = 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 M_3 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 M_3. 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 Figure 5, oxotremorine-M caused a concentration-dependent increase in phosphoinositide hydrolysis in both ileum and trachea with the maximal effect being 36,000 ± 3,000 and 35,000 ± 9,400 cpm, respectively, when expressed as cpm of [^{3}H]inositolphosphates. The pEC_{50} 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*-

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FHHSiD shifted the concentration-response curve of oxotremorine-M to the right 13.0- and 12.6-fold in ileum and trachea, respectively. The pK_B values of *p*-FHHSiD were estimated from these shifts, and these values are listed in Table 1. The pK_B 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 as compared to the trachea. When calculated using a simple competitive model, we estimated that the observed pK_B 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 pharmacokinetic (i.e., uptake process for *p*-FHHSiD) as well as pharmacodynamic (i.e., tissue differences in M_3 muscarinic receptors) explanations (Eglen et al., 1990). To address the latter possibility, we investigated the effects of *p*-FHHSiD on oxotremorine-M-mediated phosphoinositide hydrolysis in bovine trachea and guinea pig ileum. We used bovine trachea because the guinea pig trachea is too small to measure phosphoinositide hydrolysis feasibly. Competitive antagonism studies have shown that the phosphoinositide hydrolysis is mediated by the M_3 muscarinic receptor in ileum (Candell et al., 1990) and trachea (Roffel et al., 1990). We found that *p*-FHHSiD exhibited similar potency for blocking muscarinic receptor-mediated phosphoinositide hydrolysis in the ileum and trachea. Thus, these data show that *p*-FHHSiD does not discriminate between M_3 muscarinic receptors in guinea pig ileum and bovine trachea. This observation is highly relevant because it is thought that the M_3 receptor mediates contraction directly in guinea pig ileum and trachea (see reviews by Eglen et al. (1996) and Ehlert et al. (1997)). It is conceivable that *p*-FHHSiD might exhibit different

affinities for bovine and guinea pig tracheal M_3 receptors; however, the agreement between the observed pK_B value for phosphoinositide hydrolysis in bovine trachea and the observed and true pK_B values for antagonizing contraction in the guinea pig trachea argues against this possibility.

Since both M_2 and M_3 muscarinic receptors have a contractile role in smooth muscle (see review by Ehlert (2003)) and since *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 ileal selectivity of *p*-FHHSiD relative to trachea. However, we found that another muscarinic antagonist (4-DAMP) with ten-fold higher affinity for M_3 receptors over M_2 lacked ileal 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; Stengel et al., 2000; Matsui et al., 2002; Stengel et al., 2002). Nevertheless, the relatively small M_2 receptor-mediated contractions observed in M_3 receptor knockout mice are greater in trachea (Stengel et al., 2002) as compared to ileum (Matsui et al., 2000), suggesting a greater role for the M_2 receptor in the trachea. In guinea pig, M_2 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 ileal selectivity of *p*-FHHSiD relative to trachea cannot be explained by the differential contribution of the M_2 receptor to contraction.

Perhaps the strongest data to suggest an alternative mechanism for the tissue selective effects is shown in Figure 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_D = 7.30) as compared to M_2 (pK_D = 6.09). Moreover, its binding affinities (pK_D*'s*) 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 Figure 6 (see 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_2 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_D and the low affinity M_2 pK_D However, as just described, the observed pK_B in the ileum exceeds this range, which strongly suggests an alternative, non-muscarinic 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 M_3 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 M_3 receptors. Collectively, the data show that *p*-FHHSiD does not discriminate markedly between M_3 muscarinic receptors in the ileum and trachea.

Although our data show little difference between the binding affinity of *p*-FHHSiD for recombinant M_3 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 non-receptor 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 M_3 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, anti-muscarinic 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; Tacke et al., 2001). Thus, it is possible that the different effects of *p*–FHHSiD (i.e., competitive antagonism and non-muscarinic receptor mediated inhibition) observed in this study are mediated by different enantiomers. This question is difficult to address because enantiomers of silanols 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) non-muscarinic 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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LEGENDS FOR FIGURES

Figure 1: *The effects of p-FHHSiD on the contractile response to oxotremorine-M in the guinea pig ileum (a) and trachea (b).* *a*: The contractile response to oxotremorine-M was measured in the ileum in the absence (❍) and presence of *p*-FHHSiD at concentrations of 0.03 μM (Δ) and 0.1 μM (❑). *b*: The contractile response to oxotremorine-M was measured in the trachea in the absence (❍) and presence of *p*-FHHSiD at concentrations of 0.3 μM (Δ) and 1.0 μM (❑). The data represent the mean contractile measurements ± SEM from four to five experiments.

Figure 2: *The 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*: The contractile response to oxotremorine-M was measured in the ileum in the absence (❍) and presence of atropine (10 nM) (●). *b*: The contractile response to oxotremorine-M was measured in the ileum in the presence of *p*-FHHSiD (0.03 μM) (Δ) and *p*-FHHSiD (0.03 μM) plus atropine (10 nM) (▲). *c*: The 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*: The contractile response to oxotremorine-M was measured in the trachea in the absence (❍) and presence of atropine (10 nM) (●). *e*: The contractile response to oxotremorine-M was measured in the trachea in the presence of *p*-FHHSiD (0.3 μM) (Δ) and *p*-FHHSiD (0.3 μM) plus atropine (10 nM) (▲). *f*: The 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 ± SEM from four to five experiments.

Figure 3: *The effects of 4-DAMP and atropine on the contractile response to oxotremorine-M in the guinea pig ileum. a*: The contractile response to oxotremorine-M was measured in the ileum in the absence (❍) and presence of 4-DAMP at concentrations of 3.0 nM (Δ) and 10 nM (❑). *b*: The contractile response to oxotremorine-M was measured in the absence (❍) and presence of atropine (10 nM) (●). *c*: The contractile response to oxotremorine-M was measured in the presence of 4-DAMP (3.0 nM) (Δ) and 4-DAMP (3.0 nM) plus atropine (10 nM) (▲). *d*: The 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 ± SEM from four experiments.

Figure 4: *The effects of p-FHHSiD on the contractile response to histamine in the guinea pig ileum (a) and trachea (b). a*: The contractile response to histamine was measured in the ileum in the absence (❍) and presence of *p*-FHHSiD at concentrations of 0.3 μM (Δ) and 1 μM (❑). *b*: The contractile response to histamine was measured in the trachea in the absence (❍) and presence of *p*-FHHSiD at concentrations of 0.3 μM (Δ) and 1.0 μM (❑). The data represent the mean contractile measurements ± SEM from four experiments.

Figure 5: *The effects of p-FHHSiD on oxotremorine-M-stimulated phosphoinositide hydrolysis in the guinea pig ileum (a) and bovine trachea (b).* The assays were carried out in the absence (❍) and presence of *p*-FHHSiD (1.0 μM) (●). The data represent the mean values ± SEM from four experiments.

Figure 6: *Comparison of the dissociation constants of p-FHHSiD for muscarinic receptors determined in binding assays on CHO cells expressing M_2 and M_3 muscarinic receptors (a) and in functional assays on ileum and trachea (b).* The binding data are from Esqueda et

al. (1996), whereas the functional data are from Table 1. The $pK_{B\ obs}$ and $pK_{B\ true}$ values refer to the estimates calculated using equation 1 or equation 2, respectively.

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Table 1: The dissociation constants of *p*-FHHSiD estimated in functional assays on the ileum and trachea.[a]

	Ileum		*Trachea*	
Assay	*Observed* pK_B [b]	*Resultant* pK_B [c]	*Observed* pK_B [b]	*Resultant* pK_B [c]
Contraction				
Low *p*-FHHSiD [d]	7.84 ± 0.045 [f]	7.17 ± 0.20	6.90 ± 0.057	6.71 ± 0.17
High *p*-FHHSiD [e]	8.07 ± 0.041 [g]	7.25 ± 0.11	6.89 ± 0.075	6.82 ± 0.078
Phosphoinositide hydrolysis				
p-FHHSiD (1 µM)	7.08 ± 0.046		7.06 ± 0.12	

[a] The mean estimates ± SEM are shown and were calculated from the data presented in Figures 1, 2 and 4.

[b] Negative logarithm of the observed K_B value calculated according to equation 1.

[c] Negative logarithm of the observed K_B value calculated according to equation 2.

[d] The concentrations of *p*-FHHSiD in the ileum and trachea were 0.03 and 0.3 µM, respectively.

[e] The concentrations of *p*-FHHSiD in the ileum and trachea were 0.1 and 1.0 µM, respectively.

[f] Significantly different from the corresponding resultant pK_B in the ileum, P = 0.011

[g] Significantly different from the corresponding resultant pK_B in the ileum, P = 0.029

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Table 2: The dissociation constants of 4-DAMP and atropine estimated in contractile assays on the ileum.[a]

	Ileum	
Assay	*Observed* pK_B [b]	*Resultant* pK_B [c]
Contraction		
Atropine (10 nM)	9.04 ± 0.071	NA
4-DAMP (3 nM)	9.17 ± 0.12	9.04 ± 0.16
4-DAMP (10 nM)	9.09 ± 0.14	9.15 ± 0.28

[a] The mean estimates ± SEM are shown and were calculated from the data presented in Figure 3.

[b] Negative logarithm of the observed K_B value calculated according to equation 1.

[c] Negative logarithm of the observed K_B value calculated according to equation 2.

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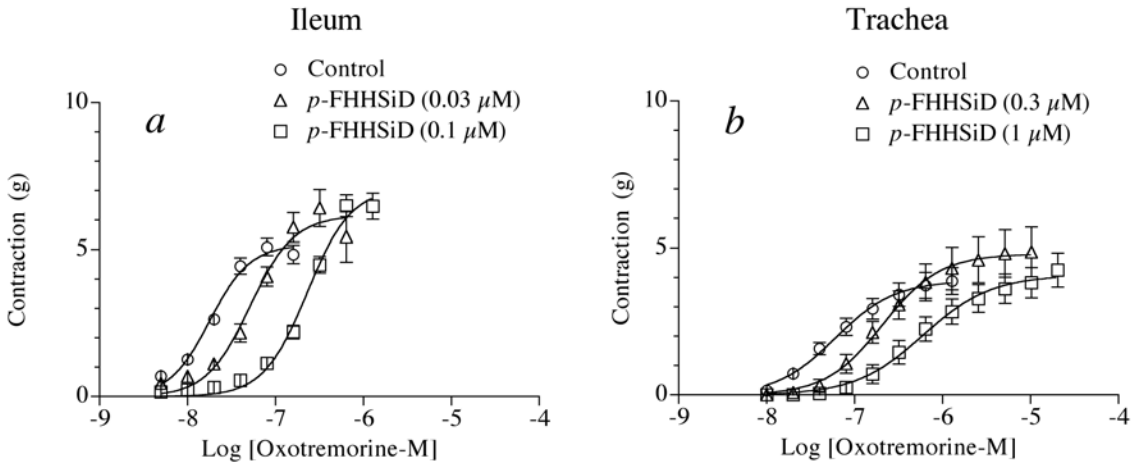

Figure 1

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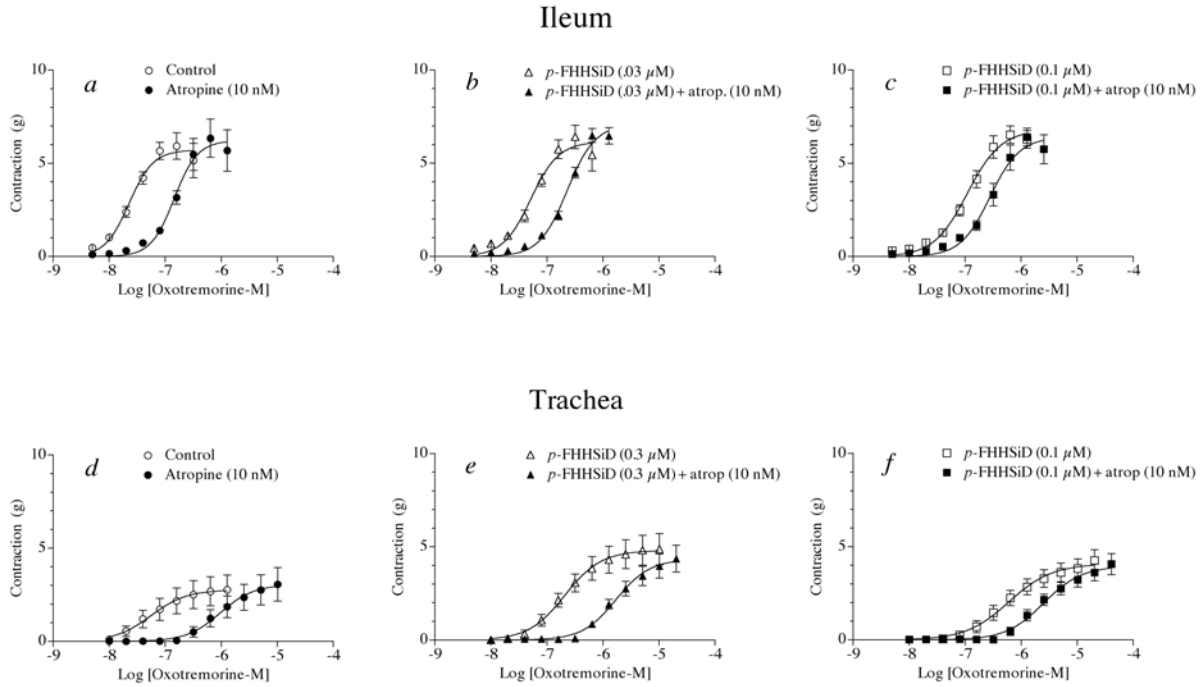

Figure 2

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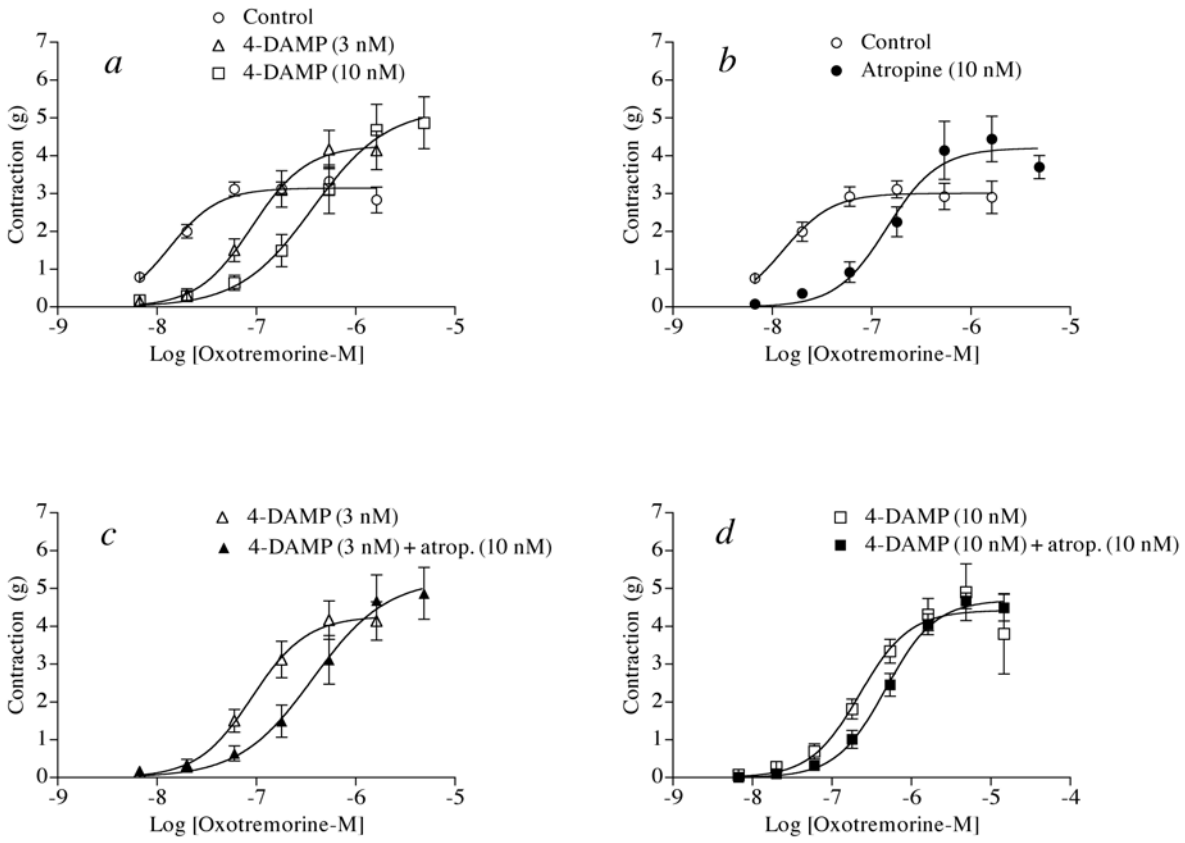

Figure 3

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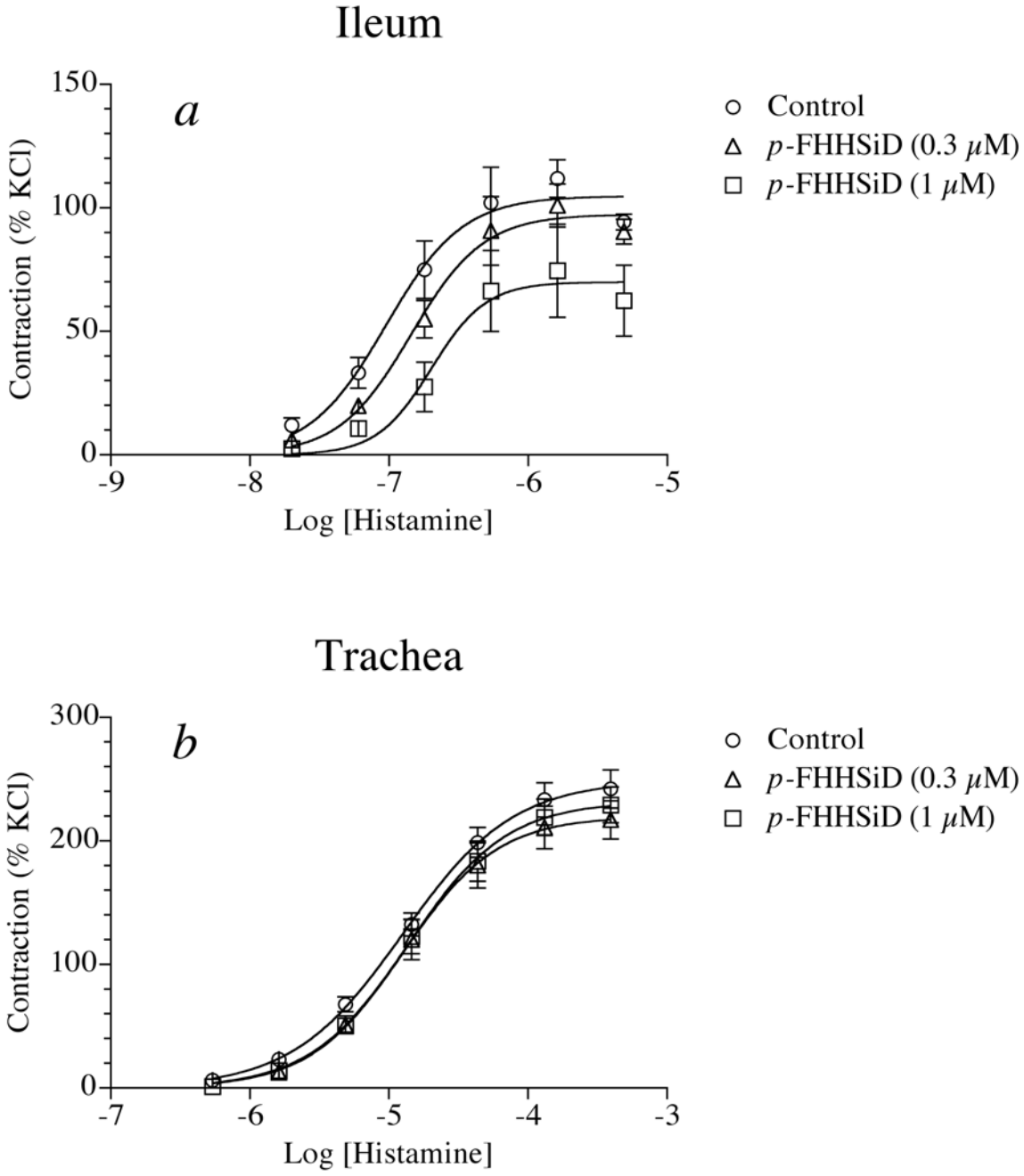

Figure 4

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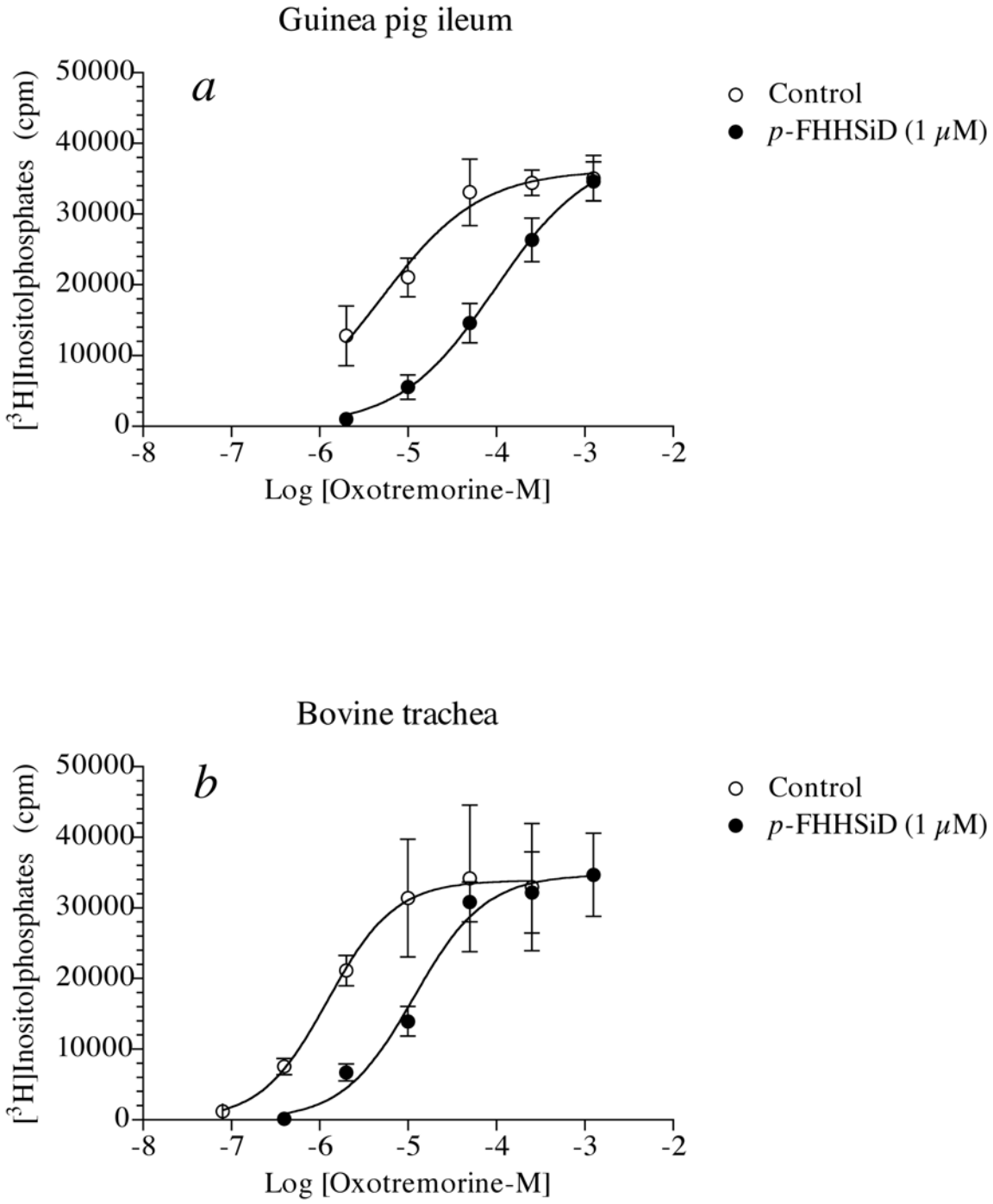

Figure 5

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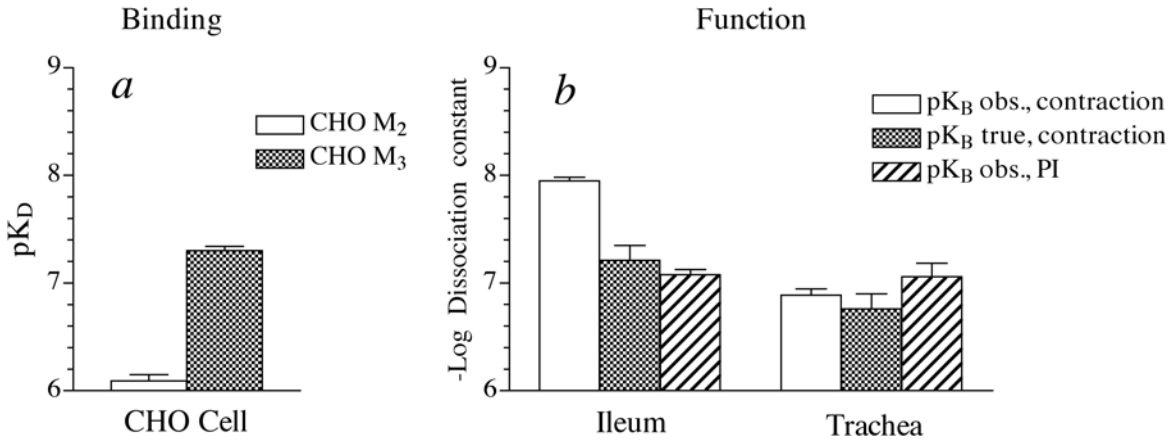

Figure 6