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Differential Coupling of Subtypes of the Muscarinic Receptor to Adenylate Cyclase and Phosphoinositide Hydrolysis in the Longitudinal Muscle of the Rat Ileum

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SUMMARY

The binding affinities of selective muscarinic antagonists were compared with their ability to block receptor-mediated inhibition of adenylate cyclase and stimulation of phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum. When measured by competitive inhibition of the binding of *N*-[^{3}H]methylscopolamine, the binding properties of selective muscarinic antagonists were consistent with a two-site model. Approximately 84% of the binding sites (major sites) had high affinity for the M_2-selective antagonists methoctramine and AF-DX 116 (11[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyr-ido[2,3-b][1,4]benzodiazepine-6-one), whereas the remainder of the sites (minor sites) had high affinity for hexahydrosiladifenidol and its *para*-fluoro derivative. There was good agreement between the estimates of the dissociation constants of muscarinic antagonists for the major binding site and those measured by antagonism of the adenylate cyclase response. There was also good agreement between the dissociation constants of muscarinic antagonists for the minor binding site and those measured by antagonism of the phosphoinositide response and the contractile response. Our data indicate that there are at least two types of muscarinic receptors in the longitudinal muscle of the ileum, the more abundant being an M_2 receptor, which mediates an inhibition of adenylate cyclase activity, and the less abundant being an M_3 receptor, which triggers contraction and phosphoinositide hydrolysis.

A variety of evidence now indicates that muscarinic receptors can be divided into three categories on the basis of their selectivity for antagonists (1–6). M_1 muscarinic receptors have high affinity for pirenzepine and are abundant in brain and sympathetic ganglia (4, 7). The M_2 subtype has high affinity for methoctramine and AF-DX 116 (11[[2-[(diethylamino)-methyl]1 - piperidinyl]acetyl] - 5,11 - dihydro - 6*H*-pyrido[2,3-b] [1,4]benzodiazepine-6-one) and accounts for most, if not all, of the muscarinic receptors in the mammalian myocardium (5, 6, 8). M_3 muscarinic receptors have high affinity for *p*-FHHSiD and trigger responses in smooth muscle and exocrine glands (9, 10). To date, five genes for the muscarinic receptor have been cloned (11–15) and designated as m_1, m_2, m_3, m_4, and m_5 by Bonner *et al.* (13, 15). On the basis of the binding properties of the expressed recombinant receptors and the distribution of their mRNAs, it appears that the m_1, m_2, and m_3 subtypes of the muscarinic receptor correspond to the M_1, M_2, and M_3 pharmacological classes, respectively (16–19). The other two subtypes of the muscarinic receptor (i.e., m_4 and m_5) have not been sufficiently characterized to allow an unambiguous pharmacological classification. The results of studies in which the cloned subtypes of the muscarinic receptor were expressed in cells previously lacking muscarinic receptors now indicate that the m_1, m_3, and m_5 subtypes couple preferentially to phosphoinositide hydrolysis, whereas the m_2 and m_4 subtypes mediate an inhibition of adenylate cyclase (20, 21).

The isolated guinea pig ileum has long been used as an assay for muscarinic activity, and it is now clear that the M_3 receptor elicits contraction in this tissue (22). However, until recently, the overall binding properties of muscarinic receptors in the guinea pig ileum appeared to be the same as those of the pharmacologically distinct cardiac M_2 muscarinic receptor (23, 24). This enigma was recently sorted out by Giraldo *et al.* (25) and Michel and Whiting (26), who found that, although the ileum contains an abundance of M_2 receptors, it also contains a small population of sites having binding properties that could account for the pharmacological specificity of the contractile response.

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ABBREVIATIONS: *p*-FHHSiD, *para*-fluorohexahydrosiladifenidol; HHSiD, hexahydrosiladifenidol; NMS, *N*-methylscopolamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; KRB, Krebs-Ringer bicarbonate.

In the present study we have investigated the binding properties and signaling mechanisms of muscarinic receptors in the longitudinal muscle of the rat ileum. The results of our binding studies with the rat are consistent with previous work with the guinea pig, indicating that the longitudinal muscle of the ileum contains a preponderance of M_2 muscarinic receptors and a small amount of receptors having binding properties not inconsistent with M_3 receptors. We have also found that the more abundant site (M_2 site) mediates an inhibition of adenylate cyclase activity, whereas the less abundant site triggers phosphoinositide hydrolysis.

Materials and Methods

Radioligand binding assay. Male Sprague-Dawley rats (200–300 g) were sacrificed by decapitation, and the heart and ileum were quickly dissected out and placed in ice-cold 0.9% saline. The ileum was cleaned, and the longitudinal muscle was separated from the underlying circular muscle by the method of Rang (27). The longitudinal muscle was minced with scissors and homogenized with a Potter Elvehjem homogenizer in a buffer containing 30 mM Na HEPES, pH 7.5, 100 mM NaCl, and 0.5 mM EGTA. The homogenate was filtered through three layers of cheesecloth. The heart was perfused through the aorta with ice-cold saline and minced with scissors, as described for the ileum. Cardiac and ileal homogenates were centrifuged at 30,000 × *g* for 10 min, and the pellets were resuspended in fresh buffer containing, in addition, 1.0 mM DTT and 10 mM $MgCl_2$. The final concentration of the homogenate solution was 0.02 g of tissue (based on original wet tissue weight)/ml of buffer.

The binding of the muscarinic antagonist [^{3}H]NMS (71.3 Ci/mmol; DuPont New England Nuclear, Boston, MA) was measured by a rapid filtration method similar to that described previously (28). Tissue homogenate (0.5 ml) was incubated for 30 min at 25°, in a final volume of 1.0 ml containing 30 mM Na HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM DTT, [^{3}H]NMS, and various muscarinic antagonists, as indicated in Results. There is some evidence indicating that methoctramine may prolong the time required for [^{3}H]NMS to reach equilibrium at muscarinic receptors (29); consequently, the incubation time for methoctramine was extended to 2 hr to ensure that equilibrium was achieved. Membrane-bound [^{3}H]NMS was trapped by rapid filtration of the tissue suspension over glass fiber filters (GF/B; Whatman, Inc., Clifton, NJ) using a cell harvester (Brandel, Gaithersburg, MD). The filters were rinsed with three aliquots (3.0 ml each) of ice-cold 0.9% saline. All assays were run in triplicate, and nonspecific binding was defined as the residual binding in the presence of 10 μM atropine.

Adenylate cyclase activity. Muscarinic receptor-mediated inhibition of adenylate cyclase activity was measured in homogenates of the longitudinal muscle of the ileum, using a procedure similar to that described previously (28). Ileal homogenate was prepared as described above, except that the buffer consisted of 30 mM Na HEPES, pH 7.5, and 0.25 M sucrose. Following centrifugation, the homogenate was resuspended at a concentration of 50 mg of tissue (based on original wet tissue weight)/ml of buffer containing, in addition, 2.0 mM DTT. The ileal homogenate was used immediately in the adenylate cyclase assay.

An aliquot of ileal homogenate (0.05 ml) was incubated at 37° for 6 min, in a final volume of 0.2 ml containing 30 mM Na HEPES, pH 7.5, 100 mM NaCl, 62.5 mM sucrose, 0.5 mM DTT, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cyclic AMP, 0.5 mM isobutylmethylxanthine, 0.1 mM GTP, 0.01 mM ATP, 0.5 μCi of [α-^{32}P]ATP, 5.0 mM creatinine phosphate, 30 units/ml creatinine phosphokinase, 0.5% bovine serum albumin, and various muscarinic drugs, as described in Results. The reaction was started by the addition of the tissue and was stopped by the addition of 0.1 ml of a solution containing 40 mM ATP, 1.4 mM cyclic AMP, and 0.1 mM sodium dodecyl sulfate, titrated to pH 7.5 with Tris base. After the reaction was stopped, 0.8 ml of water was added, and [^{32}P]cAMP was recovered by sequential chromatography on Dowex AG 50W-X4 and alumina, as described by Salomon *et al.* (30). [^{32}P]cAMP in the eluate from the alumina columns was measured by Cerenkov counting.

When adenylate cyclase assays were run in the presence of a muscarinic antagonist, the ileal homogenate was allowed to first incubate with the antagonist for 10 min before the assay was begun. This method could potentially lead to an overestimation of the affinity of slowly equilibrating antagonists (e.g., NMS), because there may be insufficient time for the antagonist to reestablish equilibrium in the presence of the agonist (31). However, this potential error would not be expected to be very great, because we collected data at moderate dose ratios (approximately 10 to 30) and also because oxotremorine-M-mediated inhibition of adenylate cyclase activity exhibits a receptor reserve for the half-maximal response. This latter conclusion is based on the observation that the EC_{50} value of oxotremorine-M for inhibition of adenylate cyclase in the longitudinal muscle of the ileum is similar to that measured in the heart, which is known to exhibit a receptor reserve for the half-maximal responses to oxotremorine-M (32).

Phosphoinositide hydrolysis. Male Sprague-Dawley rats (150–250 g) were sacrificed by decapitation, and their ileums were dissected out rapidly. The ileum was cleaned, and the longitudinal muscle was removed as described above and placed in KRB buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 10 mM glucose) gassed with O_2/CO_2 (19:1). Strips of the longitudinal muscle were placed on the stage of a McIlwain tissue chopper and chopped twice at 350 μm, with the stage being rotated 90° between each run. The slices were put in KRB buffer immediately and incubated for 20 min at 37°. The slices were washed three times by allowing them to settle and replacing the supernatant with fresh KRB buffer. An aliquot (0.05 ml) of gently packed ileal slices was added to a small plastic tube containing a final volume of 0.30 ml of KRB buffer with *myo*-[^{3}H] inositol (0.5–1 μCi; 0.1–0.3 μM) and LiCl (5 mM). When investigated, a muscarinic antagonist was also present during this initial incubation. The tubes were gassed with O_2/CO_2 (19:1), capped, and incubated for 30 min at 37°. An aliquot (0.01 ml) of the highly efficacious agonist oxotremorine-M was added, and the reaction was allowed to proceed for 90 min at 37°. The tubes were gassed with O_2/CO_2 every 30 min. The extraction and separation of inositol phosphates were done using the simplified method of Berridge *et al.* (33). Briefly, the reaction was stopped by the addition of 1.13 ml of a mixture of chloroform and methanol (1:2, v/v), with subsequent homogenization with a Polytron. Water (0.37 ml) and chloroform (0.37 ml) were added and, following mixing and centrifugation, an aliquot (1 ml) of the aqueous phase was transferred to a conical centrifuge tube with an additional 2 ml of water. An aliquot (0.5 ml) of a slurry (50%, w/v) of Dowex AG 1X-8 (100–200 mesh) was added, and the tube was mixed and centrifuged. The supernatant was discarded, and 2.5 ml of *myo*-inositol (5 mM) were added. The tube was mixed and centrifuged, and the supernatant was discarded. The resin was washed three more times as just described. Finally, inositol phosphates were displaced from the resin with 0.5 ml of a mixture of ammonium formate (1.0 M) and formic acid (0.1 M). Following centrifugation, 0.45 ml of the supernatant was added to scintillation vials with 4 ml of scintillation cocktail (3a70B; Research Products International, Somerville, IL). The radioactivity was measured by liquid scintillation counting.

Isolated ileum. The potency of HHSiD and *p*-FHHSiD for antagonizing the contractile activity of oxotremorine-M was investigated in sections (2 to 3 cm) of the whole ileum of the rat, mounted longitudinally in an organ bath containing KRB buffer at 37° that was gassed with O_2/CO_2 (19:1). Isometric contractions were measured with a force displacement transducer and polygraph. The resting tension of the ileum was adjusted to a load of 0.5 g, and the ileum was allowed to equilibrate for 40 min. Three test doses of oxotremorine-M were added to ensure that the preparation gave reproducible responses. After each test dose the ileum was washed with fresh KRB buffer and incubated for 5 min. The EC_{50} value of oxotremorine-M was estimated using a

cumulative method, with 7 to 10 concentrations of oxotremorine-M spaced geometrically every 0.33 log units. After an EC_{50} value for oxotremorine-M was obtained, the ileum was washed three times and incubated for 45 min before additional measurements were made. The KRB buffer was replaced every 15 min. When present, antagonists were incubated with the ileum for 45 min before the EC_{50} value of oxotremorine-M was determined.

Calculations. The binding parameters of muscarinic antagonists were determined by measurement of their competitive inhibition of the binding of [^{3}H]NMS. The data were fitted to one- and two-site competitive binding equations by nonlinear regression analysis, as described previously (28).

$$Y = \frac{P}{1 + [I]/K'} \quad (1)$$

In this one-site equation, Y denotes the percentage of [^{3}H]NMS bound specifically, P denotes the estimate of specifically bound [^{3}H]NMS in the absence of competitor, and K' denotes the apparent dissociation constant of the competitor. According to the consequences of this one-site model, K' is also equivalent to the IC_{50} value (i.e., concentration of competitor required for half-maximal displacement of specific [^{3}H]NMS binding). In the following two-site equation,

$$Y = P\left(\frac{a}{1 + [I]/K'_H} + \frac{1 - a}{1 + [I]/K'_L}\right) \quad (2)$$

a denotes the proportion of the specifically bound [^{3}H]NMS that is bound to the high affinity site and K'_H and K'_L denote the apparent dissociation constants of the competitor for the high and low affinity sites, respectively. The dissociation constants (K) were calculated from the apparent dissociation constants (K') using the following equation:

$$K = \frac{K'}{1 + [[^3\text{H}]\text{NMS}]/K_{\text{NMS}}} \quad (3)$$

in which [[^{3}H]NMS] denotes the molar concentrations of [^{3}H]NMS used in the competitive binding assay and K_{NMS} denotes the dissociation constant of [^{3}H]NMS. The dissociation constant of [^{3}H]NMS (K_{NMS}) was calculated from the IC_{50} value (K') of the NMS/[^{3}H]NMS competition curve, using the following equation, which is a special case of Eq. 3.

$$K_{\text{NMS}} = \text{IC}_{50} - [[^3\text{H}]\text{NMS}] \quad (4)$$

The dissociation constants of muscarinic antagonists were also estimated by measurement of their ability to antagonize oxotremorine-M-mediated inhibition of adenylate cyclase activity, stimulation of phosphoinositide hydrolysis, and contraction of the ileum. The dissociation constant (K_B) was calculated from the following equation:

$$K_B = \frac{[I]}{DR - 1} \quad (5)$$

in which $[I]$ denotes the concentration of the antagonist, and DR denotes the ratio of the EC_{50} value of oxotremorine-M (concentration of oxotremorine-M required for half-maximal response) measured in the presence of the antagonist divided by that measured in the absence of the antagonist. The EC_{50} values of oxotremorine-M for inhibition of adenylate cyclase activity were estimated by nonlinear regression analysis of the data according to a logistic equation, as described previously (28). The EC_{50} value of oxotremorine-M for stimulation of phosphoinositide hydrolysis was estimated by fitting the data to the following logistic equation by nonlinear regression analysis:

$$y = B + \frac{X^n S_{\max}}{X^n + \text{EC}_{50}^n} \quad (6)$$

in which y denotes measurements of phosphoinositide hydrolysis, B denotes measurements of basal phosphoinositide hydrolysis, S_{max} denotes the maximum stimulation of phosphoinositide hydrolysis, X denotes the molar concentration of oxotremorine-M, and n denotes the Hill coefficient. The values of S_{max} are expressed as a percentage of B in the text. The EC_{50} values of oxotremorine-M for inhibition of adenylate cyclase activity and stimulation of phosphoinositide hydrolysis were estimated by regression analysis, sharing the same estimate of the maximum response between the control data and the data measured in the presence of the muscarinic antagonist.

Results

Radioligand binding. The competitive inhibition of the specific binding of [^{3}H]NMS by various nonlabeled muscarinic antagonists was measured in the longitudinal muscle of the ileum (see Fig. 1, C and D). The competitive inhibition of [^{3}H]NMS binding by nonlabeled NMS was consistent with a one-site model having a dissociation constant of 0.78 nM and a binding capacity of 0.69 pmol/mg of protein. In contrast, nonlinear regression and analysis of variance showed that the competitive binding curves of the various selective muscarinic antagonists used in this study were inconsistent with a simple one-site model but could be described adequately by a two-site model. The M_2-selective antagonists AF-DX 116 and methoctramine had high affinity for the majority of the binding sites in the ileum (72 and 91%, respectively), whereas HHSiD, *p*-FHHSiD, and pirenzepine had high affinity for only 19, 14, and 11% of the sites, respectively. Table 1 lists the dissociation constants of the various nonlabeled muscarinic antagonists. The simplest interpretation of our data is that the longitudinal muscle of the ileum contains at least two types of muscarinic binding sites. The more abundant site (major site) has high affinity for AF-DX 116 and methoctramine, and the less abundant site (minor site) has high affinity for HHSiD and *p*-FHHSiD and intermediate affinity (i.e., K_D = 0.083 μM) for pirenzepine. According to this postulate, the estimate of the

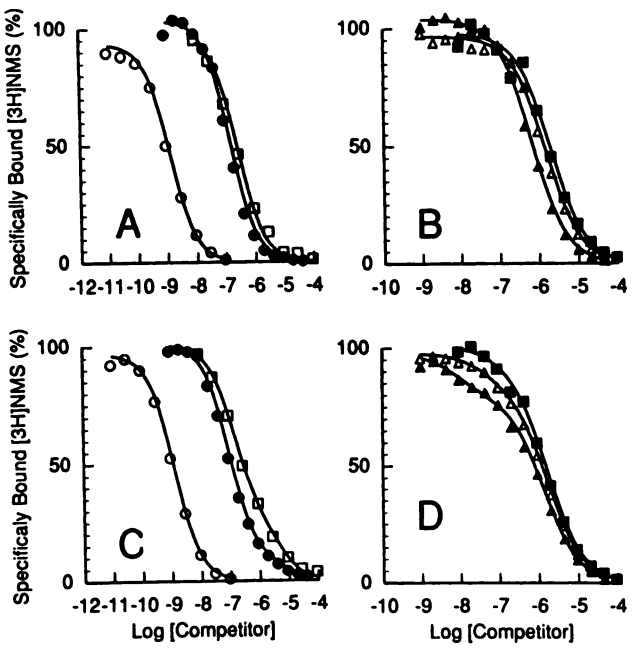

Fig. 1. Competitive inhibition of specific [^{3}H]NMS binding by NMS (○), methoctramine (●), AF-DX 116 (□), HHSiD (▲), *p*-FHHSiD (△), and pirenzepine (■) in the heart (A and B) and longitudinal muscle of the rat ileum (C and D). Each *point* represents the mean binding of six experiments, each done in triplicate. The average standard error was approximately 2%. The concentration of [^{3}H]NMS was 0.5 nM.

TABLE 1

Binding parameters of selective muscarinic antagonists in the heart and longitudinal muscle of the ileum

The parameters were calculated from data shown in Fig. 1.

Antagonist	Ileum			Heart, pK_D
	pK_D		Major site	
	Major site	Minor site		
			%	
AF-DX 116	7.04	5.59	72	6.76
HHSiD	6.08	8.27	81	6.38
p-FHHSiD	5.97	7.53	86	6.04
Methoctramine	7.22	5.23	91	7.04
Pirenzepine	5.92	7.08	89	5.90
NMS	9.12[a]			9.10

[a] The nonselective antagonist NMS bound to all sites with uniform affinity.

proportion of major sites should be the same for all of the selective antagonists used. However, Table 1 shows that the estimate of the proportion of major sites varies from 72 to 91%. We have no satisfactory explanation for this variability, and we suspect it is related to experimental error, day to day variation in preparations of ileal homogenates, differential proteolysis of binding sites, the contribution of a third muscarinic site in very low density (see Discussion), and, perhaps, the allosteric effects of high concentrations of AF-DX 116 (34) and methoctramine (29)

Our competitive binding data in the longitudinal muscle of the rat ileum are consistent with previous reports (25, 26) showing that the longitudinal muscle of the guinea pig ileum contains two types of muscarinic binding sites, with the more abundant site (70%) having a pharmacological specificity similar to that of the mammalian cardiac muscarinic receptor. Consequently, we ran competitive binding experiments in the heart so that it would be possible to compare the binding properties of cardiac muscarinic receptors with those of the major site in the ileum. Fig. 1, A and B, shows the competitive inhibition of the specific binding of [^{3}H]NMS to the heart by various nonlabeled muscarinic antagonists. Regression analysis showed that the antagonist/[^{3}H]NMS competition curves were consistent with a one-site model having high affinity for the M_2-selective antagonists AF-DX 116 and methoctramine and relatively low affinity for HHSiD, *p*-FHHSiD, and pirenzepine. Table 1 lists the dissociation constants of the various muscarinic antagonists in the heart. The data in Table 1 show that there is good agreement between the dissociation constants of AF-DX 116, HHSiD, *p*-FHHSiD, methoctramine, and pirenzepine for cardiac muscarinic receptors and their dissociation constants at the major site in the ileum.

Adenylate cyclase. Oxotremorine-M caused a concentration-dependent inhibition of adenylate cyclase activity in homogenates of the longitudinal muscle of the ileum, with the maximum inhibition and ED_{50} value being approximately 30% and 1 μM, respectively. The ability of AF-DX 116 (1.0 and 10 μM), *p*-FHHSiD (10 μM), methoctramine (0.62 μM), NMS (10 nM), and pirenzepine (10 and 100 μM) to antagonize oxotremorine-M-mediated inhibition of adenylate cyclase activity was investigated in homogenates of the longitudinal muscle of the rat ileum (see Fig. 2). In general, these antagonists caused parallel rightward shifts in the concentration-effect curve of oxotremorine-M. A change in the slope of the concentration-effect curve was noted in the presence of methoctramine; however, nonlinear regression and analysis of variance showed that

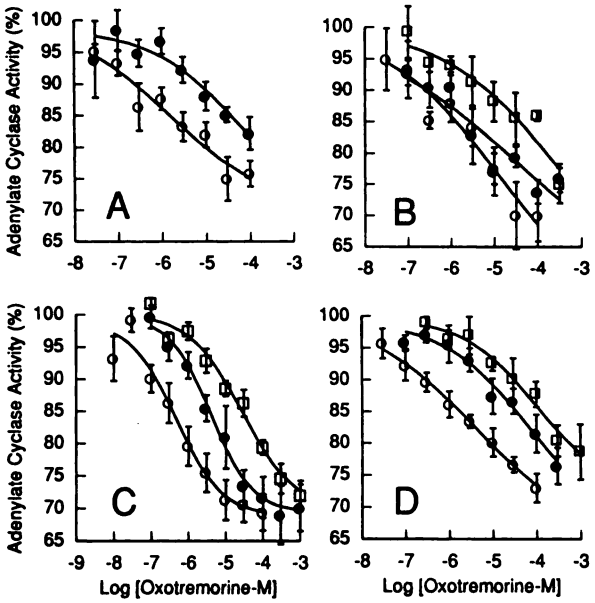

Fig. 2. Competitive antagonism of oxotremorine-M-mediated inhibition of adenylate cyclase activity by NMS (A), *p*-FHHSiD and methoctramine (B), AF-DX 116 (C), and pirenzepine (D) in the longitudinal muscle of the rat ileum. Each *point* represents the mean ± standard error of four to six experiments, each done in triplicate. Adenylate cyclase activity was measured in the presence of various concentrations of oxotremorine-M in the absence (○) and presence of various antagonists: A, NMS (10 nM) (●); B, *p*-FHHSiD (10 μM) (●) and methoctramine (0.62 μM) (□); C, AF-DX 116 (1.0 and 10 μM) (●, □); D, pirenzepine (10 and 100 μM) (●, □). The overall Hill coefficient of the oxotremorine-M concentration-effect curve was approximately 0.7.

TABLE 2

Estimates of dissociation constants (K_B) of selective muscarinic antagonists for muscarinic receptors coupled to adenylate cyclase in the longitudinal muscle of the rat ileum

The parameters were calculated from the data in Fig. 2. The average K_B values ± standard error are shown.

Antagonist	Antagonist concentration	Dose ratio	pK_B
	μM		
AF-DX 116	1.0	8.6	6.88 ± 0.17
	10	60	6.77 ± 0.21
p-FHHSiD	10	4.6	5.56 ± 0.18
Methoctramine	0.62	31	7.68 ± 0.31
Pirenzepine	10	20	6.29 ± 0.21
	100	68	5.82 ± 0.07
NMS	0.010	37	9.55 ± 0.31

this effect was insignificant ($F_{(1,6)} = 4.011$, $p = 0.0973$). The dissociation constant (K_B) of each antagonist was calculated from the dose ratio, using Eq. 5, as described in Materials and Methods. Two concentrations of both AF-DX 116 and pirenzepine were used in these experiments; consequently, two K_B values were calculated for each of these antagonists. There was no significant difference ($p > 0.05$) between the two values calculated for AF-DX 116 (0.13 and 0.17 μM) and pirenzepine (0.51 and 1.50 μM), which is consistent with the consequences of competitive inhibition. Table 2 lists the K_B values of the various muscarinic antagonists. The rank order of potency of the selective antagonists for preventing the adenylate cyclase response was methoctramine > AF-DX 116 > pirenzepine > *p*-FHHSiD. There was good agreement between the dissociation constants of AF-DX-116, *p*-FHHSiD, methoctramine, and pi-

renzepine for the major binding site (K_D) and the dissociation constants calculated by antagonism of the adenylate cyclase response (K_B).

Phosphoinositide hydrolysis. Oxotremorine-M caused a concentration-dependent accumulation of inositol phosphates in slices of the longitudinal muscle of the rat ileum, with the maximal effect being a 2- to 3-fold increase over basal levels. The EC_{50} value of oxotremorine-M for this effect was approximately 3 μM. The concentration-effect relationship of oxotremorine-M was shifted to the right in a nearly parallel fashion, without a reduction in maxima, upon the addition of AF-DX 116 (11 μM), HHSiD (1.0 μM), *p*-FHHSiD (1.0 μM), methoctramine (30 μM), and pirenzepine (3.0 μM) (see Fig. 3). Nonlinear regression and analysis of variance showed no significant increase in residual error when the concentration-effect curves were fitted simultaneously, sharing the same estimate of the Hill coefficient between the control curve and the curve measured in the presence of the antagonist. Methoctramine (30 μM) by itself caused a very small stimulation (13 ± 5.0%) of phosphoinositide hydrolysis. A similar effect has been reported by Lee *et al.* (35) in the cerebral cortex. In contrast, the other competitive antagonists caused a small reduction in the basal level of phosphoinositide hydrolysis. This small decrease was probably due to a blockade of the effect of endogenous acetylcholine. This explanation is reasonable, because phosphoinositide hydrolysis was measured using intact slices of the ileum, which may release acetylcholine. We felt that this small effect of endogenous acetylcholine would have only a negligible effect on the estimate of the dose ratios. Consequently, we estimated the K_B values of the antagonists using Eq. 5, as described in Materials and Methods, and Table 3 lists the results of these calculations. In contrast to that observed in the adenylate cyclase assay, the order of potency of the antagonists for antagonizing the phosphoinositide response was HHSiD > *p*-FHHSiD > pirenzepine > AF-DX 116 > methoctramine. There was good agreement between the dissociation constants of AF-DX 116, HHSiD, *p*-FHHSiD, methoctramine, and pirenzepine for the minor binding site (K_D) and their respective K_B values calculated by antagonism of the phosphoinositide response. We noted some discrepancy between the K_B value of NMS measured by antagonism of phosphoinositide hydrolysis and inhibition of adenylate cyclase activity. We think that this discrepancy may be due, in part, to experimental error and, in part, to the inability of NMS to reestablish equilibrium in the adenylate cyclase assay (see Materials and Methods).

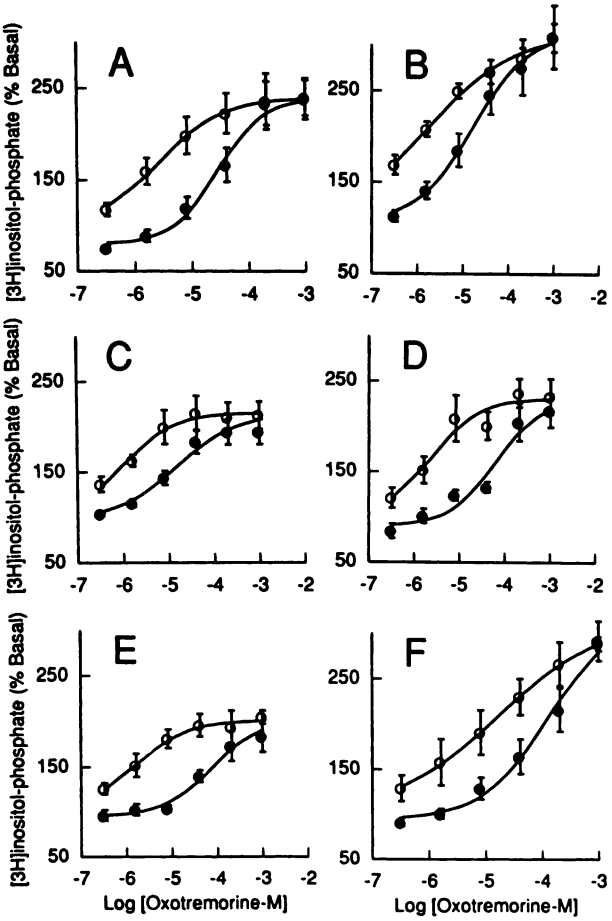

Fig. 3. Competitive antagonism of oxotremorine-M-mediated stimulation of phosphoinositide hydrolysis in slices of the longitudinal muscle of the rat ileum by AF-DX 116 (A), methoctramine (B), *p*-FHHSiD (C), pirenzepine (D), HHSiD (E), and NMS (F). Each *point* represents the mean of six experiments, each done in triplicate. Oxotremorine-M-stimulated phosphoinositide hydrolysis was measured in the absence (○) and presence (●) of AF-DX 116 (11 μM), methoctramine (30 μM), *p*-FHHSiD (1.0 μM), pirenzepine (3.0 μM), HHSiD (1.0 μM), and NMS (10 nM). The overall Hill coefficient of the oxotremorine-M concentration-effect curve was approximately 1.0.

Isolated ileum. In order to determine whether they antagonistic activity of HHSiD and *p*-FHHSiD in the rat ileum was similar to that reported in the guinea pig, we measured the ability of these compounds to antagonize oxotremorine-M-mediated contractions of the rat ileum. Oxotremorine-M contracted the ileum of the rat with an EC_{50} value of approximately 0.13 μM. Both HHSiD and *p*-FHHSiD shifted the oxotremorine-M concentration-effect curve to the right in a parallel fashion, without affecting the maximum response. Two concentrations of each antagonist were investigated, and K_B values were calculated from the dose ratios using Eq. 5. The two K_B values calculated for each antagonist at 0.1 and 1.0 μM were similar to one another, indicating a consistency with the consequences of competitive inhibition (see Table 4). The negative logarithm of the K_B values of HHSiD and *p*-FHHSiD determined here in the rat ileum are similar to those reported previously in the guinea pig (10).

Discussion

Several investigators have demonstrated that the predominant muscarinic binding site in the guinea pig ileum has pharmacological properties similar to those of the mammalian car-

TABLE 3

Estimates of the dissociation constants (K_B) of muscarinic antagonists for muscarinic receptors coupled to phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum

The parameters were calculated from data in Fig. 3. The average K_B values ± standard error are shown.

Antagonist	Antagonist concentration	Dose ratio	pK_B
	μM		
AF-DX 116	11	13	5.96 ± 0.19
HHSiD	1.0	51	7.70 ± 0.27
p-FHHSiD	1.0	14	7.12 ± 0.11
Methoctramine	30	11	5.55 ± 0.19
Pirenzepine	3.0	26	6.92 ± 0.20
NMS	0.01	7.6	8.82 ± 0.27

TABLE 4

Estimates of the dissociation constants (K_B) of selective muscarinic antagonists for muscarinic receptors eliciting contraction of the ileum

Antagonist	Antagonist concentration	Dose ratio	pK_B
	μM		
HHSiD	0.1	13	8.06
	1.0	160	8.20
p-FHHSiD	0.1	4.5	7.55
	1.0	32	7.49

diac muscarinic receptor and that the minor site is akin to that found in exocrine glands (25, 26). The results of our competition binding experiments on the longitudinal muscle of the rat ileum are consistent with this previous work with the guinea pig. Accordingly, our results show that the major site has high affinity for the cardioselective antagonists AF-DX 116 and methoctramine and low affinity for pirenzepine, *p*-FHHSiD, and HHSiD, whereas the minor site has high affinity for HHSiD and *p*-FHHSiD, intermediate affinity for pirenzepine, and low affinity for AF-DX 116 and methoctramine.

The pharmacological specificity of the major site in the ileum is consistent with that of an M_2 (m_2) muscarinic receptor, and there is strong evidence that supports this conclusion. For example, there is good agreement between the dissociation constants estimated for AF-DX 116, HHSiD, *p*-FHHSiD, methoctramine, and pirenzepine at the major site and those calculated for muscarinic receptors in the rat heart (see Table 1), which is known to contain a homogeneous population of M_2 muscarinic receptors (8, 9). When expressed in *Xenopus* oocytes (16) and CHO-K1 cells (19), cloned porcine and rat m_2 muscarinic receptors exhibit binding properties consistent with those of the mammalian cardiac muscarinic receptor, and Northern blot analysis has shown an abundance of m_2 mRNA in both the mammalian heart and small intestine (18).

The binding properties of the minor site in the longitudinal muscle of the rat ileum indicate that it consists primarily of M_3 muscarinic receptors. This conclusion is based on the agreement between the dissociation constants of antagonists for the minor binding site and those measured by antagonism of the contraction of the guinea pig ileum, which is a convenient assay system for M_3 muscarinic receptors [see Lambrecht *et al.* (22)]. Accordingly, there is good agreement between the negative logarithm of the K_D values of the M_3-selective antagonists HHSiD and *p*-FHHSiD for the minor binding site (8.27 and 7.53, respectively; see Table 1) and the pA_2 values of these antagonists in the isolated guinea pig ileum [7.96 and 7.84, respectively; see Lambrecht *et al.* (36)]. The latter values also agree with the average negative logarithm of the K_B values of HHSiD and *p*-FHHSiD that we estimated in the isolated rat ileum (8.13 and 7.53, respectively; see Table 4). It should be pointed out that HHSiD has high affinity for both M_1 and M_3 receptors (36). There is also good agreement between the estimates of the negative logarithm of the K_D values of AF-DX 116, methoctramine, and pirenzepine for the minor binding site (5.59, 5.23, and 7.08, respectively; see Table 1) and their pA_2 values for antagonizing contractions of the guinea pig ileum [6.44, 5.4 and 6.88, respectively; see Giachetti *et al.* (5), Giraldo *et al.* (6), and Lambrecht *et al.* (36)]. Moreover, the latter values agree reasonably well with the negative logarithm of the concentration of AF-DX 116, methoctramine, and pirenzepine required for half-maximal occupancy of [^{3}H]NMS binding sites in salivary glands [5.53, 5.35, and 6.89, respectively; see Hammer *et al.* (3, 37) and Giraldo *et al.* (6)], which are thought to contain a majority of M_3 muscarinic receptors (9).

In our analysis of the competitive binding experiments, we have assumed that the radioligand [^{3}H]NMS binds with equivalent affinity to all of the muscarinic sites in the ileum. This assumption was based on our observation that the NMS/[^{3}H]NMS competition curve was consistent with a simple one-site model. However, it is possible that [^{3}H]NMS might have a small difference in its affinity for the major and minor sites in the ileum, which was not detectable in our binding experiments. Other investigators have shown that NMS has higher affinity for M_3 receptors (minor site), as compared with M_2 receptors (major site) (9, 19). Such a condition will lead to an underestimation of the affinity of the minor site for competitive antagonists and an overestimation of its relative abundance. However, because it has been shown that the ability of NMS to discriminate between M_2 and M_3 sites is reduced to only a 2-fold difference in affinity in a physiological buffer similar to that used here (3), we think our estimates of the binding parameters of the antagonists are reasonable.

Apparently, it is primarily the major site in the longitudinal muscle of the rat ileum that mediates an inhibition of adenylate cyclase activity, whereas the minor site elicits phosphoinositide hydrolysis. The basis for this conclusion rests on the excellent agreement between the dissociation constants of the selective muscarinic antagonists measured by radioligand binding to the major and minor sites and the K_B values estimated by antagonism of the adenylate cyclase response and the phosphoinositide response, respectively. The excellent agreement between these parameters is shown in Fig. 4. These observations are consistent with our postulate that the major and minor sites represent the M_2 and M_3 subtypes of the muscarinic receptor, respectively, because previous studies have shown that the cloned m_2 subtype couples preferentially to adenylate cyclase, whereas the m_3 subtype couples preferentially to phosphoinositide hydrolysis (20).

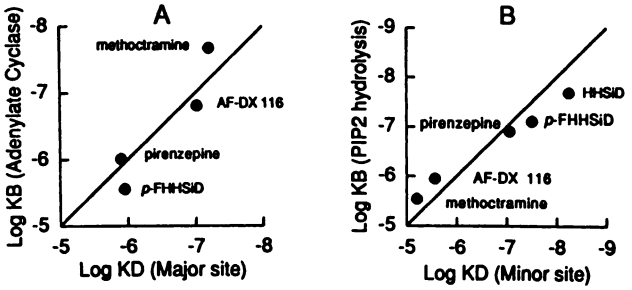

Fig. 4. Comparison between the binding parameters of muscarinic antagonists and their antagonism of oxotremorine-M-mediated inhibition of adenylate cyclase (A) and stimulation of phosphoinositide (*PIP2*) hydrolysis (B). A, The K_B value of each antagonist measured by antagonism of the adenylate cyclase response is plotted against its respective K_D for the major binding site in the ileum. In the case of AF-DX 116 and pirenzepine, the average K_B values have been plotted. The data are from Tables 1 and 2. B, The K_B value of each antagonist measured by antagonism of the phosphoinositide response is plotted against its respective K_D for the minor binding site in the ileum. The data are from Tables 1 and 3. *Diagonal lines*, the line of identity (i.e., $y = x$). Analysis of variance showed that the variance estimate based on the deviations between the mean K_B values and the line of equivalence was not significantly greater than that estimated by replicate measurements of K_B (A: $F_{(4,20)} = 0.430$, $p = 0.785$; B: $F_{(5,25)} = 0.641$, $p = 0.671$), indicating that the data were adequately fitted by the line $y = x$ in both A and B.

One potential source of error in our experimental strategy is that the binding experiments were carried out at 25° and the phosphoinositide assays were run at 37°. However, this difference is unlikely to introduce any problems in our interpretation of the data in Fig. 4B, inasmuch as the binding affinities of pirenzepine and other muscarinic antagonists only change by 1.25- to 1.1-fold over this modest range in temperature (38).

Although our results provide compelling evidence that the major and minor sites in the ileum are composed mainly of M_2 and M_3 muscarinic receptors, respectively, we cannot rule out the possibility that the longitudinal muscle of the ileum contains a small contribution of another receptor subtype. In this regard, Northern blot analysis of the guinea pig small intestine has revealed a relatively large amount of m_2 mRNA, a small amount of m_3 mRNA, and a trace amount of m_1 mRNA (18). These results are generally consistent with our postulate that the ileum contains M_2 (major site) and M_3 (minor site) muscarinic receptors but suggest that there may be a very small amount of M_1 receptors present that we were unable to detect. This condition would not be surprising, because it is difficult to discriminate between two types of sites with radioligand binding methods when one of the sites is in low abundance or when the ratio of dissociation constants of the selective ligand for the two sites is less than 6-fold (39). Although the K_B value of pirenzepine for antagonizing phosphoinositide hydrolysis in the ileum was approximately equal to that expected for an M_3 response, it was also only 6.5-fold greater than that expected for an M_1 response [i.e., 0.02 μM; see Hammer *et al.* (3)]. Thus, we cannot rule out the possibility of a very small population of M_1 receptors in our preparation that contributes to the phosphoinositide response.

As might be anticipated from the foregoing discussion, we noted good agreement between the K_B values of HHSiD and *p*-FHHSiD for antagonism of the phosphoinositide response and those estimated by antagonism of the contractions of the isolated ileum. There is also good agreement between the K_B values of AF-DX 116, methoctramine, and pirenzepine for antagonism of the phosphoinositide response and those calculated by other investigators in studies of the contraction of the isolated guinea pig ileum. The agreement between the parameters just described is shown in Fig. 5. This relationship is consistent with the idea that phosphoinositide hydrolysis is an early event in the signaling pathway for calcium mobilization and the consequent contraction of smooth muscle. It is likely that the initial phasic contraction of the ileum is triggered by phosphoinositide hydrolysis, because this contraction depends on an intracellular source of calcium (40). However, it should be emphasized that it is primarily the later tonic phase of contraction that contributes to the measured response in the isolated ileum and this phase of contraction relies on an extracellular source of calcium (40) whose mobilization may not be directly linked to phosphoinositide hydrolysis.

A comparison of the EC_{50} values of oxotremorine-M for eliciting contraction and phosphoinositide hydrolysis indicates that this agonist is 20-fold more potent at contracting the ileum than at causing an accumulation of inositol phosphates. A similar relationship between phosphatidylinositol turnover and contraction has long been known to exist for carbachol in the guinea pig ileum (41). These results illustrate that the train of events between receptor activation and smooth muscle contraction is characterized by a considerable degree of signal

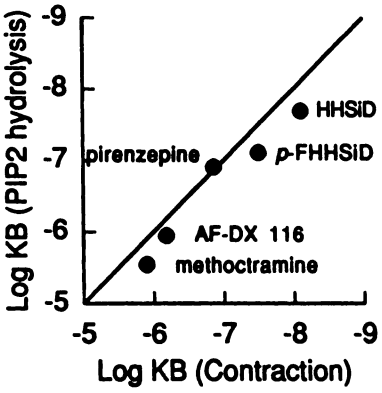

Fig. 5. Comparison of the dissociation constants of muscarinic antagonists determined by antagonism of the phosphoinositide (*PIP2*) response and the contractile response. The K_B value of each antagonist measured by antagonism of the phosphoinositide response is plotted against its respective K_B value calculated by antagonism of the contraction of the ileum. In the case of HHSiD and *p*-FHHSiD, the average K_B values from Table 4 have been plotted. The data are from Tables 3 and 4. The K_B values of pirenzepine, AF-DX 116, and methoctramine for antagonism of the contraction of the guinea pig ileum are from Lambrecht *et al.* (22), Eberlein *et al.* (48), and Melchiorre *et al.* (49). *Diagonal line*, the line of identity, $y = x$.

amplification between phosphoinositide hydrolysis and contraction of the smooth muscle. In other words, it requires only a small amount of phosphoinositide hydrolysis to contract the ileum. This situation explains why many partial agonist analogs related to oxotremorine are potent stimulants of the isolated guinea pig ileum but have little or no effect on phosphoinositide hydrolysis when this response is measured by the [^{3}H]inositol prelabeling method (42, 43). Thus, it can be seen that phosphoinositide hydrolysis is a relatively insensitive assay for agonist activity, and it is possible that a muscarinic agonist could trigger an important physiological response (e.g., exocrine secretion, contraction of smooth muscle) through a phospholipase C-linked m_1, m_3, or m_5 muscarinic receptor without producing a detectable response in the standard phosphoinositide hydrolysis assay. Caution should, therefore, be exercised when the phosphoinositide response is used as a screen for determining the specificity of agonists for subtypes of the muscarinic receptor.

The relatively high density of M_2 muscarinic receptors in the longitudinal muscle of the ileum raises the question of their function. In this regard, it should be borne in mind that this tissue contains a heterogeneous population of cells that includes the myenteric plexus. Thus, some of the M_2 receptors may be on parasympathetic ganglia in the ileum. The binding properties of various other preparations of smooth muscle, including the trachea (44), cerebral arteries (45), and uterus (46), indicate that the majority of the muscarinic receptors in these tissues are also M_2 receptors. These observations suggest that at least some of the M_2 receptors are on the smooth muscle and may influence contraction. Many receptors that cause a relaxation of smooth muscle also stimulate adenylate cyclase (47), suggesting a causal relationship between the elevation in cyclic AMP and the relaxation of smooth muscle. It is possible that M_2 muscarinic receptors prevent this relaxation via an inhibition of adenylate cyclase activity. Accordingly, it has long been known that activation of muscarinic receptors in intact cell preparations of the guinea pig ileum inhibits the rise in cyclic AMP accumulation elicited by activation of β-adrenergic recep-

tors with the smooth muscle relaxant isoproterenol (47). Thus, there may be two parallel pathways for muscarinic contraction of smooth muscle, a direct M_3 receptor-mediated contraction via phosphoinositide hydrolysis and an indirect M_2 receptor-mediated inhibition of the relaxation elicited by other receptors. The development of selective M_2 muscarinic agonists should provide a useful means of probing the role of M_2 receptors in smooth muscle.

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