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# Muscarinic Receptors in Canine Colonic Circular Smooth Muscle. I. Coexistence of $M_2$ and $M_3$ Subtypes

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## SUMMARY

The parasympathetic neurotransmitter acetylcholine, acting postsynaptically at the smooth muscle muscarinic receptor, is a principle determinant of colonic motility. In order to elucidate the receptor signal-transduction events responsible for muscarinic receptor-induced contraction of colonic circular smooth muscle, we present here and in the accompanying work studies designed to characterize the muscarinic receptors present in colon and to determine their biochemical coupling. Muscarinic receptor subtypes in canine colonic circular smooth muscle were characterized using radioligand binding techniques. The nonselective muscarinic receptor antagonist radioligand [$^3$H]quinuclidinyl benzilate ([$^3$H]QNB) binds rapidly and reversibly to a single class of saturable sites in colon circular smooth muscle membranes, with an affinity ($K_D$) for the antagonist radioligand of 79.8 ± 12.6 pM and a density of 123.3 ± 18.7 fmol/mg of protein. Experiments using membranes prepared from isolated cells purified from the circular smooth muscle layer of canine colon ($K_D$ = 102.4 ± 13.5 pM) confirm the smooth muscle origin of the binding and yield a receptor density of 124,340 receptors/cell. The order of potencies of selective muscarinic receptor antagonists in competition with [$^3$H]QNB for binding to colonic receptors is 4-diphenylacetoxy-*N*-methylpiperidine methobromide > methoctramine > AF-DX 116 > pirenzepine. Unlike other antagonists tested, pirenzepine competition of [$^3$H]QNB binding is biphasic. The high and low affinities deduced from nonlinear fit of the binding data in colon correlate very well with affinities determined for pirenzepine in mixtures of both submandibular gland ($M_3$) and atrium ($M_2$), indicating the presence of two muscarinic receptor subtypes (82% $M_2$, 18% $M_3$) in colon circular smooth muscle. The muscarinic agonist carbachol binds to both high and low affinity sites in colon, and addition of guanine nucleotide (100 μM GTPγS) shifts the agonist competition curve to the right, without eliminating high affinity binding sites. Agonist competition studies with a known ratio of $M_2$ and $M_3$ receptors, obtained by mixing pure $M_2$ and $M_3$ populations, predict the result obtained in colon. cDNA probes specific for each of the muscarinic receptors m1 through m4 were hybridized to colon RNA in a Northern blot analysis. Only m2 and m3 probes hybridized to colon RNA, suggesting the presence of both M2 and M3 receptors. Our data demonstrate that the colon circular smooth muscle contains muscarinic receptors of both the $M_2$ and $M_3$ subtypes, which may be coupled to disparate signal transduction pathways important in the physiological actions of acetylcholine in this tissue.

Three subtypes of muscarinic receptors, $M_1$, $M_2$, and $M_3$, have been defined principally on the basis of the action of antagonists, in either radioligand binding or functional studies (1, 2). The correlation between these subtypes and those defined by amino acid sequence, $m_1$, $m_2$, $m_3$, and $m_4$, has also been proposed (1). Because there is no highly selective antagonist for one receptor subtype over all others, the pharmacological characterization of a muscarinic receptor subtype follows from its spectrum of affinities for a number of antagonists.

Where they have been examined, $M_1$, $M_2$, and $M_3$ receptor subtypes display high, low, and intermediate affinities, respectively, for pirenzepine, whereas AF-DX 116 and methoctramine bind with relatively higher affinity to receptors of the $M_2$ subtype (1–3). The muscarinic antagonist 4-DAMP, on the other hand, is thought to be selective for receptors of the $M_3$ subtype (2). Although it was originally thought that $M_1$, $M_2$, and $M_3$ receptor subtypes were distributed in a tissue-specific manner in neuronal tissue, cardiac muscle, and exocrine gland or smooth muscle, respectively, this is not the case. Indeed, the coexistence of more than one receptor subtype has been demonstrated in many tissues and cells studied, including smooth muscle (4–10).

The presence of more than one muscarinic receptor subtype in tissues such as smooth muscle suggests the possibility that each receptor subtype is coupled to a disparate effector. Indeed,

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**ABBREVIATIONS:** 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methobromide; AF-DX 116, 11-2[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one; $B_{max}$, maximal radioligand binding (receptor density); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; G protein, guanine nucleotide-binding protein; kb, kilobase; $K_D$, affinity constant; QNB, quinuclidinyl benzilate; KRB, Krebs-Ringer bicarbonate solution.

studies of tissues thought to contain only $M_2$ receptors suggest coupling to inhibition of adenylate cyclase (1, 11), whereas studies of $M_3$ receptors confirm a role for phosphoinositide metabolism in their action (1). Thus, the coexistence of two receptor subtypes in a tissue, such as colonic smooth muscle, that contracts in response to acetylcholine released from parasympathetic nerve endings suggests that both receptors would be occupied by agonist and thus both receptor subtypes would play a role in contraction of the tissue. On the other hand, contractile studies in smooth muscles where more than one muscarinic receptor subtype is thought to exist suggest that only $M_3$ receptors play a role in contraction (12). The role of more than one muscarinic receptor subtype involved in regulation of muscle contraction is further complicated by the knowledge that in mammalian tissues thought to contain only one subtype, such as cardiac muscle, more than one biochemical effector (inhibition of adenylate cyclase and stimulation of phospholipase C) is regulated by receptor occupation by agonist (1, 11, 13). Thus, this study and the accompanying work (31) were undertaken to determine the subtype or subtypes of muscarinic receptor present in the colon, the likely role of G proteins in coupling muscarinic receptors to response, and the changes produced in biochemical effectors in colonic circular smooth muscle after agonist stimulation.

## Experimental Procedures

**Tissue and cell preparation.** Mongrel dogs of either sex were anesthetized with pentobarbital sodium (30 mg/kg). A segment of proximal colon (6–14 cm from the ileocecal sphincter), the submandibular gland, and the right and left atria were removed and kept in oxygenated KRB. After washing out of fecal material, the colon was cut into 2- × 3-cm pieces and pinned out in a dissecting dish bathed with fresh KRB. The longitudinal smooth muscle was carefully removed and the circular smooth muscle was separated from the submucosa by the method of Smith *et al.* (14). In order to isolate the colonic circular smooth muscle cells, muscle strips were minced and digested in calcium-free Hanks' solution containing (in mg/ml) collagenase type II, 1.3; bovine serum albumin, 2.0; trypsin inhibitor, 2.0; and ATP, 0.56. The digestion was carried out with gentle agitation at 37° for 30 min. The supernatant was collected and replaced alternatively with calcium-free Hanks' solution and buffer containing enzyme, every 20 min until the tissue was totally digested. The intact cells, pooled from successive supernatants, were collected by unit gravity sedimentation and resuspended in fresh Hanks' buffer to which 10% fetal calf serum was added.

**Membrane preparation.** Membranes from either tissues or cells were prepared in a similar fashion; tissues and cells were washed three times with ice-cold hypotonic buffer A (50 mM Tris base, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4) and homogenized in 10 volumes of the same buffer. The homogenate was filtered through a nylon cloth (500 μm) and centrifuged at 40,000 × *g* for 60 min at 4°. The resulting supernatant was discarded, and the pellet was quickly frozen in liquid nitrogen and stored as a frozen powder at −80° for 1–2 weeks, until used in radioligand binding studies.

**Radioligand binding studies.** Binding of the nonselective muscarinic receptor antagonist [$^3$H]QNB was measured by a rapid filtration method similar to that described previously (15). Membrane pellets were resuspended in buffer A to yield approximately 0.4 mg/ml protein, as determined by the method of Bradford (16). Radioligand binding assays were carried out in a volume of 500 μl, consisting of 440 μl of membrane suspension, 50 μl of radioligand, and 10 μl of buffer or drug. Saturation binding experiments used concentrations of [$^3$H]QNB from 20 to 5000 pM, and nonspecific binding was determined by the addition of 1 μM atropine. Equilibrium binding, carried out at 30° for 75 min, was stable for 120 min and completely reversible. Bound and free radioligand were separated by rapid vacuum filtration over Whatman GF/C filters that had been pretreated with 0.3% polyethylenimine, as described by Bruns *et al.* (17). The filters were washed with two 5-ml aliquots of ice-cold buffer (5 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM EGTA) and counted at 45% efficiency in a Beckman LS 6000IC liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). In experiments using mixed membranes of atrium and submandibular gland, receptor density was determined, as fmol of receptor/mg of protein, in separate saturation studies with [$^3$H]QNB (atrium = 216 ± 14.3 fmol/mg, submandibular gland = 863 ± 57.8 fmol/mg; mean ± standard error, four experiments), and the percentage mixture of receptors (82:18) was constructed accordingly. All determinations were performed in triplicate, and specific binding of [$^3$H]QNB at 0.3 nM was examined in competition studies using increasing concentrations of unlabeled agonists and antagonists.

**Northern blot analysis.** Poly(A)$^+$ RNA was prepared from a pooled sample of circular smooth muscle from five animals, using the FAST TRACK kit (Invitrogen, San Diego, CA), according to the manufacturers instructions. cDNA probes specific for each of the muscarinic receptors, m1, m2, m3, and m4 (18), were provided by Tom Bonner (Laboratory of Cell Biology, National Institute of Mental Health). RNA was size fractionated on a 1.0% agarose/formaldehyde gel and transferred to Immobilon filters (Millipore Corp., Boston, MA), which were baked and prehybridized in 50% formamide at 42°, overnight (19). Hybridization to $^{32}$P-labeled probes was performed overnight under the same conditions. The filters were washed in a high stringency fashion, three times in 2× standard saline citrate buffer (0.3 M NaCl, 0.03 M sodium citrate, 0.1% sodium dodecyl sulfate, pH 7) for 5 min each at room temperature and then twice for 30 min each in 0.2× standard saline citrate, 0.1% sodium dodecyl sulfate, at 65°, to assure specificity of labeling. Autoradiography was performed at −80° with intensifying screens.

**Data analysis.** Saturation and competition binding curves were analyzed using the computer program GraphPAD InPlot (version 3.0; GraphPAD Software, San Diego, CA). This program uses nonlinear least-squares regression to fit binding data to equations that adhere to the laws of mass action. For saturation binding data, nonlinear curve fitting was used to generate both the $K_D$ and $B_{max}$ values, in preference to the linear transform. Both methods yield similar results (15). For competition binding data, the nonlinear least-squares approach fits the data to either one or two classes of binding sites and assists in determining whether the two-site model is significantly better than the one-site fit of the data (*F* test). Data are expressed as the mean ± standard error. Statistical significance was determined by Students *t* test, where a *p* value of $<0.05$ was considered significant.

**Buffers and drugs.** KRB contains (in mM) $Na^+$, 137.4; $K^+$, 5.9; $Ca^{2+}$, 2.5; $Cl^-$, 134; $HCO_3^-$, 15.5; $H_2PO_4^-$, 1.2; and dextrose, 11.5; calcium-free Hanks' solution contains (in mM) KCl, 0.4; $KH_2PO_4$, 0.06; NaCl, 8; $Na_2HPO_4$, 0.05; $NaHCO_3$, 0.35; $MgSO_4$, 0.1; phenol red, 0.01; and dextrose, 1. Buffers were oxygenated with a mixture of oxygen/carbon dioxide (97:3), producing a pH of 7.3–7.4 at 37°. The stable muscarinic agonist carbachol and the antagonists pirenzepine and atropine, as well as GTPγS, EGTA, Tris base, bovine serum albumin, soybean trypsin inhibitor, pertussis toxin, and ATP, were purchased from Sigma Chemical Co. (St. Louis, MO). Methoctramine and 4-DAMP were obtained from Research Biochemicals Incorporated (Natick, MA). AF-DX 116 was supplied by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, CT). [$^3$H]QNB (45.7 Ci/mmol) was obtained from DuPont-NEN (Boston, MA). Collagenase type II was from Worthington Biochemicals (Freehold, NJ).

## Results

Studies of the binding of [$^3$H]QNB to membranes prepared from colon circular smooth muscle tissue and circular smooth muscle cells indicated that the binding of this nonselective

antagonist was specific and saturable (Fig. 1). Nonspecific binding represented about 5% of the total binding at concentrations of [$^3$H]QNB near the $K_D$ (80 pM). In membranes from both tissues and cells, saturation isotherms using [$^3$H]QNB were best described by an interaction of the radioligand with a single class of binding sites. Although $K_D$ values for the antagonist radioligand in the membranes of the tissues (79.8 ± 12.6 pM) and the cells (102.4 ± 13.5 pM) were similar, the density of receptors, based on protein, was significantly greater ($p < 0.01$), as expected, in the isolated cells (438.2 ± 37.1 fmol/mg of protein), compared with tissues (123.3 ± 18.7 fmol/mg of protein). This enrichment confirms the smooth muscle origin of the receptors we describe and serves to illustrate the density of ~20 muscarinic receptors/$\mu m^2$ on each smooth muscle cell (124,340 sites/cell; average surface area, 6087 $\mu m^2$).

Because our interest in studying muscarinic receptors in colonic smooth muscle required assessment of both agonist and antagonist binding, we sought to determine the role, if any, of guanine nucleotides in regulating antagonist radioligand binding. Our own studies of adenosine $A_1$ receptors in smooth muscle (15) and the studies of others using [$^3$H]QNB binding to cardiac $M_2$ muscarinic receptors (20, 21) have reported increased binding of an antagonist radioligand in the presence of guanine nucleotides. An analysis of the presence of muscarinic subtypes by radioligand binding methods would be complicated if guanine nucleotides increased radioligand (antagonist) binding, unless the identity of such sites were known.

Such a result is surprising, in light of our understanding of the role of antagonists, and suggests the possibility that a population of sites exist that do not normally bind the antagonist. In the present study, not only did GTPγS regulate the agonist binding to muscarinic receptors in colon circular smooth muscle, as expected, but it also regulated the binding of the antagonist radioligand. Addition of 100 μM GTPγS produced a significant increase in [$^3$H]QNB specific binding, from 121 to 150 fmol/mg of protein ($p < 0.05$), without changing $K_D$ for the radioligand (Fig. 2A; Table 1). After pretreatment of tissues with pertussis toxin, known to ADP-ribosylate $G_i$, the G protein coupling the $M_2$ receptor to inhibition of adenylate cyclase (1), GTPγS no longer increased the binding of the antagonist (Fig. 2B; Table 1). Indeed, after pertussis toxin pretreatment, binding of the antagonist radioligand (148.5 ± 15.5 fmol/mg) was the same as that seen in the presence of GTPγS in control tissues (149.5 ± 11.8 fmol/mg). These results are consistent with the presence of trapped endogenous agonist competing for binding to muscarinic receptors, because addition of GTPγS lowers the affinity of the receptor for agonist and allows increased binding of the antagonist radioligand. Pertussis toxin serves to produce the same effect as guanine nucleotide and, thus, GTPγS no longer produces increased binding of the radioligand in pertussis toxin-pretreated tissues. Armed, then, with evidence that guanine nucleotides do not alter the affinity of the muscarinic sites for the antagonist radioligand, we have characterized the muscarinic receptors present in colonic smooth muscle.

In order to investigate the presence of muscarinic receptor subtypes in canine colonic circular smooth muscle, the muscarinic antagonists pirenzepine, 4-DAMP, AF-DX 116, and methoctramine were used in radioligand binding competition studies with [$^3$H]QNB. As shown in Fig. 3, the rank order of the affinities ($K_I$) values of muscarinic receptors in colon circular smooth muscle for the antagonists used was 4-DAMP (38 nM) > methoctramine (210 nM) > AF-DX 116 (648 nM) > pirenzepine (2422 nM). The same rank order of potency has been obtained for these antagonists in binding to $M_2$ muscarinic receptors in a number of other tissues and cell types (1–3). To further characterize the muscarinic receptor subtypes in colonic circular smooth muscle, we examined the relative affinities of these antagonists in competition with [$^3$H]QNB for binding in the canine atrium (Fig. 4A) and submandibular gland (Fig. 4B), which exhibit homogeneous populations of $M_2$ and $M_3$ muscarinic receptors, respectively (2). In the atrium AF-DX 116 ($K_I$ = 683 nM) was more potent than pirenzepine ($K_I$ = 1588 nM), whereas for the $M_3$ muscarinic receptor (submandibular gland) pirenzepine ($K_I$ = 34.3 nM) was 120-fold more potent than AF-DX 116 ($K_I$ = 4070 nM). The affinity of 4-DAMP determined

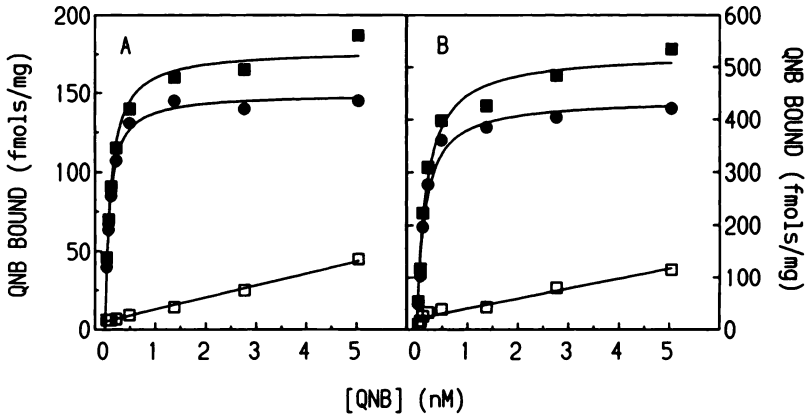


**Fig. 1.** Saturation binding of [$^3$H]QNB to muscarinic receptors in canine circular smooth muscle membranes and cells. Increasing concentrations of [$^3$H]QNB (0.02–5 nM) were incubated with colonic circular smooth muscle membranes (A) and isolated circular smooth muscle cells (B), as described in Experimental Procedures. Specific [$^3$H]QNB binding (●) was defined as the arithmetic difference between total binding (■) and nonspecific binding (□) observed in the presence of 1 μM atropine. Analysis of the specific binding data by nonlinear computer-based methods (fit to a rectangular hyperbola) confirmed that [$^3$H]QNB bound to a single class of binding sites in both tissues and cells. The receptor affinities ($K_D$) derived from the computer fit of the binding data are 91 pM for tissue (A) and 113 pM for cells (B). The $B_{max}$ is 136 fmol/mg in tissue and is enriched 3.25-fold in cells (442 fmol/mg of protein), equaling approximately 124,340 receptors/cell. Data points in both A and B represent the mean of triplicate determinations in a single experiment, repeated six and three times, respectively. Mean data are described in the text.

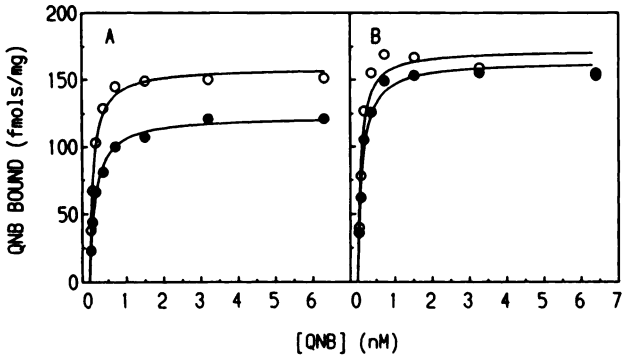


**Fig. 2.** Effects of guanine nucleotide on antagonist radioligand binding. Membranes, prepared from colonic circular smooth muscle as described in the text, were incubated with increasing concentrations of [$^3$H]QNB, in the presence (○) or absence (●) of 100 μM GTPγS. In control treated tissues (A), the maximum binding ($B_{max}$) of [$^3$H]QNB was increased from 123 to 158 fmol/mg of protein, whereas $K_D$ values were similar (0.12 to 0.11 nM) in the absence and presence of the guanine nucleotide. In pertussis toxin (100 ng/ml, 90 min)-pretreated tissues (B), the increase in $B_{max}$ caused by GTPγS was abolished (165 to 172 fmol/mg of protein). Values are the mean specific binding in two experiments performed in triplicate. Similar data were obtained in three additional experiments.

in membranes of the atrium (20.4 nM) was similar to that in colon and about 10-fold lower than that in submandibular gland (2 nM). The antagonist affinities determined in colon circular smooth muscle (Fig. 5) were highly correlated to those in atrium ($r^2 = 0.99$, $p < 0.05$) but not to those in submandibular gland ($r^2 = 0.31$). These results support the presence of $M_2$ muscarinic receptors in the colon circular smooth muscle.

Unlike other antagonists tested, analysis of competition curves using pirenzepine indicated that it may interact with more than one class of binding site. Curves were best fit by computer as a sum of interaction with two classes of sites, with 82% of the total population of sites having a $K_I$ of 4 μM and the remainder having a $K_I$ of 26 nM (Fig. 6A; Table 2). This result raises the possibility that $M_2$ receptors may coexist with another muscarinic receptor subtype in colon circular smooth muscle. To explore this possibility, competition of [$^3$H]QNB binding by pirenzepine was examined in membranes of both atrium ($M_2$) and submandibular gland ($M_3$). In both of these tissues, the competition curves for pirenzepine were best fit assuming the presence of a single class of binding sites (Fig. 6C). The $K_I$ for pirenzepine binding to muscarinic receptors in atrium ($M_2$) and submandibular gland ($M_3$) correlated very well with the low and high affinities found for pirenzepine in colonic smooth muscle (Table 2).

Believing that [$^3$H]QNB may be binding to both $M_2$ and $M_3$ receptors in the colon, we sought to predict the result found with pirenzepine in the colon by mixing pure populations of $M_2$ and $M_3$ receptors in proportions determined by the presence of high and low affinity binding in colon. Competition of [$^3$H]QNB binding by pirenzepine in mixed membranes of the atrium (82% $M_2$ receptors) and submandibular gland (18% $M_3$ receptors) yielded results indistinguishable from those determined in colon (Fig. 6B; Table 2). To exclude the possibility that the biphasic pirenzepine competition found in colon was due to contamination of smooth muscle membranes by a non-muscle cell type possessing an $M_3$ muscarinic receptor, pirenzepine competition of [$^3$H]QNB binding was also examined in membranes from isolated colonic smooth muscle cells. The competition curve for pirenzepine binding in cell membranes was best fit by assuming the presence of two sites, of high (102 nM) and low affinity (1169 nM). The proportions of high affinity (17.8%) and low affinity (82.2%) sites calculated from the isolated cell data are in excellent agreement with those determined in smooth muscle tissue.

The ability of the muscarinic agonist carbachol to compete for specific [$^3$H]QNB binding to receptors in colonic circular smooth muscle was studied in the absence and presence of the stable GTP analog GTPγS. In the absence of GTPγS, competition curves for carbachol were best fit by computer as a sum of binding to two classes of sites (Fig. 7A; Table 3). In the presence of 100 μM GTPγS, the competition curve for carbachol was steepened and shifted to the right, consistent with a role for a G protein in coupling of the muscarinic receptors to response in the cell (Fig. 7A; Table 3). Unlike atrium and submandibular gland, in which the high affinity binding of carbachol was totally abolished by addition of 100 μM GTPγS (data not shown), addition of 100 μM GTPγS in agonist competition studies using colonic smooth muscle decreased the population of high affinity binding sites for carbachol from 50.1% to 27.1% (Fig. 7A; Table 3). Biphasic competition curves in the presence of a saturating concentration of guanine nucleotide suggest the presence of more than one receptor-G protein complex, with distinct affinities for agonist. If distinct muscarinic receptor-G protein complexes have disparate affinities for agonist, then examination of agonist competition curves in mixed populations of two receptor types might reveal biphasic plots in the presence of GTPγS. As shown in Fig. 7B and Table 3, in the presence of 100 μM GTPγS there is an excellent agreement between the values for affinity obtained from the computer fit of competition curves for carbachol in membranes from circular smooth muscle and those determined with combined membranes of atrium and submandibular gland.

Although radioligand binding studies such as those we performed offer a persuasive case for the presence of two muscarinic receptor subtypes in colonic circular smooth muscle, the identity of the subtypes as $M_2$ and $M_3$ receptors is less assured.

TABLE 1

**Effect of pertussis toxin on GTPγS-regulated antagonist binding in canine colonic circular smooth muscle membranes**

Saturation binding of [$^3$H]QNB to membranes of colon circular muscle was measured in the absence or presence of 100 μM GTPγS, and the effect of pertussis toxin (100 ng/ml, 6 hr) on binding of the radioligand was determined. $K_D$ and $B_{max}$ values were determined by nonlinear computer fitting, as described in Experimental Procedures. Data are means ± standard errors of three or four experiments determined in triplicate.

| | −GTPS | | +GTPS | |
|---|---|---|---|---|
| | $K_D$ | $B_{max}$ | $K_D$ | $B_{max}$ |
| | pM | fmol/mg | pM | fmol/mg |
| Control | 90.0 ± 17.8 | 120.5 ± 13.1 | 72.5 ± 17.5 | 149.5 ± 11.8* |
| Pertussis toxin | 80.0 ± 20.1 | 148.5 ± 15.5 | 65.0 ± 15.1 | 153.5 ± 18.5 |

* Statistically significant increase, compared with −GTPγS control conditions ($p < 0.05$).

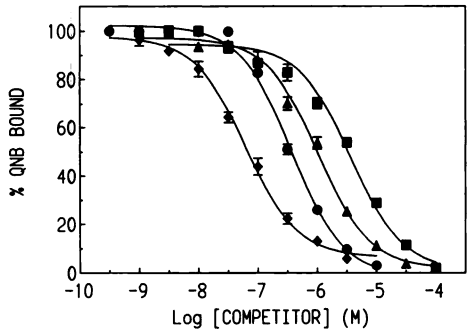


**Fig. 3.** Competition of [$^3$H]QNB binding by subtype-specific muscarinic antagonists in colon circular smooth muscle membranes. Membranes prepared from circular smooth muscle, as described in Experimental Procedures, were incubated with 0.03 nM [$^3$H]QNB, in the presence or absence of increasing concentrations of 4-DAMP (♦), methoctramine (●), AF-DX 116 (▲), or pirenzepine (■). Each data point represents the mean ± standard error of three experiments, each performed in triplicate. Data, normalized to binding in the absence of competitor (100%) and that occurring in the presence of 1 μM atropine (0%), are expressed as the percentage of maximal [$^3$H]QNB binding. *Curves* represent the computer-generated best fit of the data, assuming the presence of a single class of binding sites. This fit was superior (*F* test) to the two-site fit of the data for each of the antagonists except pirenzepine (see Fig. 6).

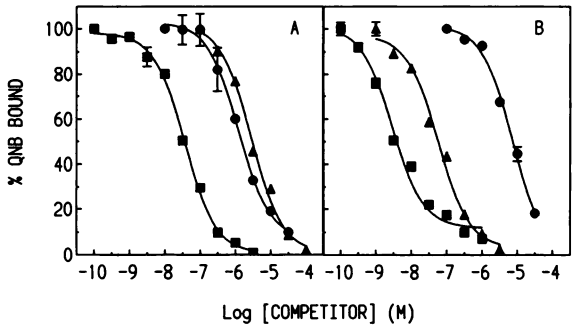


**Fig. 4.** Competition of [$^3$H]QNB binding by subtype-specific muscarinic antagonists in canine atrium and submandibular gland. Membranes were prepared from each tissue as described for circular smooth muscle in Experimental Procedures and were incubated with increasing concentrations of subtype-selective muscarinic antagonists. Competition of [$^3$H]QNB (0.03 nM) binding in atrium (A) and submandibular gland (B) was performed in triplicate, and data points represent the mean ± standard deviation of two such experiments. Data, normalized to binding in the absence of competitor (100%) and that occurring in the presence of 1 μM atropine (0%), are expressed as the percentage of maximal [$^3$H]QNB binding. The antagonists tested are 4-DAMP (■), AF-DX 116 (●), and pirenzepine (▲). *Curves* represent the computer-generated best fit of the data, assuming a single class of binding sites. The slopes of the fits in each case are not significantly different from unity. The rank order and absolute affinities of receptors in atrium are consistent with a single population of $M_2$ receptors, whereas the $K_i$ values are consistent with $M_3$ receptors being the exclusive binding site in submandibular gland.

Indeed, such assignment is based on the presence of single populations of such subtypes in other tissues. The availability of cDNA probes for $m_1$ through $m_4$ receptors allowed a direct analysis of RNA species present in colonic muscle. The cDNA probe specific for $m_2$ receptor RNA hybridized to a 5.4-kb transcript in colonic circular smooth muscle mRNA (Fig. 8). A transcript of identical size was detected in canine heart but not in canine brain. The same blot was stripped of the $m_2$ probe and reprobed with the $m_3$-specific cDNA. The $m_3$ probe hybridized to a 4.5-kb transcript in both canine colon (Fig. 8) and brain. An additional band labeled by $m_3$ was detected at ~3 kb in colon but was not seen in brain (data not shown). No

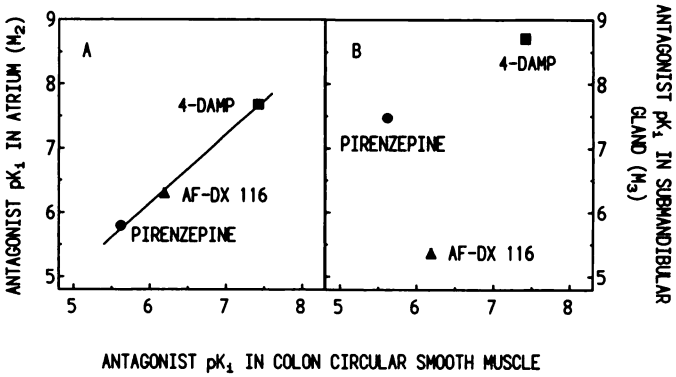


**Fig. 5.** Correlation between antagonist affinities determined in colon circular muscle and those in atrium (A) and submandibular gland (B). The affinities (p$K_i$) of muscarinic receptors for subtype-selective antagonists measured in colon circular smooth muscle membranes correlated very well with those in atrium ($r^2 = 0.99$, $p < 0.05$) but not with those in submandibular gland ($r^2 = 0.31$). Data are the mean of three experiments performed in triplicate.

hybridization was detected for the $m_1$ and $m_4$ cDNA probes to colonic circular smooth muscle RNA.

## Discussion

The finding that guanine nucleotides can enhance the binding of [$^3$H]QNB to muscarinic receptors is not without precedent. Indeed, Mattera *et al.* (20) and Boyer *et al.* (21) showed that [$^3$H]QNB binding to cardiac membranes was significantly increased by GTPγS. We have obtained similar results in studies of the $A_1$ adenosine receptor (15) in smooth muscle. Our data with purinergic receptors support the hypothesis that endogenous agonist, trapped inside microsomal vesicles during membrane preparation, is able to compete with the antagonist radioligand for binding to the receptor binding sites in outside-in vesicles. When GTPγS is added, it has access to the outwardly facing GTP binding site on the G protein; thus, it lowers the affinity of the receptor for agonist and decreases the binding of the endogenous agonist, providing for increased competition for binding by the antagonist radioligand. These data (15) are supported by estimations of the concentration of trapped agonist, its source, and the size of the outside-in population of vesicles present in the preparation.

In the present study, the increased [$^3$H]QNB binding is abolished in pertussis toxin-treated circular muscle, indicating that increased [$^3$H]QNB binding, produced by addition of guanine nucleotide, is mediated through G protein-receptor interaction. It is not necessary to our interpretation that only the pertussis toxin-sensitive G protein be involved in GTPγS-regulated [$^3$H]QNB binding. However, because it is clear that the majority of muscarinic receptors (80%) in colon circular muscle are of the $M_2$ subtype, which is coupled to a pertussis toxin-sensitive G protein (31), the majority of the increased [$^3$H]QNB binding produced by GTPγS is likely the result of binding to $M_2$ receptors. Because this increased binding produced by addition of GTPγS is not due to an altered affinity of the antagonist radioligand for the sites involved and because the majority of their number are likely to be $M_2$ receptors, we conclude that the increase in binding of the radiolabel occurring in agonist competition studies involving GTPγS does not complicate our interpretation of the data.

The finding that guanine nucleotides increase the binding of

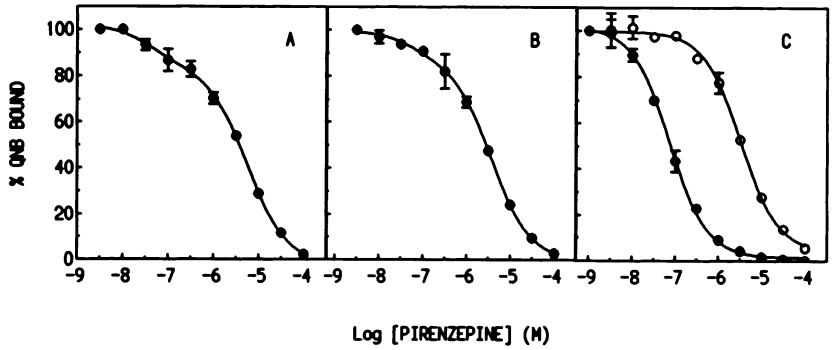


**Fig. 6.** Pirenzepine competition of radioligand binding in gastrointestinal smooth muscle, cardiac muscle, and exocrine gland from the dog. Competition of 0.03 nM [$^3$H]QNB binding by pirenzepine in membranes of colon circular muscle (A), combined membranes (mixed on the basis of receptor number) of atrium (82%) and submandibular gland (18%) (B), and membranes of atrium (○) and submandibular gland (●) alone (C). Data, normalized to binding in the absence of competitor (100%) and that occurring in the presence of 1 μM atropine (0%), are expressed as the percentage of maximal [$^3$H]QNB binding. The binding parameters determined are summarized in Table 1. Each *point* represents the mean ± standard error of two or three experiments, each done in triplicate. The concentration of [$^3$H]QNB was 0.3 nM.

TABLE 2

**Binding of pirenzepine in colon circular smooth muscle, atrium, and submandibular gland**

Pirenzepine affinities and proportions of high and low affinity sites ($K_H$, $K_L$, $R_H$, and $R_L$, respectively) in competition for [$^3$H]QNB (0.3 nM) binding were determined by nonlinear computer fit, as described in Experimental Procedures. Proportions of $M_2$ receptors from atrial membranes and $M_3$ receptors from submandibular gland membranes were mixed, as described in the text, to match the proportions of receptors determined in colon. Data are the mean ± standard error of three experiments determined in triplicate.

| Tissues | Antagonist affinity | | Site population | |
|---|---|---|---|---|
| | $K_H$ | $K_L$ | $R_H$ | $R_L$ |
| | nM | μM | % | |
| Colon | 26.2 ± 4.6 | 3.6 ± 0.5 | 18.0 ± 1.2 | 82.0 ± 1.2 |
| Atria (82%) + submandibular gland (18%) | 30.7 ± 5.5 | 1.9 ± 0.2 | 13.1 ± 1.3 | 86.9 ± 1.4 |
| Atria | | 1.6 ± 0.3 | | 100 |
| Submandibular gland | 36.7 ± 3.1 | | 100 | |

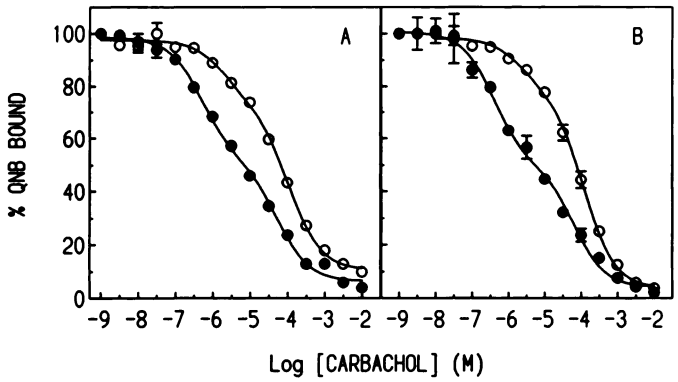


**Fig. 7.** Agonist competition of [$^3$H]QNB binding in homogeneous smooth muscle membranes and mixed membranes of atrium and submandibular gland. Competition of [$^3$H]QNB binding by the muscarinic agonist carbachol was performed in the absence (●) and presence (○) of 100 μM GTPγS, in membranes of colonic circular muscle; (A) and the mixed membranes of atrium (82% $M_2$ receptors) and submandibular gland (18% $M_3$ receptors) (B). The specific binding parameters are presented in Table 3. Data, normalized to binding in the absence of competitor (100%) and that occurring in the presence of 1 μM atropine (0%), are expressed as the percentage of maximal [$^3$H]QNB binding. Each *point* represents the mean ± standard error of three experiments, each performed in triplicate. *Curves* are computer-generated best fits of the data modeled to two classes of binding sites in the presence and absence of GTPγS.

antagonists due to the presence of endogenous agonist that is not washed away is not unlikely, despite the usual notion that acetylcholine is not stable, particularly in a crude homogenate that would be expected to contain cholinesterase activity. We think it likely, in fact, that significant acetylcholine is released from nerve contained in the bulk smooth muscle and that it becomes trapped inside vesicles and made unavailable to the action of cholinesterase. Our conclusion is supported by additional evidence not shown here. First, addition of acetylcholinesterase directly to the radioligand assay under conditions designed to open vesicles removes the effect of GTPγS to alter radioligand binding. Second, incorporation of the acetylcholinesterase inside the vesicles, by preparing membranes in the presence of the enzyme, also abolishes the increase in radioligand upon subsequent addition of guanine nucleotide. Finally, the effect of GTPγS is not seen in membranes prepared from cells, suggesting that endogenous agonist does not survive the rigors of cell isolation.

Muscarinic receptors in canine colonic circular smooth muscle were characterized by radioligand binding assay techniques using the nonselective muscarinic receptor antagonist [$^3$H]QNB. The binding of [$^3$H]QNB to the circular smooth muscle was highly specific and could be described by interaction of the radioligand with a single class of binding sites, displaying a $K_D$ in close agreement with those determined for muscarinic receptors in a number of other tissues and cell types (4, 20, 21). Although the receptor density ($B_{max}$) determined by [$^3$H]QNB binding was higher in isolated smooth muscle cells than in the tissue from which the cells were prepared, the $K_D$ values for [$^3$H]QNB were not different. This result is to be expected if the receptors identified in the tissue enjoy a smooth muscle origin. Indeed, we calculate the presence of ~20 muscarinic receptors/μm$^2$ on isolated colonic myocytes.

Competition of specific [$^3$H]QNB binding by a series of

TABLE 3

**Effect of GTPγS on agonist binding to colonic circular smooth muscle, atrium, and submandibular gland muscarinic receptors**

Carbachol affinities and proportions of high and low affinity sites ($K_{i_H}$, $K_{i_L}$, $R_H$, and $R_L$, respectively) in competition for [$^3$H]QNB (0.3 nM) binding, in membranes prepared as described in Experimental procedures, were determined in the absence or presence of 100 μM GTPγS. The parameters (mean ± standard error) were calculated from data (three experiments) shown in Fig. 7.

| | $K_{i_H}$ | $K_{i_L}$ | $R_H$ | $R_L$ |
|---|---|---|---|---|
| | μM | μM | % | % |
| Colon | | | | |
| −GTPγS | 0.27 ± 0.01 | 28.15 ± 3.32 | 50.1 ± 2.2 | 49.9 ± 2.1 |
| +GTPγS | 1.12 ± 0.22 | 56.41 ± 5.51 | 27.1 ± 1.1 | 72.9 ± 1.1 |
| Atria + submandibular gland (82:18) | | | | |
| −GTPγS | 0.19 ± 0.03 | 31.15 ± 4.33 | 52.1 ± 1.7 | 47.9 ± 1.7 |
| +GTPγS | 1.08 ± 0.41 | 56.64 ± 8.12 | 20.6 ± 2.8 | 79.4 ± 2.8 |


**Fig. 8.** Northern blot analysis of colonic smooth muscle RNA, using $m_2$ and $m_3$ cDNA probes. Northern blot containing poly(A)$^+$ RNA from canine colonic circular smooth muscle was hybridized to muscarinic cDNA probes. *hm2*, human $m_2$ receptor cDNA fragment containing 0.5 kb from the i3 region of the $m_2$ receptor primary structure. *hm3*, human $m_3$ receptor cDNA fragment containing 0.7 kb from the i3 region of the $m_3$ receptor primary structure. *Numbers to the left*, molecular weight markers, in kb, obtained by running an RNA ladder (BRL) alongside the tissue-derived RNAs.

muscarinic antagonists satisfied the criteria for $M_2$ muscarinic receptors in the circular muscle. Although the absolute affinity of a drug for its receptor may vary according to assay conditions and from tissue to tissue, the relative potency of different drugs towards the same receptor is less variable and, thus, presents a more consistent criterion for comparison. The rank order of potencies for the antagonists used in the present study was 4-DAMP > methoctramine > AF-DX 116 > pirenzepine. The same rank order of the antagonists has been reported for $M_2$ muscarinic receptors in both binding and functional studies (1–3). Whereas 4-DAMP and methoctramine are generally found to be more potent than AF-DX 116 and pirenzepine for binding to either $M_2$ or $M_3$ muscarinic receptors, AF-DX 116 is consistently seen as more potent than pirenzepine for binding to the $M_2$ muscarinic receptor in all tissues and cell types thus far examined. The presence of $M_2$ muscarinic receptors in the colon circular smooth muscle was further supported by a parallel study, conducted with identical procedures, undertaken to examine the affinities of these antagonists for the muscarinic receptors known to exist in the atrium and submandibular gland. The excellent correlation between the antagonist affinities determined in colonic circular smooth muscle and those in atrium, which has a homogeneous population of $M_2$ muscarinic receptors (2, 22), provided strong evidence for the presence of $M_2$ muscarinic receptors in colon circular muscle.

The curves for pirenzepine competition of [$^3$H]QNB binding in atrium or submandibular gland could be best described as binding to a single class of sites, distinct in each tissue. The nonlinear regression analysis of the data obtained for pirenzepine competition of [$^3$H]QNB binding in colonic smooth muscle revealed the coexistence of two muscarinic binding sites, with ~20% of the total receptor population having high affinity for pirenzepine and the remainder having low affinity for the antagonist. It has been well documented that atrium and submandibular gland contain homogeneous populations of $M_2$ and $M_3$ muscarinic receptors, respectively (2). The high and low affinities for pirenzepine determined in colon circular muscle correlated very well with those determined in submandibular gland and atrium, indicating that the major and minor sites in the circular muscle consisted of ~80% $M_2$ and ~20% $M_3$ muscarinic receptors. The ability of pirenzepine to discriminate 20% of the total population of binding sites for [$^3$H]QNB as $M_3$ receptors was confirmed by examination of pirenzepine competition of [$^3$H]QNB binding in membranes composed of a mixture containing $M_2$ receptors from atrium (82%) and $M_3$ receptors (18%) from submandibular gland. Computer-assisted analysis of pirenzepine competition of [$^3$H]QNB binding in the mixed membranes found 87% $M_2$ and 13% $M_3$ receptors (Table 2).

The finding that colonic circular smooth muscle contains multiple muscarinic receptors is not surprising. The coexistence of $M_2$ and $M_3$ muscarinic receptors in smooth muscle has also been demonstrated in ileum (8), trachea (4), stomach (5), and coronary artery (7). The ratio of $M_3$ to $M_2$ muscarinic receptors determined by pirenzepine competition in the isolated smooth muscle cells we prepared was the same as that obtained in the tissue, thus confirming the smooth muscle origin of both $M_2$ and $M_3$ receptors and further showing that both receptors exist on the same cells.

The competition curves for AF-DX 116 in the circular muscle are best described by interaction with a single class of binding sites. This is distinct from data reported in rat ileum and bovine trachea, in which AF-DX 116 was able to distinguish both high and low affinity binding sites (4, 8). In the present study, however, the affinity of AF-DX 116 for $M_2$ muscarinic receptors (atrium) was only 5-fold higher than its affinity for $M_3$ muscarinic receptors (submandibular gland). Although many studies have demonstrated a 20–35-fold difference in the affinities of the cardiac and glandular muscarinic receptors for AF-DX 116 (23–25), less selectivity has also been reported (3, 26, 27). The ability of an antagonist to resolve two distinct classes of binding sites is dependent on the magnitude of difference in apparent affinity of receptors for the agent in question and the relative proportions of the two sites. Indeed,

to resolve mixtures of receptors containing a small contribution of the minor constituent (~20%), a selectivity ratio of 30–100-fold is required (28). The difference in the affinities of AF-DX 116 for $M_3$ and $M_2$ muscarinic receptors in the present study is too small to detect the contribution of binding to $M_3$ muscarinic receptors in colon circular smooth muscle. The antagonist 4-DAMP, which displayed a 10-fold selectivity for $M_3$ over $M_2$ muscarinic receptors, is similarly unable to differentiate the individual contributions of $M_2$ and $M_3$ receptors. In the present study, the affinity of the glandular muscarinic receptor for pirenzepine was ~45-fold greater than its affinity for the cardiac muscarinic receptor, thus providing for its ability to detect the minor contribution of $M_3$ binding sites in the circular muscle. The affinity of the glandular ($M_3$) muscarinic receptor determined in the present study was higher than that reported by Doods *et al.* (2) but similar to that measured by Gil and Wolfe (6). Such differences are likely due to species differences and/or assay conditions. Recently, Candell *et al.* (8) demonstrated that pirenzepine could discriminate $M_2$ (84%) and $M_3$ (16%) muscarinic receptors in rat ileum smooth muscle. The affinity of $M_3$ muscarinic receptors in ileum toward pirenzepine is similar to that described here for colonic smooth muscle.

It is interesting that the high affinity binding sites for carbachol in colonic circular smooth muscle are only partially abolished by addition of what we know to be saturating concentrations (15) of GTPγS in smooth muscle binding studies, whereas the high affinity agonist binding sites in atrium and submandibular gland are totally abolished by addition of GTPγS. Guanine nucleotide regulation of muscarinic agonist binding has been demonstrated in the majority of tissues where muscarinic binding sites have been detected (1). It is not clear, however, whether the presence of guanine nucleotide-insensitive high affinity sites for agonist is due the presence of two receptor subtypes with disparate affinities for agonist or due to a population of receptors, with a high affinity for agonist, that are not coupled to G proteins.

Henis *et al.* (29) have recently reviewed the literature on muscarinic receptors, and their work provides a useful discussion of the possible explanations for GTP-resistant high affinity agonist binding. We believe that our results in the colon, that is, that high affinity sites exist in the presence of saturating concentrations of GTPγS, are not satisfactorily explained by assuming the presence of such guanine nucleotide-resistant sites. Indeed, we find that, although additional high affinity sites for agonist persist in the presence of 100 μM GTPγS, these sites are shifted significantly rightward from the high affinity sites seen in the absence of GTPγS (Table 3).

The explanation for the heterogeneity in agonist binding we measure in the presence of GTPγS follows from the notion that muscarinic receptors coupled to disparate G proteins have distinct affinities for agonist. This notion is supported by recent evidence of Wess *et al.* (30) that cloned $m_2$ and $m_3$ muscarinic receptors display distinct affinities for the agonist carbachol. Further evidence in support of this explanation comes from our own data. In the present study, high affinity binding sites for carbachol were totally abolished by GTPγS in both atrium and submandibular gland studied individually. When membranes from atrium and submandibular gland were mixed ~80:20 (based on receptors/mg of protein), GTPγS only partially abolished the high affinity binding sites for carbachol (52.1% to 20.6%). In fact, in the presence of GTPγS both the high affinity and the low affinity sites determined in the absence of guanine nucleotide were shifted significantly rightward. Computer-based nonlinear fit of these data suggested the presence of high and low affinities (20.6% high affinity, $K_I$ = 1.08 μM; 79.4% low affinity, $K_I$ = 56.64 μM) in the mixed membranes. These data are in nearly exact agreement with data obtained for carbachol competition of [$^3$H]QNB binding in colonic membranes in the presence of 100 μM GTPγS (27.1% high affinity, $K_I$ = 1.12 μM; 72.9% low affinity, $K_I$ = 56.41 μM) and fit the notion that $M_2$ and $M_3$ receptors couple to disparate G proteins that confer a distinct affinity of each receptor for the agonist carbachol.

The presence of both $m_3$ and $m_2$ cDNA labeling of colonic RNA confirms our radioligand binding data. The probes, obtained from Dr. Tom Bonner, have been used to label muscarinic receptor RNAs (18). The presence of only $m_2$ hybridization in canine heart confirms our use of this tissue in the radioligand binding studies as a positive control for the presence of $M_2$ receptors. The absence of either $m_1$ or $m_4$ hybridization in colon, along with the presence of an $m_3$ transcript in colon and brain, confirms our interpretation that $M_2$ and $M_3$ receptors coexist in colonic smooth muscle. The presence of an additional $m_3$-specific band in colonic RNA not found in brain is intriguing but not readily explained. What is clear, however, is that both $m_2$ and $m_3$ cDNAs hybridize with colonic RNA in a fashion that supports the interpretation that $M_2$ and $M_3$ receptors are present in colon and that the apparent abundance of RNAs is in general agreement with the receptor populations quantified by radioligand-binding methods.

We believe that the data provided here establish that the circular smooth muscle of the canine proximal colon possesses both $M_2$ and $M_3$ receptors and that these receptors are likely to couple to distinct G proteins. In addition, we provide evidence that the effects of GTPγS to increase antagonist binding seen in this tissue are not likely to be the result of special conformations of the muscarinic receptor induced by guanine nucleotide. Rather, we believe that this phenomenon, seen in many different tissues and cells and relevant to a large group of receptors, is likely to be due to the presence, in the membranes, of trapped muscarinic agonist.

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