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Muscarinic Receptors in Canine Colonic Circular Smooth Muscle. II. Signal Transduction Pathways

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SUMMARY

We have, in the accompanying work, demonstrated the coexistence of M_2 and M_3 muscarinic receptors in the circular smooth muscle of canine colon. In the present study, the effects of muscarinic receptor stimulation on phosphoinositide turnover and adenylate cyclase activity were examined. In *myo*-[^{3}H]inositol-labeled circular smooth muscle strips, carbachol caused a concentration-dependent (EC_{50} = 5 μM) increase in [^{3}H]inositol phosphate production. The more M_3 receptor-selective muscarinic antagonist pirenzepine (K_B = 53 nM) was ~60 times more potent than the more M_2-selective agent AF-DX 116 (K_B = 3 μM) in blocking carbachol-elicited accumulation of [^{3}H]inositol phosphates. The carbachol-stimulated increase in [^{3}H]inositol phosphate accumulation was not affected by pretreatment of the tissue with pertussis toxin (200 ng/ml, 3 hr). Within the first minute, carbachol (100 μM) caused a rapid and transient increase of [^{3}H]inositol 1,4,5-trisphosphate production that oscillated continuously in the presence of agonist (120 min). The accumulation of [^{3}H]inositol 1,3,4-trisphosphate was also extremely rapid, reaching a peak at 15 sec. The accumulation of [^{3}H]inositol monophosphate was delayed and progressively increased over 30 min. [^{3}H]Inositol 1,3,4,5-tetrakisphosphate, although not detectable in the first minute, accumulated to significant levels over 30 min in the presence of agonist. Addition of carbachol in the adenylate cyclase assay caused inhibition of forskolin-stimulated [^{32}P]cAMP production and blocked forskolin-stimulated cAMP accumulation in the intact tissue. The inhibitory effects of carbachol on adenylate cyclase were blocked by atropine, AF-DX 116, and 4-diphenylacetoxy-*N*-methylpiperidine methobromide but were unaffected by the more M_3-selective agent pirenzepine (1 μM). Pretreatment of tissues with pertussis toxin completely eliminated M_2 receptor-mediated inhibition of adenylate cyclase activity, without altering inositol 1,4,5-trisphosphate accumulation. We conclude that muscarinic receptor stimulation of inositol trisphosphate production is mediated by the M_3 receptor coupled to a pertussis toxin-insensitive GTP-binding protein and results in the rapid formation of inositol tetrakisphosphate, whereas inhibition of adenylate cyclase activity is mediated by the M_2 subtype of muscarinic receptor coupled to the pertussis toxin-sensitive GTP-binding protein G_i.

In the accompanying work (1), we have demonstrated that M_2 and M_3 subtypes of the muscarinic receptor coexist in canine colon circular muscle and are present on isolated smooth muscle cells. Understanding the receptor signaling that occurs after agonist occupation of muscarinic receptors in the colon is important because this is the principal manner in which colonic motility is enhanced. Activation of muscarinic receptors in numerous tissues and cells leads to a variety of physiological outcomes. The coupling of muscarinic receptors to these outcomes appears to occur via a limited number of biochemical responses, including stimulation of PI hydrolysis and inhibition of adenylate cyclase activity (2).

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Generally, each subtype of muscarinic receptor is thought to couple to a particular effector mechanism (3), although this designation may not be exclusive (4). It is well known that muscarinic receptors of the M_3 subtype are efficiently coupled to the stimulation of PI hydrolysis via a PTx-insensitive G protein, leading to activation of PLC. The M_2 receptor subtype, on the other hand, preferentially inhibits adenylate cyclase activity through a PTx-sensitive G protein (3).

Inhibition of adenylate cyclase activity results in a reduction in cAMP and cAMP-dependent protein kinase activity within cells (5). Such an action in smooth muscle leads to a functional antagonism of the relaxation produced by cAMP action and thus attends contraction of the tissue. Hydrolysis of PIP_2 leads to the production of two important second messenger molecules, $Ins(1,4,5)P_3$, which is thought to release intracellular

ABBREVIATIONS: PI, phosphatidylinositol; 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; 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; $Ins(x_1\text{-}x_n)P_y$, *myo*-inositol phosphate isomer (x = position of phosphates; y = number of phosphates); K_D, affinity constant; K_B, antagonist affinity constant; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; QNB, quinuclidinyl benzilate; HPLC, high performance liquid chromatography; PTx, pertussis toxin.

calcium from sarcoplasmic stores in smooth muscle, and diacylglycerol, which activates protein kinase C (6). Phosphorylation of Ins(1,4,5)P_3 by inositol 3-kinase yields Ins(1,3,4,5)P_4 (7). Recent evidence suggests that Ins(1,3,4,5)P_4, in the presence of Ins(1,4,5)P_3, is involved in regulating the influx of extracellular calcium into cells (6, 8) through sarcolemmal channels.

In colonic smooth muscle, the intracellular events that are responsible for the initiation and maintenance of contraction of the tissue after muscarinic receptor activation are not entirely clear. The preponderance of M_2 receptors (80%) present in rat and guinea pig ileum (9, 10) and canine colon (1), along with their coupling to adenylate cyclase inhibition, suggests that this receptor subtype is likely to play a role in the contractile actions of acetylcholine, whereas the minor component of the muscarinic receptor population, the M_3 receptor, is thought to be coupled to PLC activation, generation of $InsP_3$, and thus release of intracellular Ca^{2+}. This biochemical event alone (Ca^{2+} release), however, is not thought to be sufficient to sustain smooth muscle contraction in phasic muscles such as colon (11). Indeed, a role for Ca^{2+} entry into the cell is thought to explain the contractile actions of acetylcholine in gastrointestinal smooth muscle (12). Furthermore, selective antagonists used in contraction studies suggest that M_3 but not M_2 receptors are responsible for contraction (9).

Thus, the present study was conducted to examine the coupling of M_2 and M_3 muscarinic receptors in canine colonic circular smooth muscle to PI hydrolysis and adenylate cyclase activity.

Experimental Procedures

Materials. Carbachol, PTx, pirenzepine, atropine, isoproterenol, forskolin, GTPγS, ATP, cAMP, GTP, creatine phosphate, creatine phosphokinase, and cAMP-dependent protein kinase were purchased from Sigma (St. Louis, MO). The imidazolididine cAMP-phosphodiesterase inhibitor Ro 20-1724 was a gift of Hoffmann LaRoche Inc. (Nutley, NJ). 4-DAMP was from Research Biochemicals Inc. (Natick, MA). AF-DX 116 was supplied by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, CT). [^{3}H]QNB (45.7 Ci/mmol), [^{3}H]cAMP (44.5 Ci/mmol), [^{32}P]ATP (3000 Ci/mmol), and *myo*-[^{3}H]inositol (58.1 Ci/mmol) were obtained from DuPont-NEN (Boston, MA). Anion exchange resin was obtained from Bio-Rad Laboratories (Richmond, CA).

PI hydrolysis. Canine colonic circular smooth muscle was prepared as described previously (1). The tissue was dissected into 50-mg pieces and placed into oxygenated labeling buffer of the following composition (in mM): NaCl, 118.0; KCl, 4.7; KH_2PO_4, 0.6; Na_2HPO_4, 0.6; $MgCl_2$, 1.2; dextrose, 5.0; $CaCl_2$, 0.5; Tris base, 10.0; and $NaHCO_3$, 5.0. Tissues were incubated with *myo*-[^{3}H]inositol (200 μCi/ml) for various times at 35° and washed with six changes of fresh buffer over 30 min, to remove radiolabel. Control experiments (data not shown) demonstrated that lowering the glucose concentration in the labeling buffer from 20 to 5 mM favored the incorporation of *myo*-[^{3}H]inositol into PIP_2.

After labeling, tissues were challenged with the muscarinic agonist carbachol, in the presence of Li^+ (10 mM), and the reactions were terminated by quickly freezing tissues in liquid nitrogen. The tissues were then homogenized in 2 ml of ice-cold $CHCl_3/CH_3OH/HCl$ (66:33:1) and centrifuged at 400 × *g* for 10 min, and the aqueous phase was collected and lyophilized to dryness. [^{3}H]InsPs were separated by HPLC as described by Doggwiler and Buxton (13), with slight modification. The compounds were separated on a Whatman Partisil 10 SAX analytic column with a 5-min isocratic elution at 100% H_2O, followed by a 30-min linear gradient from 100% 0.05 M ammonium phosphate to 100% 2 M ammonium phosphate. The identities of the [^{3}H]InsPs were verified by comparison with authentic standards obtained from DuPont-NEN (Boston, MA). Quantification was achieved by determining peak area, as recorded in cpm by the Radiomatic flow scintillation counter. The organic phase from the tissue extracts was lyophilized, reconstituted in 1 ml of a methylamine solution containing $CH_3NH_2/H_2O/CH_3OH/CH_3(CH_2)_3OH$ (24:16:40:10), and incubated for 45 min at 53°. Samples were then suspended in 1.0 ml of H_2O, and the acyl moieties were removed by washing with 1.0 ml of $CH_3(CH_2)_3OH$/petroleum ether/$HCO_2C_2H_5$ (20:4:1) solution, vortexing, and separation of the aqueous phase for HPLC separation, as described (13). The incorporation of *myo*-[^{3}H]inositol into the inositol-containing phospholipids PI, PIP, and PIP_2 reached steady state by 3 hr (Fig. 2).

Cyclic AMP assay. Formation of cAMP in canine colonic circular smooth muscle was measured as previously described (14). Briefly, tissues were homogenized in 5% trichloroacetic acid, and the deproteinized supernatant was purified over Dowex AG 50W-X4 (200–400 mesh). [^{3}H]cAMP (0.25 pmol) was added to determine the recovery after purification. Cyclic AMP content was determined by the protein binding assay of Gilman (15). Values are expressed as pmol of cAMP/mg of protein, determined by the dye-binding method of Bradford (16).

Adenylate cyclase assay. Adenylate cyclase activity was determined by the method of Salomon (17). The circular muscle was homogenized in a buffer containing 50 mM Tris base and 1 mM EGTA, pH 7.4, and was filtered through a nylon cloth (500 μm). Assay mixtures (final volume of 50 μl) contained 50 mM Tris·HCl, pH 7.4, 1 mM ATP, 2 mM cAMP, 0.1 mM GTP, 0.1 mM Ro 20-1724, 5 mM $MgCl_2$, 100 mM NaCl, 5 mM creatine phosphate, 50 units/ml creatine phosphokinase, and 2–4 × 10^6 cpm of [^{32}P]ATP. The reaction was started by addition of tissue homogenate and was carried out at 37° for 15 min. The reaction was stopped by addition of 100 μl of stopping solution containing 2% sodium dodecyl sulfate, 45 mM ATP, and 1.5 mM cAMP. The [^{32}P]cAMP generated during the reaction was purified from [^{32}P]ATP by sequential chromatography on Dowex AG 50W-4X and alumina columns. [^{3}H]cAMP (10,000 cpm) was added to determine recovery. Results were expressed as pmol of cAMP/mg of protein/min.

Radioligand binding assay. The competition of [^{3}H]QNB binding by the agonist carbachol was determined as previously described (1). The colon smooth muscle homogenate was prepared as described above, except that the buffer contained 10 mM $MgCl_2$. Competition of specific [^{3}H]QNB (0.3 nM) binding was examined in the presence of increasing concentrations of carbachol. Binding was carried out at 30° for 75 min, and bound and free radioligand were separated by rapid filtration of the suspension over Whatman GF/C filters, using a Brandel harvester.

Data analysis. The dissociation constants (K_B) of antagonists for inhibition of responses elicited by carbachol were calculated from the rightward shift of the control curves, according to the formula $K_B = [B]/CR - 1$, where CR is the ratio of EC_{50} values in the presence and absence of antagonist and $[B]$ is antagonist concentration (18). The competition radioligand binding data were analyzed using the computer program GraphPAD InPlot (version 3.1) (GraphPAD Software, San Diego, CA). For agonist 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). Results were expressed as mean values ± standard errors, and the differences were evaluated for statistical significance ($p < 0.05$) by Student's t test.

Results

Treatment of colon circular muscle with the muscarinic agonist carbachol (100 μM) for various periods revealed time-dependent accumulation of [^{3}H]InsPs, with an apparent steady state being reached at 30 min (Fig. 1). Although measurement of the specific activity of the inositol lipid pool in this smooth muscle is not described here, it is evident from the data of Fig. 2 that an apparent steady state of incorporation of [^{3}H]inositol was reached at 3 hr of incubation with label. Measurement of radioactivity remaining in the incubation medium at 3 hr (data

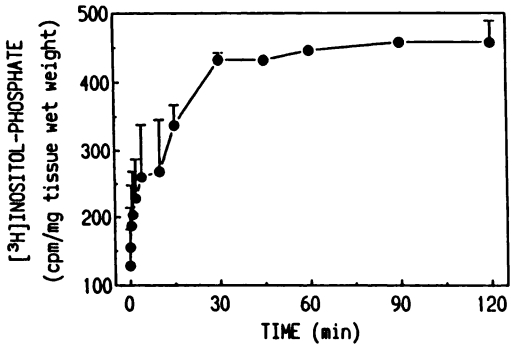

Fig. 1. Muscarinic stimulation of [^{3}H]InsP accumulation in canine colon circular smooth muscle. Individual [^{3}H]InsPs were separated and quantified as described in the text, and the areas under the peaks, in cpm, for the isomers were added together to generate total InsPs. Values, in radioactivity cpm, were normalized to tissue weight. Data are mean values ± standard errors of three experiments performed in triplicate.

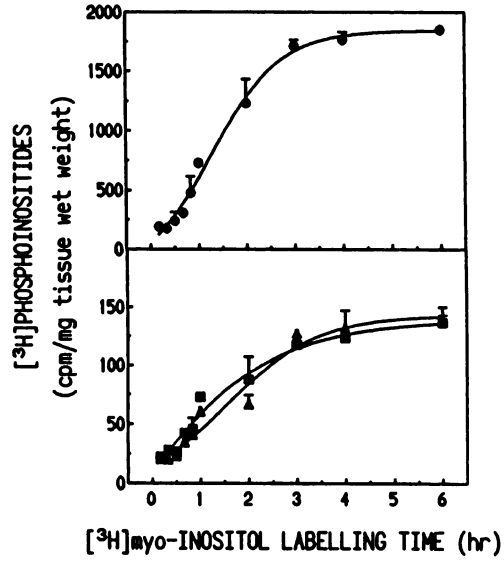

Fig. 2. Time course of *myo*-[^{3}H]inositol labeling of phosphoinositides in canine colon circular smooth muscle. Labeled phosphoinositides were extracted and separated by HPLC on strong anion exchange resin, as described in the text. Areas under the HPLC peaks, in cpm, are expressed in terms of tissue weight. The identity of each lipid was verified by deacylation of standards. Samples were incubated with *myo*-[^{3}H] inositol (200 μCi/ml) in the absence of muscarinic agonist for various times as shown. Values for PI (●) (*upper*), as well as PIP (■) and PIP_2 (▲) (*lower*), are the mean ± standard error of two experiments performed in triplicate.

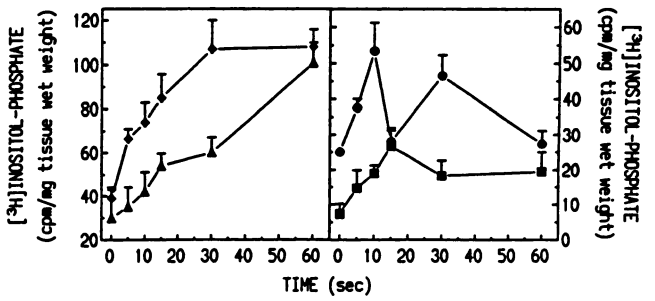

Fig. 3. Accumulation of InsP isomers after muscarinic stimulation of colonic smooth muscle. Individual [^{3}H]InsPs, [^{3}H]$InsP_1$ (▲), [^{3}H]$InsP_2$ (◆), [^{3}H]Ins(1,3,4)P_3 (■), and [^{3}H]Ins(1,4,5)P_3 (●), were separated and quantified as the areas under the peaks, in cpm, as described in the text. Values for $InsP_1$ are the sum of Ins(1)P_1 plus Ins(4)P_1 and for $InsP_2$ are the sum of Ins(1,4)P_2 plus Ins(1,3)P_2. These individual isomers changed in parallel and are presented together for simplicity. Values, in radioactivity cpm, were normalized to tissue weight. Data are mean values ± standard errors of three experiments performed in triplicate.

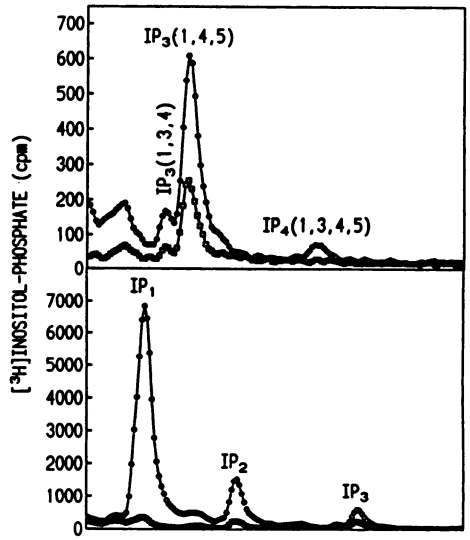

Fig. 4. HPLC separation of [^{3}H]InsPs from canine colon circular smooth muscle and effects of acetylcholine. Strips of colonic smooth muscle were labeled with *myo*-[^{3}H]inositol, and InsPs were extracted as described in the text. Both control (□) and carbachol (100 μM)-stimulated (○) tissues were incubated under identical conditions for 30 min before extraction. *Lower*, the initial 8.4 min of the elution profile, containing [^{3}H] inositol and [^{3}H]glycerophosphoinositol, have been subtracted for clarity ($InsP_1$ elution, 10.8 min; $InsP_2$ elution, 14.4 min). *Upper*, the peaks of [^{3}H]Ins(1,3,4)P_3 (18.5 min), [^{3}H]Ins(1,4,5)P_3 (19.4 min), and [^{3}H] Ins(1,3,4,5)P_4 (23.8 min) are amplified. Data are the HPLC elution profile from a single representative experiment. Similar data from several experiments are presented in Table 1; data for $InsP_4$ production are presented in the text.

not shown) provided assurance that this equilibrium was not due to significant reduction in the availability of substrate.

Within the first minute after addition of muscarinic agonist to [^{3}H]inositol-labeled tissues, a rapid increase in [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ is seen, followed by a later increase in [^{3}H]$InsP_1$ (Fig. 3). The accumulation of [^{3}H]Ins(1,4,5)P_3 reached its peak at 10 sec and subsequently declined to near basal levels at 15 sec. This increase in [^{3}H]Ins(1,4,5)P_3 was biphasic, peaking again at 30 sec after addition of agonist and returning to baseline once more at 60 sec. Further oscillation in the appearance of $InsP_3$ was seen for up to 120 min in the continued presence of agonist. While the oscillation continued, [^{3}H]$InsP_3$ values doubled and returned to baseline with a periodicity from 10 to 30 min that varied in each experiment (data not shown). [^{3}H]Ins(1,3,4)P_3 also accumulated very rapidly, but its peak was delayed 5 sec, relative to that of [^{3}H]Ins(1,4,5)P_3. The accumulation of [^{3}H]Ins(1,3,4,5)P_4 was not detectable at early times. However, at 30 min after addition of agonist, [^{3}H] Ins(1,3,4,5)P_4 had increased to 13.2 ± 2.7-fold above baseline (three experiments).

Fig. 4 illustrates the HPLC separation of [^{3}H]InsPs in control and carbachol-stimulated (30 min) circular smooth muscle extracts. The accumulation of InsPs after muscarinic stimulation was dramatic, as seen in Fig. 4, *lower trace*. Apparent in the section of the trace presented in Fig. 4, *upper*, is the accumulation of [^{3}H]Ins(1,3,4,5)P_4. The appearance of $InsP_4$, confirmed by addition of bona fide standards, confirms the presence of the $InsP_3$ 3-kinase in this smooth muscle and suggests

that a role for $InsP_4$ may exist in this tissue. The accumulation of [^{3}H]InsPs after carbachol stimulation was effectively blocked by 1 μM atropine (Table 1) but was unaffected by pretreatment with PTx (200 ng/ml, 3 hr) under conditions known to inactivate muscarinic receptor coupling to adenylate cyclase in this muscle (see Fig. 7).

The concentration dependence of the carbachol effect on PI hydrolysis is illustrated in Fig. 5. The EC_{50} for carbachol stimulation of InsP accumulation is 5 μM. This value is in agreement with the affinity of the M_3 receptor for agonist (1.12 μM) measured in the presence of guanine nucleotide in agonist competition studies (1). The maximum [^{3}H]InsPs accumulation of 3.2-fold, relative to its basal level, was reached at 100 μM carbachol. The concentration-dependent accumulation of [^{3}H] InsPs produced by carbachol was competitively antagonized by pirenzepine and AF-DX 116, with dissociation constants (K_B) of 53 nM and 3 μM, respectively. These data are in agreement with the notion that the M_3 receptor mediates the increase in PIP_2 hydrolysis we measure.

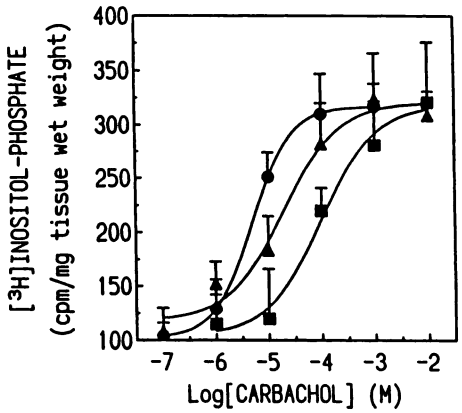

Fig. 5. Carbachol-stimulated concentration-dependent accumulation of [^{3}H]InsPs in canine colonic circular smooth muscle. [^{3}H]Inositol-labeled tissues were incubated with increasing concentrations of the muscarinic agonist carbachol for 30 min at 35°, in the presence of the more M_2-selective antagonist AF-DX 116 (10 μM) (▲) or the more M_3 receptor-selective antagonist pirenzepine (1 μM) (■) or in the absence of antagonist (●). Data for each InsP isomer were added together to produce total InsPs and are expressed relative to tissue wet weight. Curves are simple fits to mean data, using the equation for a sigmoid curve. Values at 1×10^7 M carbachol are not significantly different from results obtained in the absence of agonist. Data are mean values ± standard errors of three experiments performed in duplicate.

TABLE 1

Stimulation of InsP accumulation by carbachol in colonic circular smooth muscle and effects of atropine and PTx

myo[^{3}H]Inositol-prelabeled tissues were incubated, in the presence of 10 mM LiCl, with 100 μM carbachol (CCh) for 30 min. InsPs were extracted and separated by HPLC, as described in the text. Where appropriate, PTx (200 ng/ml) was added during the labeling period. Data are mean values ± standard errors of four experiments performed in triplicate.

	Total InsPs	Ins(1,3,4) P_3	Ins(1,4,5) P_3
	cpm/mg of tissue wet weight		
Control			
Basal	179 ± 31	10 ± 2	24 ± 2
CCh	707 ± 51[a]	21 ± 2[a]	46 ± 5[a]
Atropine			
Basal	199 ± 17	12 ± 2	27 ± 3
CCh	175 ± 13	13 ± 1	22 ± 2
PTx			
Basal	225 ± 16		21 ± 2
CCh	906 ± 28[a]		47 ± 3[a]

[a] Statistically significant increase, compared with basal ($p < 0.05$).

Carbachol also mediated the inhibition of cAMP formation in the circular muscle, consistent with the role of a receptor subtype distinct from that involved in PI hydrolysis. The receptor-independent adenylate cyclase agonist forskolin (10 μM) elevated tissue cAMP levels 2.4-fold over basal in the circular muscle at 10 min. As shown in Fig. 6, carbachol produced a concentration-dependent inhibition (46%) of forskolin-stimulated cAMP formation, with an IC_{50} value of 1.3 μM. Atropine (1 μM) shifted the concentration-response curve to the right in a parallel manner. The dissociation constant (K_B) for atropine inhibition of the carbachol response was 17 nM. The inhibitory effect of carbachol on forskolin-induced cAMP formation was completely blocked by pretreatment of tissues with 200 ng/ml PTx for 3 hr (Fig. 7), as expected for M_2 receptor-mediated inhibition of adenylate cyclase through the G protein G_i. Similar results were obtained when direct measurements of colonic smooth muscle adenylate cyclase activity were made. Carbachol produced a 27% inhibition of the forskolin-stimulated smooth muscle adenylate cyclase enzyme, with an apparent affinity (IC_{50} = 1.6 μM) similar to that seen in the whole tissue for inhibition of cAMP accumulation (Fig. 8). Again, pretreatment of tissues with PTx (200 ng/ml, 3 hr) before preparation of membranes completely eliminated the inhibitory effect induced by carbachol. The K_B value for atropine was 6.4 nM. The effects of other muscarinic antagonists were also examined and are shown in Table 2. These data confirm the M_2 nature of the inhibition of adenylate cyclase by muscarinic stimulation in colonic smooth muscle.

To further examine the involvement of a PTx-sensitive G protein in coupling circular smooth muscle muscarinic receptors to response, the effect of PTx on carbachol competition of

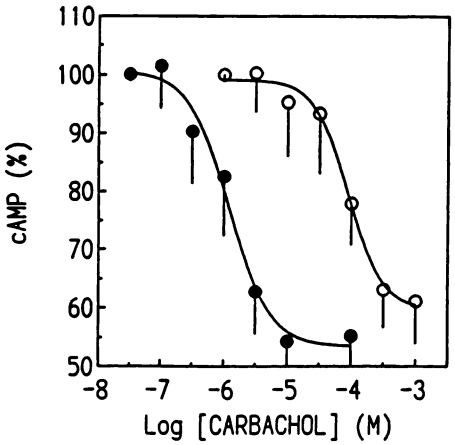

Fig. 6. Effect of carbachol on forskolin-stimulated cAMP formation in canine colonic circular smooth muscle. Strips of circular smooth muscle were incubated with forskolin (10 μM) for 10 min at 35°, and cAMP was extracted and measured as described in the text. From a basal cAMP content of 214 ± 19 pmol/mg of protein, addition of forskolin increased the tissue cAMP level to 514 pmol/mg. Forskolin-stimulated tissues were incubated with increasing concentrations of the muscarinic agonist carbachol, in the presence (○) or absence (●) of a 1 μM concentration of the muscarinic antagonist atropine. As expected for hormonal inhibition of adenylate cyclase, stimulated activity was decreased on the order of 50% at maximal concentrations of carbachol. The inhibitory effect of carbachol is presented as a percentage decrease in forskolin-stimulated cAMP formation. Data are mean values ± standard errors of seven experiments performed in duplicate.

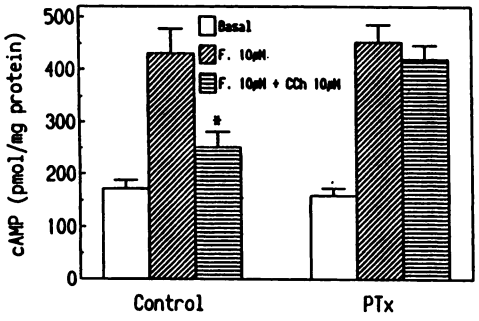

Fig. 7. Effects of PTx on carbachol inhibition of forskolin-stimulated cAMP formation in canine colonic circular smooth muscle. Tissues, preincubated in the absence or presence of 200 ng/ml PTx for 3 hr at 35°, were stimulated with 10 μM forskolin (*F*), in the presence or absence of 10 μM carbachol (*CCh*). Values, determined as described in the text, are expressed as pmol of cAMP/mg of tissue protein. Data are mean values ± standard errors of four experiments performed in duplicate. *, Significantly different from control, $p < 0.005$.

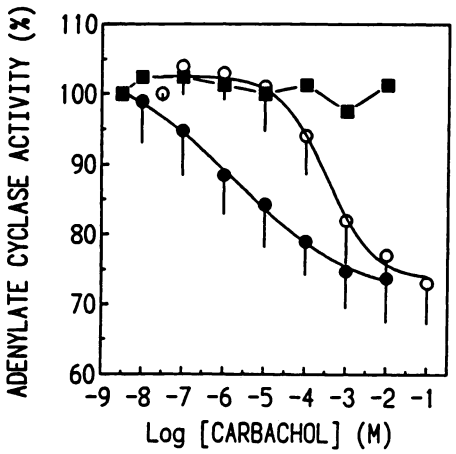

Fig. 8. Concentration-dependent inhibition by carbachol of adenylate cyclase activity in canine colonic circular smooth muscle. Adenylate cyclase activity, determined at 35° for 15 min as described in the text, was measured in circular smooth muscle membranes before (basal activity = 5.2 ± 0.6 pmol of cAMP/mg of protein/min) and after β-adrenergic stimulation by 10 μM isoproterenol (10.5 ± 1.3 pmol/mg/min). Assays were conducted with 10 μM β-adrenergic stimulation in the presence of increasing concentrations of carbachol alone (●) or in the presence of 1 μM atropine (○). The effects of carbachol were also determined after treatment of tissues with PTx (■), as described in the text. Values were normalized to percentage of basal adenylate cyclase activity. Data are mean values ± standard errors of seven experiments performed in duplicate.

TABLE 2

Effects of muscarinic receptor antagonists on carbachol inhibition of adenylate cyclase activity in canine colonic circular smooth muscle

Circular smooth muscle membranes were incubated in the adenylate cyclase assay with the β-adrenergic agonist isoproterenol (ISO) (10 μM) and carbachol (CCh) (100 μM, in the absence or presence of muscarinic receptor subtype-selective antagonists, for 15 min. Data are the mean values ± standard errors of three experiments performed in duplicate.

	Adenylate cyclase activity			
	Control	4-DAMP (1 μM)	AF-DX 116 (1 μM)	Pirenzepine (1 μM)
	pmol of cAMP/mg of protein/min			
Basal	4.1 ± 0.3	4.3 ± 0.5	4.6 ± 0.6	3.7 ± 0.3
ISO	7.7 ± 0.3	8.2 ± 0.3	8.5 ± 0.5	7.5 ± 0.4
ISO + CCh	5.6 ± 0.2[a]	7.9 ± 0.7	8.0 ± 0.9	5.5 ± 0.3[a]

[a] Statistically significant decrease, compared with isoproterenol alone ($p < 0.05$).

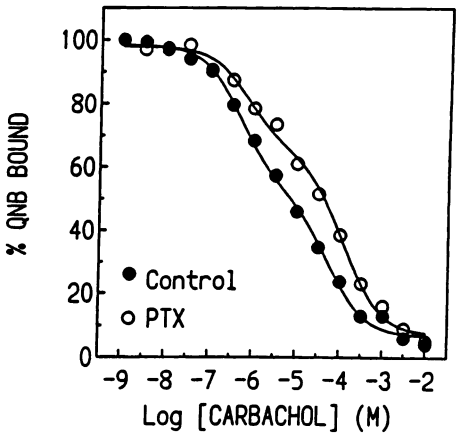

Fig. 9. Effects of PTx on agonist competition of [^{3}H]QNB binding in canine colon circular smooth muscle. Competition of [^{3}H]QNB binding by the muscarinic agonist carbachol was performed in membranes from control tissues (●) and after treatment of tissues with PTx (○), as described in the text. 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 of triplicate determinations in a representative experiment. Additional experiments, described in detail in Table 3, gave similar results. Curves are computer-generated best fits of the data modeled to two classes of binding sites.

[^{3}H]QNB binding was studied. As shown in Fig. 9 and Table 3, in the absence of the hydrolysis-resistant GTP analog GTPγS pretreatment of tissues with PTx (200 ng/ml, 3 hr) shifted the carbachol competition curve to the right and decreased the proportion of high affinity binding sites, consistent with the coupling of muscarinic receptors to adenylate cyclase via the PTx-sensitive G protein G_i. The remaining high affinity binding sites were, however, shifted further rightward upon addition of 100 μM GTPγS, demonstrating the presence of an additional muscarinic receptor population interacting with a PTx-insensitive G protein. In the presence of GTPγS, the ratio of the proportions of binding sites with high and low affinities was not different between control and PTx-pretreated tissues.

Discussion

The present study clearly demonstrates that muscarinic receptor stimulation in the circular smooth muscle of canine colon results in accumulation of [^{3}H]InsPs and inhibition of adenylate cyclase activity. Although it has been demonstrated that a single muscarinic receptor subtype can couple to more than one G protein and, therefore, mediate different signal-transduction pathways (4, 19), it is more commonly found that each subtype of muscarinic receptor couples to a particular effector mechanism (3, 20–22). In the present study, it is clear that the increase in PI hydrolysis and the inhibition of adenylate cyclase that occur after muscarinic receptor stimulation of colonic smooth muscle are mediated by disparate subtypes of muscarinic receptors. The selective inhibitory effect of PTx on carbachol-mediated inhibition of adenylate cyclase but not on the hydrolysis of PIP_2 provides strong evidence that supports this conclusion.

The presence of both PTx-sensitive and PTx-insensitive G proteins in the circular muscle is further confirmed by examination of the effect of PTx on carbachol competition of antagonist radioligand binding to the muscarinic receptors present. Pretreatment of tissues with PTx under conditions that abolish

TABLE 3

Effects of PTx on carbachol competition of [^{3}H]QNB binding in colonic circular smooth muscle

Radioligand binding was performed as described in the text. The effects of PTx on the affinities and proportions of high and low affinity binding (K_H, K_L, R_H, and R_L, respectively) in agonist competition studies ([^{3}H]QNB, 0.3 nM) using carbachol were determined in the absence and presence of 100 μM GTPγS. Data, analyzed by nonlinear computer methods described in the text, are mean values ± standard errors of four experiments performed in triplicate.

	−GTPγS		+GTPγS	
	Control	PTx	Control	PTx
K_H (μM)	0.24 ± 0.01	0.26 ± 0.10	0.79 ± 0.32	0.86 ± 0.68
R_H (%)	49.5 ± 2.03	36.9 ± 1.68[a]	25.3 ± 1.13	24.2 ± 2.82
K_L (μM)	23.7 ± 4.30	51.4 ± 7.01[a]	45.4 ± 5.52	82.3 ± 20.8
R_L (%)	50.5 ± 2.02	63.1 ± 1.68[a]	74.7 ± 1.13	75.8 ± 2.81

[a] Statistically significant difference, compared with the control ($p < 0.05$).

muscarinic receptor-mediated adenylate cyclase inhibition decreased high affinity agonist binding (Fig. 9; Table 3). Such a result demonstrates the coupling of some of the muscarinic receptors present to the PTx-sensitive G protein G_i. However, addition of a saturating concentration of GTPγS further shifted the high affinity agonist binding curve, indicating that a population of high affinity sites are coupled to effector by a G protein that is PTx insensitive. In the accompanying study (1), we demonstrated that M_2 and M_3 subtypes of muscarinic receptor coexisted in the circular muscle. It has been well documented that M_2 muscarinic receptors are coupled to a PTx-sensitive G protein and mediate inhibition of adenylate cyclase, whereas M_3 muscarinic receptors are coupled to a PTx-insensitive G protein and mediate PI hydrolysis (2). Our results are in agreement with those found in rat ileum longitudinal smooth muscle, in which M_2 and M_3 subtypes of muscarinic receptor coexist and mediate inhibition of adenylate cyclase and PI hydrolysis, respectively (9).

The analysis of our radioligand binding agonist competition curves cannot be made from the simple point of view that the 80% of receptors that are M_2 should shift to the right after PTx treatment of tissues. Indeed, the model we propose in this case would involve four components of binding in the absence of GTPγS. That is, 80% of receptors (M_2) would exist in two affinity states, high and low. The minor population of receptors present, M_3, would also exist in two affinity states for agonist. Subtle differences in the agonist affinity of the states of receptor for carbachol would thus, theoretically, reveal four agonist affinity states. Such a possibility as this cannot be modeled adequately by radioligand binding. The likelihood of such a result, however, is mirrored in our data. Two affinity states of the receptor exist in the presence of PTx, as well as in the presence of a saturating concentration of GTPγS. Furthermore, in the presence of GTPγS alone, the computer fit of the competition data predicts the presence of the same receptor populations (~80% M_2; 20% M_3) as are expected from the antagonist binding studies described in the accompanying paper (1).

The selectivity of pirenzepine in blocking carbachol-elicited PI hydrolysis without significantly affecting the inhibition of adenylate cyclase provides further evidence of the involvement of different subtypes of muscarinic receptor in responses to acetylcholine in colonic smooth muscle. In the accompanying study (1), we demonstrated that the affinity of the M_3 muscarinic receptor for pirenzepine in the circular muscle was 26 nM, a value similar to that determined for its ability to block PI hydrolysis in the present study (53 nM). This result confirms that carbachol-stimulated PI hydrolysis in the circular muscle is mediated by the M_3 subtype of the muscarinic receptor. Similar results have also been described in bovine tracheal smooth muscle and rat ileum smooth muscle (9, 23). The affinity of the M_2 muscarinic receptor in the circular muscle for pirenzepine (3.6 μM) was much lower than that of the M_3 receptor (1). We believe that this result explains why addition of 1 μM pirenzepine failed to block carbachol inhibition of adenylate cyclase. In contrast, carbachol-elicited inhibition of adenylate cyclase was blocked by the same concentration of the more M_2-selective antagonist AF-DX 116.

The rapid accumulation in colonic smooth muscle of [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$, with delayed increase in the appearance of [^{3}H]$InsP_1$, in the presence of carbachol, excluded the possibility that PI is a direct substrate for receptor-activated PLC in this tissue. The transient oscillatory nature of [^{3}H]Ins(1,4,5)P_3 accumulation in colonic smooth muscle is in close agreement with measurements of agonist stimulation of PLC activity in a number of tissues, including smooth muscle (24, 25). The finding that the Ins(1,4,5)P_3 isomer releases Ca^{2+} from intracellular stores has been demonstrated in a wide variety of cells and tissues, including smooth muscle (for review, see Ref. 6). In the present study, not only did [^{3}H]Ins(1,4,5)P_3 accumulate rapidly, but the formation of [^{3}H]Ins(1,3,4)P_3 was also extremely rapid and paralleled the changes in [^{3}H]Ins(1,4,5)P_3. This result is distinct from data in other tissues (26, 27). However, in a recent study Chilvers *et al.* (25) demonstrated that [^{3}H]Ins(1,3,4)P_3 production increased rapidly after carbachol stimulation of bovine tracheal smooth muscle. Because the only established route of Ins(1,3,4)P_3 formation is via dephosphorylation of Ins(1,3,4,5)P_4, it is reasonable to suggest that there is rapid formation of [^{3}H]Ins(1,3,4,5)P_4 in *myo*-[^{3}H]inositol-labeled colonic smooth muscle after carbachol stimulation. The reason that [^{3}H]Ins(1,3,4,5)P_4 is not detectable within the first minute after agonist addition could be due to the similar activities of Ins(1,4,5)P_3 3-kinase and the 5-phosphatase enzymes responsible for the formation and removal of Ins(1,3,4,5)P_4, respectively.

A biochemical role for Ins(1,3,4,5)P_4 in regulating extracellular calcium entry has recently been explored (6, 8, 28). Ins(1,3,4,5)P_4 alone has not been shown to raise cytosolic calcium. Instead, the presence of Ins(1,4,5)P_3 is required. In the circular smooth muscle of canine colon, Ins(1,3,4,5)P_4 may be an important second messenger that regulates the movement of extracellular calcium into the cell. This possibility is particularly pleasing because we believe that the role of $InsP_3$-mediated release of Ca^{2+} from sarcoplasmic stores in the muscle is only important for inducing contraction in the initial seconds after addition of agonist. In the continued presence of agonist,

the availability of Ca^{2+} from outside, mediated in part by $InsP_3$ conversion to $InsP_4$, could play the dominant role in the contribution of PI turnover to force generation.

The oscillatory phasic pattern of $InsP_3$ accumulation is not readily explained but may be related to the Ca^{2+}-dependent conversion of $InsP_3$ to $InsP_4$. Activation of the Ca^{2+}/calmodulin-requiring $InsP_3$ 3-kinase would diminish $InsP_3$ levels rapidly. Subsequent decreases in InP_3 and a fall in intracellular Ca^{2+} would again allow $InsP_3$ to accumulate, only to be converted again to $InsP_4$. Additional studies will be required to address this issue, as well as the possible role of $InsP_4$ in this tissue. We are intrigued that $InsP_4$ may have a target in the sarcolemmal membrane. Our preliminary results in purified plasma membranes from colonic smooth muscle demonstrate high affinity binding of $InsP_4$.

As we reported in the accompanying paper, the majority of muscarinic receptors in the circular smooth muscle of canine colon are of the M_2 subtype. Activation of these receptors inhibits adenylate cyclase activity in the circular muscle, thus removing a relaxation signal. Elevation of tissue cAMP content can lead to smooth muscle relaxation through several mechanisms (29–31). In a recent study, Supattapone *et al.* (32) demonstrated that phosphorylation of the $Ins(1,4,5)P_3$ receptor-binding protein by cAMP-dependent protein kinase decreased by 90% the ability of $Ins(1,4,5)P_3$ to release intracellular calcium. In colonic circular muscle, Carl *et al.* (33) have demonstrated, using the patch-clamp technique in single colonic smooth muscle cells, that phosphorylation of the Ca^{2+}-activated K^+ channel by cAMP-dependent protein kinase resulted in an increase in open probability of the channel. Such an increase in channel openings favors repolarization of the muscle cell and thus a decrease in the voltage-dependent entry of Ca^{2+} into the cell. Thus, activation of M_2 muscarinic receptors in the colon circular muscle may directly enhance the effect of M_3 muscarinic receptors and prevent tissue relaxation via an inhibition of cAMP production.

Lechleiter *et al.* (34) demonstrated that activation of the PTx-insensitive G protein-coupled M_3 receptor led to a rapid and transient release of intracellular calcium, whereas activation of PTx-sensitive G protein-coupled M_2 muscarinic receptors produced a slow and oscillatory calcium release. In the continued presence of muscarinic agonist, intracellular calcium continued to oscillate above basal levels. This may well help explain the contraction pattern of the colon circular smooth muscle produced by carbachol, in which an initial variable contraction is followed by a regular phasic contraction.

It is not intellectually pleasing, then, that, despite the obvious complementary role played by M_2 receptor-mediated lowering of cAMP production in the muscarinic-induced contraction of the tissue, antagonism of such contraction by muscarinic receptor subtype-selective antagonists reveals involvement of only M_3 receptors. This conclusion may be due, at least in part, to the absence of antagonists with sufficiently disparate potencies for M_2 versus M_3 receptors. This possibility is not supported by the usefulness of pirenzepine to distinguish receptor subtypes in radioligand binding studies, however. Contraction of tissues pretreated with PTx under conditions described here may help to determine the role of the M_2 receptor in colonic smooth muscle contraction, because this treatment will essentially remove the contribution of decreased cAMP production in the contractile response.

We conclude that M_3 and M_2 receptors coexist in the circular smooth muscle of canine colon and couple to distinct G proteins responsible for the coupling of these receptors to disparate effectors. We suggest that decreased cAMP accumulation and activation of $InsP_3$ production are complex interacting pathways in the muscle that are responsible for the contraction in response to acetylcholine. The presence of $InsP_4$ in the muscle after muscarinic stimulation suggests a role for $InsP_4$ in the regulation of smooth muscle contraction.

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