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Inflammation Inhibits Muscarinic Signaling in *In Vivo* Canine Colonic Circular Smooth Muscle Cells

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ABSTRACT

We investigated the effects of experimental colitis on the muscarinic signaling properties and contractile behavior of canine colonic circular smooth muscle. The hypotheses that inflammation 1) inhibits *in vivo* muscarinic receptor mediated contractions, and 2) alters receptor density or receptor-binding affinities were tested. Muscarine was infused close-intra-arterially in seven conscious dogs during normal and experimental colitis states. Colonic circular muscle contractions were recorded *via* surgically attached strain gauge transducers. Muscarine stimulated phasic contractions in a dose-dependent manner, whereas colitis was inhibited. The inhibitory concentration 50% dose of M_3 receptor inhibitor was several times lower than that of M_1, M_2, and M_4 inhibitors during normal and colitis. However, inflammation induced a significant leftward shift in the circular muscle inhibitory dose-response curve of M_2 inhibitor. Muscarinic receptor density and binding analyses in isolated circular muscle cells was done in normal and colitis states. Inflammation significantly decreased maximum binding from 4082 fmol/mg to 2708 fmol/mg, whereas affinity constant remained unaffected. The conclusions were that 1) spontaneous and muscarine-activated *in vivo* phasic contractile activity of colonic circular muscle cells is primarily mediated by M_3 receptors; 2) inflammation was associated with a shift in M_2 receptor potency, due chiefly to a decrease in receptor density; and 3) this inhibitory effect was seen in normal and inflamed states, suggesting the importance of M_2 receptor. These findings suggest that changes in muscarinic response during colitis may contribute to the abnormal motility seen with inflammatory bowel disease. **(*Pediatr Res* 52: 756–762, 2002)**

Abbreviations

Ach, acetylcholine
AUC, area under contractions
4-DAMP, 4-diphenyl acetoxy-*N*-methyl-piperidine methiodide
Methoctramine, N, N',-bis[6[[(2-methoxyphenyl) methyl]amino]hexyl]-1,8-octanediamine tetrahydrochloride
TTX, tetrodotoxin
IP_3, inositol (1, 4, 5,)-triphosphate
DAG, diacylglycerol
PKC, protein kinase C
IC_{50}, inhibitory concentration 50%
EC_{50}, excitatory concentration 50%
B_{max}, binding maximum
K_d, affinity constant
SMC, smooth muscle cells
[^{3}H]QNB, 1-quinuclidinyl[phenyl-4-^{3}H]benzilate
MPO, myeloperoxidase
Arterial, close-intra-arterial

ACh, the principal neurotransmitter of the enteric nervous system, is released not only as a result of parasympathetic activation, but also in response to many other gastrointestinal peptides (1). Atropine (nonselective muscarinic antagonist), when administered intravenously or close-intra-arterially (arterial) completely blocks all spontaneous smooth muscle contractions in the gut (1–3). In the gastrointestinal system, muscarinic receptors play a significant role in coordination of peristalsis and propulsion of luminal contents (4).

The muscarinic receptors in the gut are localized at presynaptic, postsynaptic, and prejunctional and postjunctional sites (5). These receptors on smooth muscle cells mediate contractions by G-protein coupled mechanisms, whereas those at presynaptic and prejunctional sites modulate the release of ACh by negative feedback. The activation of muscarinic receptors at postsynaptic sites stimulates postsynaptic excitatory and inhibitory neurons to release neurotransmitters. The physiologic mechanisms by which muscarinic receptors stimulate *in vivo* colonic circular muscle contractions are not completely understood.

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In the human colon, ulcerative colitis inhibits phasic contractions and increases giant migrating contractions, and similar findings are noted in canine colon (6–9). The inhibitory effect of colonic inflammation on spontaneous phasic contractions has been observed in earlier studies (9–11). *In vitro*, at the myenteric plexus level at least in rats, excitatory neuronal function may be inhibited, resulting in a decrease in the release of ACh (12), whereas at the circular muscle cell, there may be a decrease in the density or affinity of muscarinic receptors (13).

This study aimed to 1) identify the muscarinic receptor subtypes that mediate the *in vivo* colonic circular muscle contractions during normal and inflamed states; and 2) determine the effect of colonic inflammation on muscarine-stimulated contractile activity. Specifically, the hypothesis that inflammation inhibits muscarinic receptor-mediated contractions *in vivo*, and that inflammation alters receptor density and/or receptor-binding affinities was tested.

METHODS

Animal care approval. This project was approved by the Animal Care Committees at the Zablocki Veterans Affairs Medical Center and the Medical College of Wisconsin, Milwaukee. The National Institutes of Health guidelines on animal care safety have been adhered to. A minimum number of animals were used to achieve a statistically valid study.

Reagents and chemicals. The following substances were used: atropine sulfate (Eli Lilly, Indianapolis, IN, U.S.A.); hexamethonium chloride (ICN Pharmaceuticals, Cleveland, OH, U.S.A.); pirenzepine dihydrochloride, methoctramine tetrahydrochloride, and 4-DAMP (Research Biochemical International, Natick, MA, U.S.A.); and muscarine chloride, tropicamide, and TTX (Sigma Chemical, St. Louis, MO, U.S.A.). All substances were dissolved in 0.9% NaCl and kept frozen at −20°C when not used. Polyethylene glycol 3350 electrolyte mixture (Colyte, Reed and Carnick, Piscataway, NJ, U.S.A.) was used to cleanse the colon before inducing inflammation.

Collagenase type II (319U/mg, Worthington Biochemicals, Freehold, NJ, U.S.A.), soybean trypsin inhibitor (Worthington Biochemicals), and amino acid supplement (B.M.E. amino acid supplement, M.A. Bioproducts, Walkersville, MD, U.S.A.) were used for smooth muscle cell dispersion techniques. ^{3}[H]QNB (Du Pont NEN, Boston, MA, U.S.A.) was used for muscarinic receptor binding studies.

Surgical procedures. Surgical procedures were done as described previously with some modifications (14). Briefly, seven mixed breed dogs of either sex (weight range 18–24 kg, 21.3 ± 0.8 kg) were anesthetized with i.v. sodium pentobarbital (30 mg/kg), and mid-ventral laparotomy was performed. The branches of ileocolic and middle colic arteries were identified in the proximal and distal colon, respectively. One branch artery in each region was carefully dissected from the mesentery while preserving the attached neurons. A silastic catheter (ID 0.75 mm; OD 1.63 mm; Corning, Corning, NY, U.S.A.) was inserted and secured so that its tip rested 1–2 mm from the junction of the branch artery and the main artery. The boundaries of each infused segment measured approximately 5–6 cm. One electrode-strain gauge pair and two strain gauge transducers were attached to the seromuscular layer parallel to the circular muscle axis in each infused segment by 3–0 Surgilon sutures, to record circular muscle contractions. Two additional strain gauge transducers were then attached, one between the proximal and the distal infused segment, and one caudal to the distal infused segment. The lead wires of the transducers were exteriorized through an Amphenol plug embedded in a stainless steel cannula (14).

An intraluminal silastic catheter (ID 2.6 mm, OD 4.9 mm; Corning) was implanted into the colon, 5 cm distal to the ileocolonic junction. All catheters were exteriorized subcutaneously in the subscapular region and were housed safely within jackets. The arterial catheters were flushed twice daily with 2000 IU of heparin. The animals were allowed 7 d to recover from surgery before study.

Protocol for *in vivo* measurements

Experiments were done, first in the normal colon and then in the inflamed colon, with each animal serving as its own control. Contractile activity was recorded for about 1 h to identify the quiescent and contractile states. Contractile and electrical signals were recorded on a 12-channel pen recorder (model 7D; Grass Instrument Co., Quincy, MA, U.S.A.) with lower and upper cut-off frequencies set at direct current and 15 Hz, respectively.

All substances were diluted with 0.9% saline and infused at 1 mL/min arterially for 1 min. After each infusion, catheters were flushed with 0.9% saline (1 mL), infused over 1 min. All agonists were infused during a quiescent period. Catheter patency was confirmed daily, by the response of the segmental contractile activity after muscarine infusion (5 nmol/mL/min for 1min). Successive infusions (dose range of 0.1–20 nmol) were separated by intervals of at least 30–40 min to avoid tachyphylaxis and residual effects. Preliminary experiments established that contractile responses to repeated infusions of muscarine did not vary over time.

TTX (75 nmol) was infused to block sodium channel activity in enteric neurons. Hexamethonium (70 μmol) was infused to inhibit nicotinic receptors in the enteric ganglia. In some experiments, antagonists to muscarinic receptors were infused 1 min before infusion of muscarine. The relatively selective blockers pirenzepine against M_1 receptors (15–17), methoctramine against M_2 receptors (15, 17), 4-DAMP against M_3 receptors (5, 16), tropicamide against M_4 receptors (16, 17), and atropine (nonselective) were used.

Electrical field stimulation (EFS) was applied *via* the electrodes at 10 V, 10 Hz, at 1 ms pulse duration for 1 min to test intact nerve-muscle interaction.

Induction of colonic inflammation. After control experiments were completed, colonic inflammation was induced by mucosal exposure to ethanol and acetic acid, as previously described (9, 14, 18, 19). Briefly, the intraluminal catheter was irrigated with 500 mL of PEG 3350 electrolyte mixture to clear the luminal contents. After sedation with tiletamine and zolazepam (each 10 mg/kg), 95% ethanol was infused *via* the intraluminal catheter (75 mL at 5 mL/min), and into the rectum

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(40 mL *via* a silastic tube with its tip 15 cm from the anal margin); 10 min later, 10 mL and 5 mL of 75% acetic acid was infused *via* the intraluminal catheter and rectal route respectively, and 5 min later, the colon was irrigated with 100 mL of 0.9% saline through the intraluminal catheter. Experiments were begun the following day.

Myeloperoxidase assay. MPO (20, 21) and protein assays (22) were performed, as per the methods described before, on tissues obtained from normal and inflamed colon. The method of tissue collection and assay are briefly stated below. The tissues were washed thoroughly with ice-cold 0.9% saline, and debris and fat removed. Using sharp dissection, the tissue was separated into mucosa-submucosal fraction and circular muscle fraction. Each fraction was weighed and a known volume (20 times the tissue weight) of homogenizing buffer was added to the tissue fraction, and then homogenized in ground-glass homogenizing tubes with ground-glass pestles. The homogenized tissue was stored, frozen in liquid nitrogen, and then thawed. This freeze-thaw cycle was repeated three times and stored at −60°C until assay. Just before the assay, the tissue homogenate was centrifuged for 10 min at 2000 rpm, and the supernatant drawn off. The assay for MPO activity and protein assay in the supernatant was done as described (20–22).

Smooth muscle cell isolation. Smooth muscle cells were isolated according to the methods described before (23–25). Briefly, colon tissue was thoroughly washed in ice-cold 0.9% saline to clear the debris, and then placed in ice-cold oxygenated *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid (HEPES) buffer on ice. Using a sharp dissection technique, the longitudinal muscle layer was first peeled off, and next the circular muscle layer was saved. Tissue squares (5 mm × 5 mm) from the circular muscle were treated with 0.05% collagenase and 0.01% soybean trypsin inhibitor in HEPES buffer, the composition of which was (in mM) 25 HEPES, 120 NaCl, 4 KCl, 2.6 KH_2PO_4, 2 $CaCl_2$, 0.6 $MgCl_2$, 14 glucose, and 2.1% BME amino acid supplement. The tissue squares were gassed with 100% O_2 continuously and kept at 31°C for 30 min. The digested tissue was then filtered twice over a polypropylene mesh (pore size 500 μM) and washed in 10 mL of incubation buffer. The tissue was resuspended in 30 mL collagenase-free HEPES buffer and triturated to yield dispersed smooth muscle cells. Cells were then collected by gravity filtration over a polypropylene mesh, after which they were centrifuged at 1000 × *g* for 5 min, counted, and diluted to a concentration of 2 × 10^5 cells/mL.

Muscarinic receptor binding assay. The binding of [^{3}H]QNB to muscarinic receptors was measured using previously reported methods (26–28). Briefly, binding of [^{3}H]QNB to circular muscle cells was determined in duplicate samples at a range of concentrations (0.1–8 nM). Each reaction was performed at 31°C for 20 min using 10^5 cells/mL. After 20 min, the reaction was stopped by the addition of ice-cold HEPES buffer (0.5 mL), and the cells collected after centrifugation at 14,000 rpm for 10 min. The pellet was washed with 1 mL of ice-cold incubation buffer and re-spun. The supernatant was pooled into individual scintillation vials. Tissue solubilizer (0.2 mL) was added to each pellet and incubated for 20 min at 50°C or until the pellet dissolved. Each dissolved pellet was transferred into scintillation vials containing glacial acetic acid (50 μL) and 8 mL of scintillation cocktail. After mechanically shaking, all vials were left to adapt and counted in a liquid scintillation counter. Specific [^{3}H]QNB binding was determined by the difference between radioactivity bound in the absence (total binding) and the presence (nonspecific binding) of 1 μM atropine. The amount of [^{3}H]QNB bound was determined as the product of the percentage of bound counts per minute (cpm) with the total femtomoles of [^{3}H]QNB in the reaction. Saturation of specific binding sites was examined by increasing the concentration of [^{3}H]QNB from 0.1 to 8 nM. Protein estimation was done by the method of Bradford (26), from the sample of cells (10^5 cell/0.5mL). All binding data are then expressed as [^{3}H]QNB bound per milligram protein.

Data analysis. The contractile response was quantified as AUC, using a planimeter. AUC was used as a measure because area is a true measure of a varying signal (tonic and phasic components), such as contractile activity, whereas other measurements are subsets of area measurements (29). The AUC was measured from the beginning of the first contraction after the start of infusion to when the tracing returned to the baseline, and contractions ceased to occur. All data are expressed as a percentage of the maximum response in the normal colon. Values are mean ± SEM; *n* represents the number of animals. The statistical analysis was done by ANOVA with repeated measures. Multiple comparisons were done by the Fisher protected least squares difference method. A p value < 0.05 was considered statistically significant.

RESULTS

Qualitative observations. Under control conditions, animals had good appetite with normal activity and stools. During colitis, they had decreased appetite and activity, abdominal tenderness, and passed bloody, mucoid stools. In controls, the spontaneous activity consisted of periods of quiescence, short and long duration contractions, and colonic migrating motor complexes (MMCs), whereas during colitis, decreased phasic activity, and presence of giant migrating contractions (GMCs) were noted, as described before (9, 14). Tissues obtained for *in vitro* studies were edematous, ulcerated, and laden with mucosal hemorrhages and exudates, and mucosal MPO was significantly increased during colitis.

In vivo *effect of muscarine on muscarinic receptors in circular muscle.* Whether given during normal and inflamed states, 0.9% saline infusions did not alter contractile activity either in quiescent or phasic periods. Prior infusions of 75 nmol TTX or 70 μmol hexamethonium, which are effective in blocking sodium channel enteric neural conduction and nicotinic receptors, respectively, had no significant effect on the contractile response of circular muscle to muscarine (data not shown, p = NS). However, 20 nmol of atropine completely blocked the muscarinic contractile response, thus indicating that muscarine infusions given arterially acted predominantly on muscarinic receptors on smooth muscle cells. Similar findings were noted during inflammation.

Muscarinic response during inflammation. During inflammation, muscarine-mediated, dose-dependent phasic contrac-

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tile responses were attenuated significantly (Table 1; Fig. 1, *A* and *B*). Colitis significantly reduced the phasic contractile response to muscarine at all doses. Supramaximal doses were avoided as the animals in the pilot studies exhibited discomfort and the responses were less than maximal. The significant increase in EC_{50} doses during inflammation may be reflective of decrease in potency of muscarine in this state (Table 1). In addition, inflamed colon was characteristic for prolonged latency and shortened duration of phasic activity.

***Inflammation and muscarinic receptor signaling* in vivo.** To investigate the role of muscarinic receptor subtypes, the inhibition of the muscarinic control response to increasing doses of pirenzepine, methoctramine, 4-DAMP, tropicamide, and atropine was investigated in normal and inflamed colon (Fig. 2, *A* and *B*). Each inhibitor reduced the muscarinic response in a dose-dependent manner. Under normal conditions, the IC_{50} of 4-DAMP (M_3 receptor inhibitor) was 118-fold, 22.5-fold, and 146-fold smaller than that of pirenzepine, methoctramine, and tropicamide (Table 2). The IC_{50} of atropine was similar to that of 4-DAMP. Inflammation did not change the IC_{50} of 4-DAMP, pirenzepine, tropicamide, or atropine. However, the IC_{50} of the M_2 receptor inhibitor, methoctramine, during inflammation was significantly less than that during the normal state (Table 2).

Comparison of* in vivo *spontaneous contractile activity with muscarine-activated responses during normal and inflamed states. Spontaneous phasic contractions in the normal state occurred 3.4 ± 0.3 times/h *versus* 1.6 ± 0.3 times/h during inflammation ($p < 0.0001$; $n = 7$; $df = 6$). The mean duration of each episode of spontaneous phasic activity lasted significantly longer during the control *versus* the inflamed state (Table 3). The AUC for phasic and tonic activity were also significantly decreased during inflammation. Similar responses were also noted for muscarine-mediated phasic and tonic contractile activity (Table 3). These findings suggest similarities in control of spontaneous and muscarine-activated tonic and phasic contractions, both during the normal and colitis states.

***Effect of muscarinic receptor inhibition on spontaneous phasic contractions* in vivo.** Because mean duration of phasic activity lasted for about 6 min, specific muscarinic receptor inhibitors were infused 2 min after identifying the onset of phasic activity, and suppression of phasic activity was quantified for the next 4 min. The doses of the muscarinic inhibitors were 10 nmol and 200 nmol, given arterially, with 0.9% saline serving as control. At 10 nmol doses, pirenzepine and tropicamide had no impact, but 200 nmol doses resulted in inhibition (Table 4). In contrast, methoctramine inhibited the contractions at both doses. Inasmuch as 10-nmol dose of 4-DAMP totally blocked the contractile activity, much lower doses 0.5 nmol and 2 nmol were used, which significantly attenuated the response. These findings suggest the involvement of M_2 and M_3 receptors in mediating spontaneous phasic activity *in vivo*.

Table 1. *Characteristics of muscarine-induced contractions in normal and inflamed colon*

Characteristics	Normal	Colitis
EC_{50} (nmol)	1.5 ± 0.5	4.0 ± 0.7*
Latency for 5-nmol dose	16.3 ± 2.2	28.3 ± 3.1*
Duration (min)	8.1 ± 1.2	4.7 ± 0.4*
AUC (cm^2)	21.2 ± 2.7	10.6 ± 3.3*
Maximum response (%)	100.0 ± 0	49.0 ± 9.6*

Mean ± SEM, $n = 7$, * $p < 0.05$ *vs* normal.

***Myeloperoxidase activity* in vitro.** MPO-specific activity was determined in the mucosa-submucosal samples and circular muscle samples from the tissues obtained from normal and inflamed colons ($n = 8$ each). MPO activity per milligram of protein in the mucosa-submucosa during normal state was 0.869 ± 0.289, whereas during colitis it was 6.205 ± 0.881 (mean ± SE, $p < 0.001$). MPO-specific activity per milligram of protein in circular muscle during normal state was 0.165 ± 0.083 whereas during colitis it was 0.833 ± 0.281 (mean ± SE, p = NS, $\alpha = 0.05$, $\beta = 0.8$). The inflammation resulted in a 8-fold increase in mucosal-submucosal MPO activity, thus suggesting a significant mucosal disease consistently.

***Inflammation and muscarinic receptor binding* in vitro.** In this study, direct measurement of drug binding to receptors using radioligand binding studies involving saturation experiments were done (30). The percentage of specific binding at 0.1 nM $[^3H]$QNB concentration was 85.1 ± 2.3 (normal colon) *versus* 86.8 ± 2.3 (colitis), and percentage of nonspecific binding was 14.9 ± 2.3% (normal colon) *versus* 13.2 ± 2.3 (colitis). On the other hand, the percentage of specific binding at 8 nM $[^3H]$QNB concentration was 23.2 ± 2.0 (normal colon) *versus* 17.3 ± 0.8 (colitis), and percentage of nonspecific binding was 76.7 ± 1.9 (normal colon) *versus* 95.8 ± 8.2 (colitis), respectively. Thus, the sensitivity and specificity of the assays were similar at both the conditions studied.

Specific $[^3H]$QNB binding to muscarinic receptors on isolated circular muscle cells was expressed as bound radioactivity per milligram of protein, and compared between control and inflamed colon. Data were analyzed by nonlinear regression analysis with the help of GraphPad PRISM software. B_{max} (receptor density) and K_d (ligand affinity) were determined and compared between control and inflamed colon. The specific binding of $[^3H]$QNB was saturable at higher concentrations ($r = 0.8$ control and $r = 0.7$ inflammation, nonlinear regression; Fig. 3). Comparison of variation between the condition (control or inflammation) and concentration (of ligand) was significantly different (ANOVA, $p < 0.001$). Inflammation produced a significant decrease in B_{max} (4082.2 ± 281 fmol/mg control *versus* 2797.5 ± 448 fmol/mg inflammation, *t* test, $p < 0.04$, $df = 8$). However, K_d was not different from each other (0.36 ± 0.06 nM control *versus* 0.23 ± 0.06 nM inflammation, $p = 0.17$, $df = 8$).

DISCUSSION

The physiologic and pathologic role of cholinergic activation of muscarinic receptors present on the colonic smooth muscle cells *in vivo* during health or disease is not well known. The novel approach of using segmental arterial infusions facilitated investigation of the *in vivo* modulation of muscarinic receptor activation of colonic circular muscle cells. Characteristics of spontaneous circular muscle contractions were similar to muscarine-mediated contractions. Similarly, decrease in both spontaneous and muscarine-induced phasic contractile

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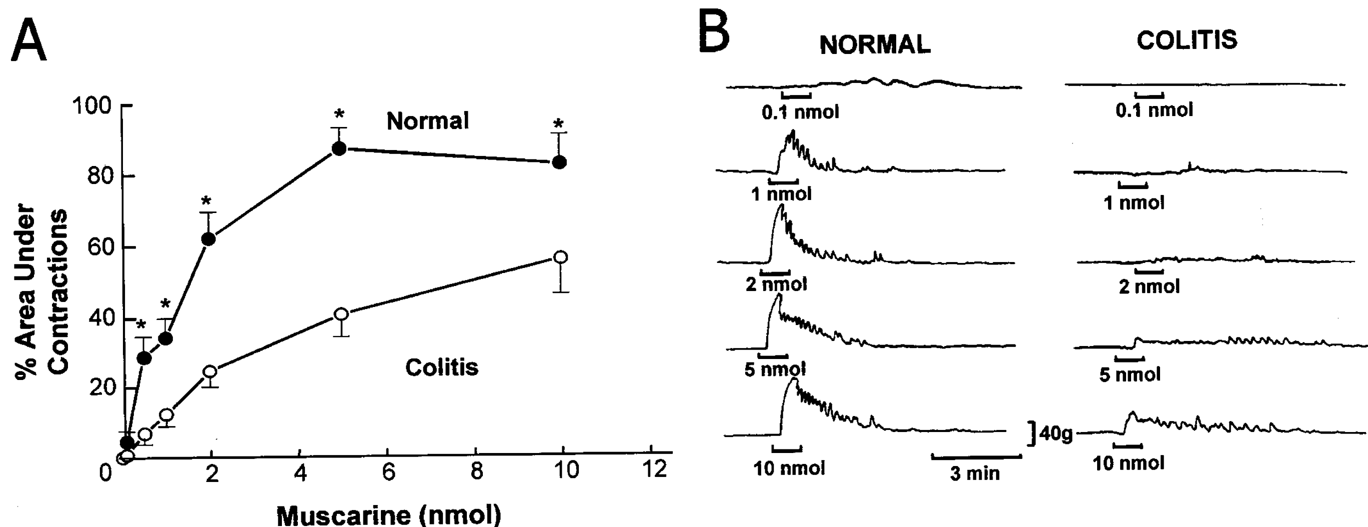

Figure 1. (*A*) Muscarinic dose response curves from normal (*closed circles*) and colitis conditions (*open circles*). The response was significantly decreased both at both lower and higher doses, $n = 6$, $*p < 0.05$. (*B*) A representative tracing of phasic contractile response with graded doses of muscarine infusion in the normal and inflamed colon in the same animal. The responses were decreased during colitis.

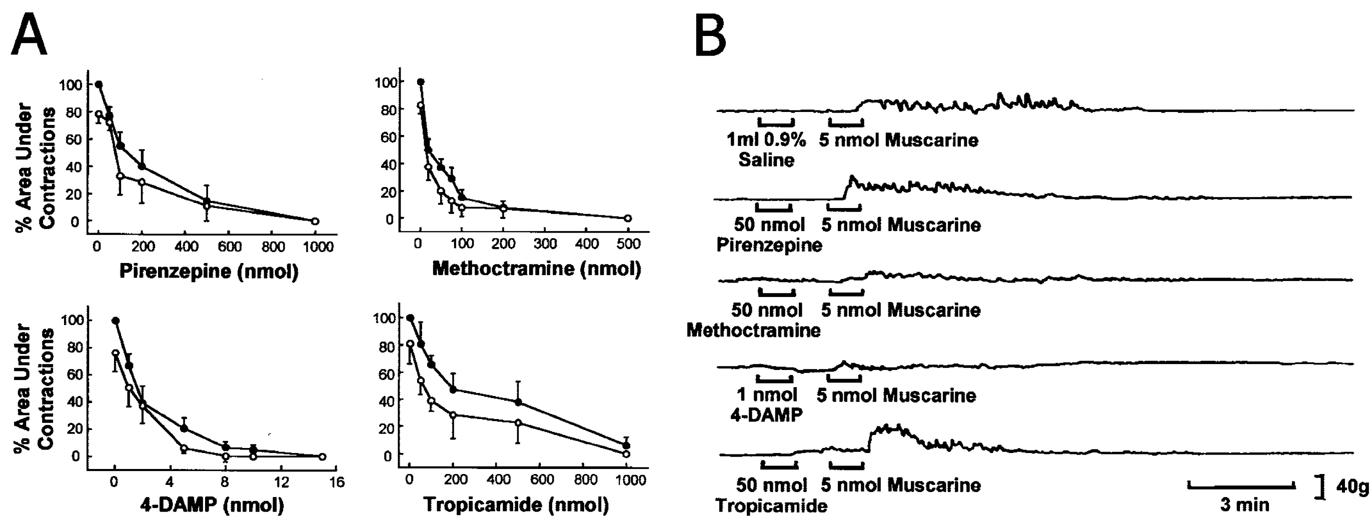

Figure 2. (*A*) *In vivo* inhibitory dose response curves to pirenzepine, methoctramine, 4-DAMP, and tropicamide against 5 nmol dose of muscarine during normal (*closed circles*) and colitis (*open circles*). The dose-response curve of methoctramine has shifted to the left at lower doses during inflammation. (*B*) A representative tracing showing the effect of muscarinic antagonists on muscarine-activated contractions in normal colon. Each antagonist was given arterially over 1 min; 1 min later, muscarine 5 nmol was infused and the response quantified. Note the potency of 4-DAMP in suppressing contractions.

Table 2. *IC_{50} (nmol) of muscarinic receptor inhibitors before and during colitis in vivo*

Muscarinic inhibitors	Before colitis	During colitis
Pirenzepine	236 ± 100*	156 ± 104*
Methoctramine	45 ± 12*	15 ± 3.5*†
4-DAMP	2.05 ± 0.6	1.74 ± 0.5
Tropicamide	291 ± 99*	174.4 ± 65.5*
Atropine	2.9 ± 1.2	1.4 ± 0.7

Mean ± SEM; $n = 4$ or 5. * $p < 0.05$ *vs* 4-DAMP and atropine; † $p < 0.05$ *vs* before colitis.

activity in all animals was characteristic of colitis, thus suggesting a critical role for cholinergic mediation of phasic activity in normal and colitis states.

Because inhibition of muscarinic receptors by atropine blocked both spontaneous and muscarine-induced contractions, it appears that the final neurotransmitter may still be ACh. These findings are consistent with those found by others, in that atropine also blocked substance-P mediated contractions to some degree (14). In addition, the decrease in muscarinic response may contribute to the suppression of phasic contractions during colitis. It is possible that during colitis ACh release by myenteric plexus may be decreased as noted in ileitis (12), however exogenous arterial muscarine infusion did not stimulate increased activity during colitis. Thus, these findings suggest the myogenic response is dependent on the muscarinic receptor and postreceptor activation during normal and colitis states.

In this study, muscarinic response was not affected by prior neural or ganglionic blockade using TTX or hexamethonium, respectively, but the same dose of TTX effectively blocked response by EFS. The response to EFS was blocked by atropine also, thus supporting that muscarine infusions given arte-

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Table 3. *Characteristics of spontaneous contractile activity vs 5 nmol-muscarine-induced activity before and during colitis*

Characteristics	Spontaneous contractions		Muscarine-induced contractions	
	Normal	Colitis	Normal	Colitis
Duration (min)	6.1 ± 0.4	3.5 ± 0.6*	8.1 ± 1.2	4.7 ± 0.4*
AUC (phasic, cm^2)	13.3 ± 2.5	4.1 ± 1.6*	21.2 ± 2.7	10.6 ± 3.3*
AUC (tonic, cm^2)	7.6 ± 1.5	3.0 ± 1.0*	12.3 ± 2.0	6.7 ± 3.2*

Mean ± SEM; $n = 7$. * $p < 0.05$ *vs* normal.

Table 4. *Inhibition of spontaneous phasic contractile activity of circular muscle by muscarinic inhibitors*

Dose	Percentage of AUC after each inhibitor with 0.9% saline as 100% response			
	Pirenzapine	Methoctramine	4-DAMP	Tropicamide
0.5 nmol			58.4 ± 31*	
2.0 nmol			33.2 ± 18*	
10 nmol	101.4 ± 10	59 ± 12*		76 ± 28
200 nmol	54 ± 11*	31.1 ± 11*		56 ± 12*

Mean ± SEM; $n = 5$. * $p < 0.05$ *vs* control.

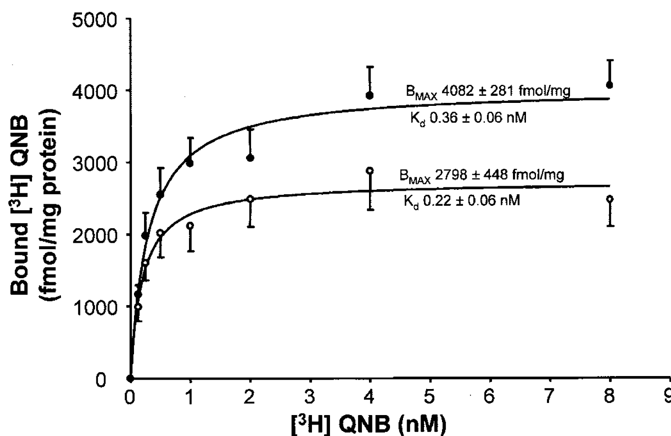

Figure 3. Specific binding of [^{3}H]QNB to isolated SMC from the normal ($n = 6$, *closed circles*) and inflamed colon ($n = 4$, *open circles*). B_{max} decreased significantly during inflammation ($p < 0.05$), whereas K_d did not.

rially activate primarily the muscarinic receptors on the smooth muscle cells, and not activate the muscarinic receptors on the postsynaptic neurons or enteric ganglia.

The contractility of circular muscle to muscarine was dose-dependent, suggesting an increasing recruitment of muscarinic receptors or postreceptor signal transduction mechanisms in mediating this response. This trend was also seen during colitis but the contractility was significantly decreased at both lower and higher doses of muscarinic agonists. It is possible that this change in response during inflammation may be due to a change in receptor affinity or density. The radioligand binding studies showed a decrease in B_{max} during inflammation, thus suggesting a decrease in receptor density. On the contrary, there was no change in K_d or receptor affinity. Based on the saturation experiments, we infer that the receptor density is decreased during inflammation; the nature of this decrease may be related to down-regulation of mRNA synthesis of the muscarinic receptors, or other signal transduction pathways (30–33).

Lu *et al.* (31) found that colitis altered specific parameters of slow waves in colon and reduced the amplitude of spontaneous spike-independent *in vitro* phasic contractions, and that this may be related to alteration in interstitial cells of Cajal function. The similarity between *in vivo* and *in vitro* contractility indicates that the reduced muscarinic response during inflammation was not due to changes in blood flow or mucosal transport, inasmuch as the muscle strips were devoid of mucosa and blood flow. Alternatively, the decrease in contractility during inflammation may be modulated due to specific muscarinic receptor subtype-mediated postreceptor modification mechanisms, such as intracellular signaling pathways mediated by G-protein activation, intracellular Ca^{2+} release, protein kinase C activation, IP_3 activation, and release and phosphorylation of myosin light chain kinases (32, 33).

A significant inhibitory response of a small dose (0.5 nmol) of M_3 receptor antagonist in decreasing *in vivo* spontaneous contractile activity is suggestive of greater potency of M_3 receptor subtype in mediating circular muscle contraction. In contrast, methoctramine, pirenzepine, and tropicamide were able to suppress contractions to the same magnitude at a dose 20-fold, 400-fold, and 400-fold higher than 4-DAMP, respectively. Because all currently available muscarinic receptor subtype inhibitors are only relatively specific, the decreased contractile effects at higher doses of these M_1 and M_4 antagonists may be due to a nonspecific inhibitory effect mediated at more than one muscarinic receptor subtypes. From this data, we may conclude that spontaneous phasic circular muscle contractions are mediated primarily by M_3 receptors, and to some extent by M_2 receptors. Similar findings were found consistent with muscarinic agonist-mediated contractions in that M_3 receptor sub-type inhibitor suppressed muscarinic agonist-mediated circular muscle contractions more than that of others, as evidenced by the fact that IC_{50} of M_3 antagonist was 22.5-fold lesser than M_2-, 118-fold lesser than M_1-, and 145-fold lesser than M_4-antagonists.

Muscarinic receptors transduce ACh signals by activating G proteins to regulate the generation of second messengers (34–36). From our studies, during inflammation there was no significant change in the inhibitory effects of M_1, M_3, and M_4 antagonists or atropine, but the IC_{50} dose of M_2 antagonist decreased significantly during inflammation. The signal transduction mechanisms for the activation of M_2 and M_3 receptors are different (36, 37). M_3 receptors activate G_q-protein that induces phosphinositide hydrolysis to synthesize IP_3 and DAG. M_2 receptors activate G_i-protein that inhibits the activation of adenylyl cyclase and, hence the synthesis of cAMP. IP_3 synthesis increases cytosolic Ca^{2+} by intracellular Ca^{2+} release, whereas cAMP inhibits IP_3 synthesis, IP_3-induced Ca^{2+} influx and stimulates Ca^{2+} uptake in intracellular stores (37). Thus, M_3 receptor activation stimulates contractions directly,

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whereas M_2 receptor activation stimulates them indirectly by inhibiting an inhibitory effect. The significant decrease in the IC_{50} dose of M_2 receptor antagonist (22.5-fold to 9-fold over 4-DAMP) during colitis suggests that the M_2 receptor stimulation may partly contribute to the phasic activity by inhibiting adenylyl cyclase. Thus, colonic circular muscle contractility may be mediated by primarily M_3 receptors and possibly M_2 receptors. Similar results were noted in cat esophageal and lower esophageal sphincter circular muscle cells in that M_2 and M_3 muscarinic receptors evoke different responses (25, 37).

The changes noted in muscarinic receptor signaling mechanisms in the circular smooth muscle in this study during colitis in our model may have been mediated by any of the inflammatory mediators studied in other animal studies. These include 1) marked increase in mucosal permeability and possible translocation of inflammatory mediators or infective organisms (9, 38); 2) neutrophil influx, MPO activity, and generation of superoxide radicals (39, 40); 3) increased production of arachidonic metabolites, immunoreactive 6-ketoprostaglandin 1α, leukotriene B_4, or thromboxane B_2 (18, 19, 41). Although ACh may be the chief neurotransmitter in the enteric nervous system, it is also likely that other putative neurotransmitters may also be affected during inflammation such as tachykinins, as seen in colitis in canine or in humans (14, 42).

In conclusion, inflammation suppresses the tonic and phasic contractile response to spontaneous and muscarinic receptor activation of circular smooth muscle *in vivo*. The primary mediator of spontaneous phasic contractions in circular muscle appears to be M_3 receptor subtype, however inflammation modulates the M_2 receptor function as indicated by significant increase in the potency of methoctramine. The decrease in phasic activity during inflammation may be due to alteration of density of muscarinic receptors or changes in postreceptor changes in signaling. The cholinergic mechanisms shown in this study may contribute to the abnormal motility seen with inflammatory bowel disease.

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