Differential inflammatory modulation of canine ileal longitudinal and circular muscle cells

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**Shi, Xuan-Zheng, and Sushil K. Sarna.** Differential inflammatory modulation of canine ileal longitudinal and circular muscle cells. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G341–G350, 1999.—The aim of this study was to identify the subtypes of muscarinic receptors that mediate in vivo and in vitro canine ileal longitudinal muscle contractions and whether their role is modulated by inflammation. Previous studies have reported that circular muscle contractions are suppressed in ileal inflammation induced by mucosal exposure to ethanol and acetic acid. We found that inflammation had no significant effect on in vivo and in vitro spontaneous or muscarinic receptor-mediated contractions of the longitudinal muscle. The longitudinal muscle contractions were mediated primarily by the M3 receptor subtype. However, the IC50 of the M2 receptor antagonist methoctramine was only 10 times greater than that of the M2 receptor antagonist 4-DAMP in the longitudinal muscle, whereas it was 224 times greater in the circular muscle. M2 receptor-coupled decrease of intracellular cAMP occurred in the longitudinal but not in the circular muscle from the normal ileum. Inflammation did not alter this coupling in the longitudinal muscle but established it in the circular muscle. In conclusion, M2 receptors may play a greater role in the mediation of longitudinal muscle contractions than circular muscle contractions. Inflammation does not alter the contractility or the relative role of muscarinic receptor subtypes in longitudinal muscle cells. However, it modulates the M2 receptor coupling to adenylate cyclase in the circular muscle.

**motility; inflammation; inflammatory bowel disease; 4-diphenylacetoxyn-methylpiperidine methiodide; methoctramine; pirenzepine; tropicamide**

**IN INTACT CONSCIOUS ANIMALS,** the contractile patterns of the longitudinal muscle layer are distinct from those of the circular muscle layer (35). When the circular muscle contracts, the longitudinal muscle is inhibited, but it does not show passive elongations. The percent duration of contractions of the longitudinal muscle is much shorter than that of the circular muscle in the fasting as well as in the fed state (35). The innervation of the two muscle layers also differs (22, 42). The motoneurons innervating the longitudinal muscle are smaller in size than those innervating the circular muscle (42). The longitudinal muscle is innervated locally, whereas the circular muscle is innervated by more distant cell bodies. Some excitatory neurotransmitters, such as neotensin, contract the circular muscle but relax the longitudinal muscle (22). In both cases, the response is mediated by the myenteric neurons.

In some species, the sources of Ca2+ utilized to contract single dispersed longitudinal and circular smooth muscle cells also differ. The longitudinal muscle cells of the guinea pig small intestine utilize Ca2+ influx through L-type Ca2+ channels and Ca2+-induced intracellular Ca2+ release from the ryanodine-sensitive stores, whereas the circular muscle cells utilize Ca2+ released from the inositol 1,4,5-trisphosphate-sensitive stores only (23, 28).

Although several differences in innervation, contractile patterns, and signal transduction pathways between the two muscle layers have been demonstrated, it is not known whether the contractions in the two muscle layers are mediated by the same or different subtypes of muscarinic receptors. A significant difference between the distribution of muscarinic receptors in the longitudinal and circular muscle layers has, however, been reported (32). Sohn et al. (40) also recently reported that ACh-induced cell shortening of enzymatically dispersed cat esophageal circular muscle cells is mediated preferentially by M2 receptors, whereas that of the lower esophageal sphincter smooth muscle cells is mediated by M3 receptors. In contrast, Preiksaitis and Laurier (31) found that carbachol- and electrical field stimulation-induced contractions in the cat esophageal circular muscle strips are mediated by M3 rather than M2 muscarinic receptors. The reasons for the discrepancy in the mediation of contractions by different muscarinic receptors in single dispersed cells and in muscle strips from the same organ of the same species are not understood.

Spontaneous ileal circular muscle contractions as well as those stimulated by cholinergic agonists in vivo and in vitro are suppressed during inflammation (7, 9, 12, 21, 25, 26, 39). Shi and Sarna (39) also found that M2 receptors play a greater role in mediating ACh-induced circular muscle contractions in the inflamed than in the normal ileum. The molecular mechanisms of this difference are, however, not understood.

In vitro studies show that muscarinic receptor-mediated tension is enhanced in the longitudinal muscle of the jejunum but decreased in the circular muscle of nematode-infected rats (9, 14). In trinitrobenzeno sulfonic acid-induced ileal inflammation, the maximal response (Emax) to carbachol is increased in the longitudinal muscle but not affected in the circular muscle (26). The Emax of carbachol in jejunal longitudinal muscle strips is also increased in Trichinella spiralis-infected rats (41). The effect of inflammation on longitudinal muscle contractions in vivo or in single dispersed...
cells and the modulation of muscarinic receptors mediating these contractions during inflammation are not known.

The aims of this study were to examine 1) whether the longitudinal muscle cell response to ileal inflammation is different from that of the circular muscle cells, 2) which subtypes of muscarinic receptors mediate the in vivo and in vitro contractions of the ileal longitudinal muscle, and 3) whether inflammation modulates the role of M2 receptors in longitudinal or circular muscle cells. In addition, if this last point is correct, is this modulation due to a change in the coupling of this receptor to adenylate cyclase? The experiments were performed on intact conscious dogs using close intra-arterial infusions of test substances in short segments of the ileum as well as on muscle strips and enzymatically dispersed single cells. Inflammation was induced by mucosal exposure to ethanol and acetic acid (21).

EXPERIMENTAL PROCEDURES

Surgical Procedures

The in vivo experiments were performed on five mixed-breed dogs, each weighing 20–26 kg (22.8 ± 2.7 kg). Under general pentobarbital sodium anesthesia (30 mg/kg iv), a midventral laparotomy was performed. An intraluminal Silastic catheter (inside diameter of 2.6 mm, outside diameter of 4.9 mm) was implanted 150 ± 10 cm proximal to the ileocolonic junction to infuse acetic acid and ethanol as described later. A stainless steel fistula was implanted 20–30 cm proximal to the ileocolonic junction to drain ethanol and acetic acid so that the inflamed segment was limited to ~130 cm.

Two mesenteric arteries in the study segment were identified and freed while the nerves were preserved (17, 20, 37, 39). A Silastic catheter (inside diameter of 0.75 mm, outside diameter of 1.63 mm) was inserted in the centripetal direction in a branch artery so that its tip rested ~2 mm from the junction of the branch artery and the main artery. The boundaries of the infused segment were identified by infusing 0.9% saline. The length of the infused segment was limited to ~6 cm by ligating branch arteries. Two strain gauge transducers were implanted perpendicularly to each other in the middle of each infused segment; one was oriented to record circular muscle contractions and the other to record longitudinal muscle contractions. The circular muscle transducer assembly also had a pair of bipolar electrodes mounted on it to apply electrical field stimulation (34). The distance between the opposing edges of the two transducers was 3–4 cm. Three additional strain gauge transducers oriented to record circular muscle contractions were implanted; one was 30 cm proximal to the intraluminal catheter, one was half-way between the two infused segments, and one was 15 cm distal to the caudal infused segment. The lead wires of the transducers were brought out through a stainless steel cannula (34).

The intraluminal and intra-arterial catheters were tunneled subcutaneously to the subcapsular region and exteriorized. The catheters were protected by jackets that the dogs wore at all times. Each intra-arterial catheter was flushed twice daily with 2,000 IU heparin. The dogs were allowed 6 days to recover from surgery.

Experimental Protocol of In Vivo Study

All in vivo experiments were conducted in the conscious state after an overnight fast. At least one duodenal phase III activity was recorded to establish the fasting state. The contractile and electrical signals were recorded on a 12-channel Grass recorder (model 7D, Grass Instrument, Quincy, MA).

All test substances were given at close intra-arterial infusions at 3 ml/min for 1 min. At the end of each infusion, the catheters were flushed with 1 ml of 0.9% saline at the same rate. All agents were infused during phase I or a quiescent period during phase II activity of the migrating motor complex (MMC) cycle. At the beginning of each experimental day, 5 µM methacholine was infused for 1 min into each catheter to confirm that the catheters were functioning. This infusion stimulated a series of phasic contractions in the perfused segment (39). A waiting period of at least 25 min was allowed between successive infusions of muscarinic agonists to avoid tachyphylaxis. Preliminary experiments established that the response to repeated infusions of agonists and antagonists was not different after this waiting period. The dose-response curve of muscarine, a muscarinic receptor agonist, was determined to be in the range of 0.1–20 µM (given at 1-min infusions). Close intra-arterial infusions of 70 µM (1 min) TTX and 70 mM (1 min) hexamethonium were used to block Na+ channel conduction in enteric neurons and nicotinic receptors in the enteric ganglia, respectively (17, 20, 37, 39).

The antagonists were infused 2 min before the infusion of the agonist. The following muscarinic antagonists were used: pirenzepine, relatively an M1 receptor antagonist (M1 > M3 > M2) (11); m ethoctramine, relatively an M2 receptor subtype antagonist (M2 > M4 > M3 > M1) (2, 33); 4-diphenyl-acetoxy-N-methylpiperidine methiodide (4-DAMP), relatively an M3 receptor antagonist (M3 > M2 > M4 > M1) (11; 16); tropicamide, a muscarinic receptor antagonist relatively selective for M4 receptors (M4 > M2 > M3 > M1) (24); and atropine, a nonspecific muscarinic receptor antagonist. All experiments were performed first in the normal ileum and then in the inflamed ileum.

Induction of Ileal Inflammation

Ileal inflammation was induced by three mucosal exposures to ethanol and acetic acid, as reported previously (20, 21). Briefly, 75 ml of 95% ethanol were infused intraluminally on day 1 during phase I of the MMC cycle. The same amount of ethanol was infused on days 2 and 3, followed 1 h later by infusions of 50 ml of 20% acetic acid. Ileal inflammation was marked by suppression of individual phasic contractions and MMC cycling and stimulation of giant migrating contractions (20, 21). Myeloperoxidase (MPO) activity, as well as neutrophil count, is increased in the muscularis externa and the lamina propria during inflammation induced by this method (20, 21). Inflammation induced by this method lasts for ~10 days.

MPO Activity and Visual Scoring of Mucosal Damage

MPO activity was determined as previously described (6, 41). Circular and longitudinal muscle layers were separated from normal and inflamed ileum. The tissue samples were weighed and homogenized in 50 mM KH2PO4 (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The homogenates were frozen in liquid nitrogen and freeze-thawed three times. After centrifugation at 2,000 rpm for 10 min, the supernatant was taken for MPO and protein determination. MPO activity was measured by adding 0.1 ml of supernatant to 2.9 ml of 10 mM phosphate buffer containing 6.7 mM guaiacol. Reaction was started by adding 0.0005% hydrogen peroxide, and absorbance at 475 nm was determined on a Beckman DU-640 spectrometer for 3 min. One
unit of MPO is defined as that required to degrade 1 µmol of peroxide per minute at 25°C. Soluble protein in the supernatant was determined using a Bio-Rad assay kit.

The degree of inflammation was also scored visually in tissue segments according to the following criteria adapted from Bel et al. (4): 0 = normal mucosa; 1 = localized hyperemia but no erosions, ulcers, or scars; 2 = linear ulcer or scattered erosion <2 mm each or ulcer scars with no significant inflammation; 3 = linear ulcer or scar with inflammation at one site >2 mm but <5 mm; 4 = two or more sites of ulcerations and/or inflammation, each up to 5 mm; 5 = two or more major sites of inflammation and ulceration of >5 mm each or one major site of inflammation and ulceration extending >1 cm along the length of the mucosa.

In Vitro Muscle Bath Experiments

A 4-cm-long segment of the ileum, ~50–100 cm proximal to the ileocolonic junction, was removed under general pentobarbital sodium anesthesia (30 mg/kg iv) from 10 normal dogs and 6 dogs that had inflammation induced as described in Induction of Ileal Inflammation. The tissues were taken on the sixth day of inflammation, when the inflammatory response and the motility effects are nearly maximal (21). The mucosa was removed, and muscle strips 3 mm × 10 mm were cut along the longitudinal muscle axis. The strips were mounted in a 3-ml muscle bath containing Krebs solution (composition in mM = 120.9 NaCl, 5.9 KCl, 14.4 NaH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, and 11.1 glucose). The bath was oxygenated with 95% O₂-5% CO₂ mixture and maintained at 37 ± 0.5°C. The Krebs solution was replaced every 10–15 min. The strips were equilibrated at 1 g tension for at least 1 h. After equilibration, stretch was applied in steps until the amplitude of spontaneous phasic contractions was maximal. The contractions were recorded on a Grass Instruments 7D polygraph with a Grass FTO3C transducer.

The area under contractions was measured with a planimeter. A motor index ratio (MIR) was determined as follows (22)

\[ \text{MIR} = \frac{A1}{A2} \]

where \( A1 \) is the area under contractions during the first 4 min after the addition of the test substances in the bath and \( A2 \) is the area during the 4 min preceding the addition of test substances. In studying the antagonists, the MIR to 10⁻⁶ M methacholine was taken as 100%.

Dispersion of Single Smooth Muscle Cells

Single smooth muscle cells were isolated separately from ileal circular and longitudinal muscle layers of four normal dogs and four dogs on the sixth day of ileal inflammation. The segments were passed over a glass tube. After light scoring with a dull scalpel blade, the longitudinal muscle layer was peeled off by using wet gauze sponges. The attached fat and connective tissue were carefully removed. The underlying circular muscle layer was further separated from the lamina propria. The circular and longitudinal muscle layers were cut into 0.5 × 0.5 cm pieces and incubated at 37°C in Hanks’ solution for 15 min. Papain (0.35 mg/ml) and diithiothreitol (0.3 mg/ml) were added into the solution, and the tissue pieces were incubated for another 15 min. After papain incubation, the tissue was washed with prewarmed HEPEs buffer and incubated at 31°C for 45 min in HEPEs buffer with 0.1 mg/ml of collagenase II and 0.1 mg/ml of soybean trypsin inhibitor gassed with 100% O₂. At the end of collagenase incubation, the tissue pieces were washed thoroughly with fresh HEPEs buffer and incubated in this buffer for 45–60 min to allow spontaneous cell dispersion from the tissue pieces under gentle to and fro motion. Free cells were collected by pouring the tissue over a 500-µm Nitex mesh, followed by centrifugation.

Measurement of Cell Contraction

Contraction of isolated smooth muscle cells was measured by scanning micrometry as described by Kuemmerle et al. (23) and Murthy et al. (28). Briefly, 0.45 ml of freshly dispensed cell suspension (10⁶ cells/ml) was incubated at 37°C for 15 min, and 50 µl of ACh (final concentration of 10⁻¹² to 10⁻⁷ M) or vehicle were added. The reaction was terminated after 40 s by adding acrolein to 1%. Kinetic studies indicated that the cell contraction in both longitudinal and circular ileal canine smooth muscle cells peaked at 40 s. The length of 50 consecutive cells from one sample was measured by scanning micrometry using the National Institutes of Health Image 1.61 program. The contractile response was expressed as percent decrease in mean cell length from the vehicle control.

Measurement of Intracellular cAMP

Circular and longitudinal muscle layers were separated from normal and inflamed ileums as described in Dispersion of Single Smooth Muscle Cells. They were cut into 2 × 2 mm pieces and incubated with the cAMP-phosphodiesterase inhibitor IBMX (10⁻⁴ M) at 37°C for 20 min. Forskolin (10⁻⁵ M) was used to activate adenylyl cyclase for 10 min. The incubation was terminated by adding two volumes of 6% ice-cold TCA, and the samples were quick-frozen in liquid nitrogen. After homogenization and centrifugation, the pellet was saved for protein measurement, whereas the supernatant was collected and extracted three times with four volumes of ethyl ether. The samples were then dried with a vacuum concentrator. cAMP levels were determined by the competitive protein binding assay using an Amersham cAMP assay kit. In experiments involving the muscarinic receptor-mediated inhibition of adenylyl cyclase, 10⁻⁸ M methacholine was added 1 min before the addition of forskolin. In the experiments with muscarinic receptor antagonists, methoctramine or 4-DAMP was added 5 min before the addition of methacholine.

Chemicals

The following substances were used: atropine (as atropine sulfate; Eli Lilly, Indianapolis, IN); hexamethonium (as hexamethonium chloride; ICN Pharmaceutical, Cleveland, OH); forskolin, pirenzepine (as pirenzepine dihydrochloride), methoctramine (as methoctramine tetrahydrochloride), and 4-DAMP (all from Research Biochemicals, Natick, MA); papain, diithiothreitol, methacholine, muscarine (as muscarine chloride), N-ethyl-2-phenyl-N-(4-pyridylmethyl)hydracrylamide (tropicamide), IBMX, TTX, and motilin (all from Sigma Chemical, St. Louis, MO). Collagenase (type II) and soybean trypsin inhibitor were purchased from Worthington Biochemicals.

Data Analysis

Quantification of in vivo longitudinal muscle contractions. Sarna (35) reported previously that in the intact small intestine, when the circular muscle contracts, the longitudinal muscle shows passive elongations. On the other hand, when the longitudinal muscle contracts, the circular muscle is quiescent, and it does not exhibit passive elongations. The amplitude and duration of circular muscle contractions and the corresponding passive longitudinal muscle elongations are linearly correlated (35). Therefore, in quantitating the contractile response of the longitudinal muscle in response to
the close intra-arterial infusion of agonists, which contract both muscle layers simultaneously, the reduction in the area of the longitudinal muscle contractions due to passive elongations must be taken into account. This was done as follows.

We found that close intra-arterial infusion of motilin stimulated pure passive elongations of longitudinal muscle that were correlated with the circular muscle contractions (Fig. 1A). The areas under circular muscle contractions and longitudinal muscle elongations in response to motilin were also highly correlated ($r = 0.92$).

In contrast to the above, close intra-arterial infusions of muscarine stimulated concurrent contractions of longitudinal and circular muscle. (Fig. 1B). The difference between motilin and muscarine is that motilin acts on presynaptic enteric neurons to stimulate contractions (38), whereas muscarine acts directly on smooth muscle receptors (see RESULTS). However, the area under longitudinal muscle contractions is reduced due to its passive elongation produced by the concurrent contractions of the circular muscle. Therefore, the actual area under the longitudinal muscle contractions, $A_L$, was determined as follows

$$A_L = r A_C + A_{LN}$$

where $r$ is the ratio of areas of passive longitudinal muscle elongations and the corresponding circular muscle contractions determined from motilin infusions. The ratio was determined for each dog because of possible variation in the sensitivities of different strain gauge transducers. $A_C$ is the area under circular muscle contractions, and $A_{LN}$ is the net area under longitudinal muscle response. The net area is the difference between the positive and negative deflections in the longitudinal muscle tracing.

Statistical analysis. All data are expressed as means ± SE. The n value represents the number of dogs for in vivo data and single cell study and number of muscle strips for in vitro data. Differences between multiple groups were determined by ANOVA for repeated or nonrepeated measures and a subsequent Student-Neuman-Keuls test. Differences between two groups were tested by the t-test. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

All dogs developed diarrhea on the third day of induction of inflammation, which continued up to day 8. The tissues were harvested on day 6. The mean mucosal damage score in the inflamed ileum was $4.3 ± 0.4$ against $0 ± 0$ in the normal ileum. Dye exclusion test with trypan blue indicated no difference in viability between the normal and inflamed longitudinal ($91 ± 1.9\%$ and $87 ± 2.3\%$, n = 3 and 4, respectively) and circular muscle cells ($92 ± 0.9\%$ and $92 ± 0.3\%$, n = 3 each, respectively). Hematoxylin and eosin staining revealed extensive leukocyte infiltration in the mucosa, submucosa, and circular muscle layer of the inflamed ileum. Leukocyte infiltration in the longitudinal muscle was sparse. However, aggregates of leukocytes were observed in the serosal layer that was thickened. The MPO activity in the inflamed circular muscle layer, $0.065 ± 0.013$ U/mg protein, was significantly greater than that in the normal circular muscle layer, $0.003 ± 0.002$ U/mg protein ($n = 6$, $P < 0.05$). The MPO activity in the inflamed longitudinal muscle layer, $0.198 ± 0.10$ U/mg protein, was 20-fold greater than that in the normal layer, $0.01 ± 0.005$ U/mg protein, but it did not reach statistical significance ($n = 6$, $P = 0.1$).

Effect of Inflammation on Spontaneous and Muscarinic Receptor-Activated Longitudinal Muscle Contractions

As reported previously (35), in the fasting state the longitudinal muscle exhibited spontaneous phasic contractions as well as negative deflections that were correlated in a 1:1 relationship with circular muscle contractions.

In normal ileum, the longitudinal muscle contractions were present only $16.3 ± 2.5\%$ of the time during daily 8-h recordings ($n = 5$) compared with $56.4 ± 3.5\%$ presence of circular muscle contractions. Inflammation had no significant effect on the percent presence of longitudinal muscle contractions, but it significantly suppressed the percent presence of circular muscle contractions. On day 6, when the inflammatory response is nearly maximum, the longitudinal muscle contractions were present $18.0 ± 4.7\%$ of the recording time, whereas the percent presence of circular muscle contractions decreased to $12.8 ± 2.1\% (P < 0.001$ vs. normal ileum). However, the mean maximum amplitude of longitudinal muscle contractions was significantly reduced from $22.5 ± 4.7\ g$ in the normal ileum to $16.0 ± 3.2\ g$ in the inflamed ileum on day 6 of inflammation ($P < 0.05$, n = 5).

Fig. 1A: close intra-arterial infusion of 1 ml of 0.2 µM motilin stimulated phasic contractions in the circular muscle but passive elongations in the longitudinal muscle. B: close intra-arterial infusion of 1 ml of 5 µM muscarine stimulated phasic contractions in both circular and longitudinal muscle in the infused segment.
There was no significant difference between the dose-response curves of longitudinal muscle contractions to close intra-arterial infusion of muscarine in the normal and the inflamed ileum (Fig. 2; \( ED_{50} = 0.73 \pm 0.21 \mu M \) in normal ileum and \( 0.66 \pm 0.16 \mu M \) in inflamed ileum, \( n = 5 \)). In contrast, we reported previously (39) that the dose-response curve of circular muscle contractions in response to cholinergic agonists is significantly suppressed during inflammation.

Locus of Action of Close Intra-Arterial Infusion of Muscarine to Stimulate Longitudinal Muscle Contractions

We first established the effectiveness of TTX and hexamethonium in blocking enteric neural contraction and nicotinic receptors, respectively. These experiments also revealed the differences between longitudinal muscle responses to enteric neural stimulation and direct smooth muscle stimulation. Close intra-arterial infusion of 0.2 \( \mu M \) motilin stimulated a series of phasic contractions in the circular muscle but only negative deflections in the longitudinal muscle (Fig. 1). Prior close intra-arterial infusion of 70 \( \mu M \) TTX, 20 \( \mu M \) atropine, or 70 mM hexamethonium almost completely blocked the circular muscle phasic contractile response to motilin (data not shown). The corresponding negative deflections in the longitudinal muscle were also absent.

In contrast to motilin, close intra-arterial infusion of muscarine stimulated phasic contractions in both longitudinal and circular muscle (Fig. 1). Prior close intra-arterial infusions of 70 \( \mu M \) TTX or 70 mM hexamethonium had no significant effect on the longitudinal muscle response to muscarine, but infusion of 20 \( \mu M \) atropine blocked the response completely (data not shown). These data indicated that muscarine, at close intra-arterial infusion, acted primarily on postjunctional smooth muscle muscarinic receptors to stimulate longitudinal muscle contractions. On the other hand, motilin acted at a presynaptic site and involved at least one nicotinic synapse to stimulate circular muscle contractions.

Muscarinic Receptor Subtypes Mediating Longitudinal Muscle Contractions

Close intra-arterial infusion of muscarine, 5 \( \mu M \times 1 \) min, was used as control for studies with muscarinic receptor antagonists. Each antagonist dose dependently inhibited the longitudinal muscle response to muscarine (Fig. 3). However, the \( ID_{50} \) of the \( M_3 \) receptor antagonist 4-DAMP was 176-fold and 183-fold smaller than that of \( M_1 \) and \( M_4 \) receptor antagonists (pirenzepine and tropicamide, respectively) but only 10-fold smaller than that of the \( M_2 \) receptor antagonist methoctramine (Table 1). The \( ID_{50} \) of 4-DAMP was not significantly different from that of the nonspecific muscarinic

![Fig. 3. Inhibitory dose-response curves of muscarinic receptor antagonists given at close intra-arterial infusions in normal ileum. 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide.](http://ajpgi.physiology.org/DownloadedFromhttp://ajpgi.physiology.org/)

**Fig. 2.** Dose-response curve of in vivo contractions to close intra-arterial infusions of muscarine was not different between longitudinal muscle layers in the normal and inflamed ileum.
Table 1. In vivo ID$_{50}$ of muscarinic antagonists

<table>
<thead>
<tr>
<th>In Vivo ID$_{50}$, µM</th>
<th>Normal ileum</th>
<th>Inflamed ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>1.8±0.3</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>Pirenzipine</td>
<td>281.8±24.1*</td>
<td>236.6±21.4†</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>16.0±3.1*</td>
<td>17.4±3.7†</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>1.6±0.3</td>
<td>2.0±0.4</td>
</tr>
<tr>
<td>Tropicamide</td>
<td>292.0±24.7*</td>
<td>218.8±17.3†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5, except for tropicamide where n = 4. *P < 0.05 vs. the ID$_{50}$ of atropine in normal ileum; †P < 0.05 vs. the ID$_{50}$ of atropine in inflamed ileum.

receptor antagonist atropine. Inflammation did not alter the ID$_{50}$ of any muscarinic receptor antagonist in the longitudinal muscle (Table 1, Fig. 4).

Effect of Inflammation on the Contractile Response of In Vitro Muscle Strips and Single Dispersed Cells

Both methacholine ($10^{-9}$ to $10^{-4}$ M) and muscarine ($10^{-9}$ to $10^{-4}$ M) exhibited similar dose-response curves in muscle strips. These responses were not affected by a prior 15-min incubation of the muscle strips with 3 µM TTX. Further in vitro muscle bath experiments were performed with methacholine only.

There was no significant difference between the dose-response curves of longitudinal muscle strips taken from the normal and the inflamed ileum in response to methacholine (Fig. 5A). The ED$_{50}$ values in the normal
Effect of Inflammation on the Coupling of M2 Receptor

The concentration-response curve to ACh was significantly suppressed when compared with that of the normal ileum (39). The inhibitory dose-response curve of methoctramine is significantly shifted to the left in the inflamed ileum compared with that in the normal ileum (39). The inhibitory dose-response curve of methoctramine is significantly shifted to the left in the inflamed ileum compared with that in the normal ileum (39). The inhibitory dose-response curve of methoctramine is significantly shifted to the left in the inflamed ileum compared with that in the normal ileum (39). M2 receptors are coupled to adenylate cyclase (1, 5, 15, 30). We, therefore, investigated whether the synthesis of cAMP in response to cholinergic stimulation differs between the longitudinal and the circular muscle cells and whether this synthesis in the two types of cells is altered during inflammation.

The basal cAMP concentrations in ileal longitudinal and circular muscle cells were not different from each other (Table 3). Ileal inflammation did not significantly alter the basal cAMP in either muscle (Table 3). Forskolin, 10 µM, increased cAMP production by 5.2 ± 1.1- and 5.3 ± 0.5-fold over basal levels in the circular and the longitudinal muscle, respectively, from the normal ileum, but these levels were not different from each other (Table 3). Also, forskolin-stimulated increases in cAMP production in the longitudinal and circular muscle of the inflamed ileum (6.9 ± 1.0- and 6.9 ± 1.6-fold, respectively) were not different from those in the normal ileum.

**Table 2. In vitro ID$_{50}$ of muscarinic receptor antagonists in longitudinal muscle strips**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>ID$_{50}$, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pirenzipine</td>
<td>(5.7 ± 0.5) × 10$^{-7}$x</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>(1.1 ± 0.2) × 10$^{-8}$</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>(1.4 ± 0.4) × 10$^{-9}$</td>
</tr>
<tr>
<td>Tropicamide</td>
<td>(6.2 ± 0.4) × 10$^{-7}$</td>
</tr>
</tbody>
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Values are means ± SE; n = 6-10 strips. *P < 0.05 vs. 4-DAMP.

**Table 3. Basal and forskolin-stimulated cAMP levels**

<table>
<thead>
<tr>
<th>cAMP Level, pmol/mg protein</th>
<th>Basal</th>
<th>10 µM Forskolin</th>
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<tbody>
<tr>
<td>Circular muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal ileum</td>
<td>338 ± 80</td>
<td>1,757 ± 372</td>
</tr>
<tr>
<td>Inflamed ileum</td>
<td>321 ± 66</td>
<td>2,216 ± 322</td>
</tr>
<tr>
<td>Longitudinal muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal ileum</td>
<td>278 ± 75</td>
<td>1,702 ± 139</td>
</tr>
<tr>
<td>Inflamed ileum</td>
<td>301 ± 55</td>
<td>2,075 ± 481</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 for each group.

In the inflamed ileum were (2.1 ± 0.2) × 10$^{-7}$ M and (3.5 ± 0.3) × 10$^{-7}$ M, respectively (n = 5, P > 0.05). We reported previously that the response of the circular muscle strips is suppressed in the inflamed ileum (39).

The amplitudes of the spontaneous contractions in longitudinal muscle strips from the normal and the inflamed ileum were also not different from each other (1.4 ± 0.2 g and 1.2 ± 0.2 g, respectively, n = 8 and 6, respectively).

Each muscarinic receptor antagonist dose dependently inhibited the contractile response of longitudinal muscle strips to 10$^{-6}$ M methacholine. The rank order of potency was the same as in the intact state (M$_3$ > M$_2$ > M$_1$ = M$_4$). The ID$_{50}$ of 4-DAMP was 407-fold, 7.8-fold, and 442-fold greater than that of pirenzipine, methoctramine, and tropicamide, respectively (Fig. 6B, Table 2).

In single cell preparations, the resting lengths of the longitudinal and circular muscle cells were 99.1 ± 3.8 and 102.1 ± 4.6 µm, respectively. The resting cell lengths in inflamed tissue were not significantly different from those in the normal tissue (97.4 ± 4.1 and 99.6 ± 4.5 µm, respectively). The concentration response of circular muscle cells from the inflamed ileum to ACh was significantly suppressed when compared with that of the cells from the normal ileum (Fig. 6A). However, the concentration-response curves to ACh in longitudinal muscle cells from the normal and the inflamed ileum were not different from each other (Fig. 6B).

**Effect of Inflammation on the Coupling of M$_2$ Receptor to Adenylate Cyclase**

A comparison of our present findings with those published previously (39) indicated that the M$_2$ receptors may play a greater role in the regulation of longitudinal muscle contractions than in circular muscle contractions in the normal ileum. The ID$_{50}$ of methoctramine is over 200-fold greater than that of 4-DAMP in the circular muscle, whereas it is only 10-fold greater in the longitudinal muscle. Also, previously published data indicate that inflammation modulates the role of M$_2$ receptors so that they play a greater role in stimulating circular muscle contractions in the inflamed ileum than in the normal ileum (39). The inhibitory dose-response curve of methoctramine is significantly shifted to the left in the inflamed ileum compared with that in the normal ileum (39). M$_2$ receptors are coupled to adenylate cyclase (1, 5, 15, 30). We, therefore, investigated whether the synthesis of cAMP in response to cholinergic stimulation differs between the longitudinal and the circular muscle cells and whether this synthesis in the two types of cells is altered during inflammation.

The basal cAMP concentrations in ileal longitudinal and circular muscle cells were not different from each other (Table 3). Ileal inflammation did not significantly alter the basal cAMP in either muscle (Table 3). Forskolin, 10 µM, increased cAMP production by 5.2 ± 1.1- and 5.3 ± 0.5-fold over basal levels in the circular and the longitudinal muscle, respectively, from the normal ileum, but these levels were not different from each other (Table 3). Also, forskolin-stimulated increases in cAMP production in the longitudinal and circular muscle of the inflamed ileum (6.9 ± 1.0- and 6.9 ± 1.6-fold, respectively) were not different from those in the normal ileum.
In normal ileal longitudinal muscle, 10 µM methacholine significantly reduced the forskolin-induced production of cAMP by 54 ± 8% (n = 4, P < 0.05; Fig. 7). Inflammation had no significant effect on the reduction of forskolin-induced cAMP by methacholine in the longitudinal muscle (50 ± 6%, P > 0.05 vs. control, n = 4; Fig. 7).

In contrast, methacholine had no significant effect on forskolin-induced production of cAMP in normal circular muscle cells (6.6 ± 10%, n = 4, P > 0.05, Fig. 7). However, in the circular muscle cells from the inflamed ileum, 10 µM methacholine significantly reduced the increase of forskolin-induced cAMP by 34 ± 5% (n = 4, P < 0.05).

**DISCUSSION**

Our findings show that ileal inflammation, which significantly suppresses in vivo and in vitro spontaneous and muscarinic receptor-mediated contractions in the circular muscle (36, 39), has no significant effect on these contractions in the longitudinal muscle. The consistent lack of effect of inflammation on longitudinal muscle in the intact conscious state, in muscle strips and in single dispersed cells, indicates that the cellular processes in these cells in response to muscarinic receptor activation may not be altered by inflammation.

The percent duration of spontaneous in vivo longitudinal muscle contractions as well as the amplitude of these contractions stimulated by exogenous administration of cholinergic agonists did not change during inflammation. However, the mean maximum amplitude of spontaneous in vivo contractions was decreased during ileal inflammation. This may be because exogenous cholinergic agonists, in the intact ileum as well as in muscle strips and single cells, act directly on smooth muscle cells, which have a response that is not altered by inflammation. On the contrary, the spontaneous contractions in the intact state depend on the release of endogenous ACh from the enteric neurons (37). Intravenous or close intra-arterial administration of atropine completely blocks these contractions (36, 37). Collins et al. (8) have reported that ACh release in response to electrical field stimulation or veratridine is decreased during Trichinella spiralis-induced inflammation in the rat jejunum. Experiments in our lab indicate that during ethanol/acetic acid-induced inflammation ACh release in response to veratridine or electrical field stimulation is also decreased in the longitudinal muscle-myenteric plexus preparations from the canine ileum (38). Therefore, the decrease in the mean maximum amplitude of spontaneous contractions is likely due to a decrease in the amount of endogenous release of ACh and not due to a change in the excitation-contraction coupling on the longitudinal muscle. Our finding that the percent presence of longitudinal muscle contractions does not change during inflammation suggests that the percent duration of spontaneous ACh release may not change during ileal inflammation.

Whereas most studies indicate that the in vitro response of small intestinal circular muscle to cholinergic agonists is suppressed during inflammation (7, 9, 12, 21, 25, 26, 39), the response of the longitudinal muscle may be species and region specific. It may also depend on the method of inducing inflammation. The contractility of the rat jejunal longitudinal muscle is increased during inflammation induced by T. spiralis or Nippostrongylus brasiliensis in the jejunum (14), but it is decreased in the ileal and colonic longitudinal muscle (13, 18, 27). Our data show that the response of the canine ileal longitudinal muscle during ethanol/acetic acid-induced inflammation is not altered. The precise mechanisms for the differences in the response of the longitudinal and circular muscle to inflammation are not understood, but several factors, such as differences in the signal transduction pathways between the two cell types (23, 28), differences in their reaction to specific inflammatory response mediators (19), the differential utilization of M2 and M3 receptors by the two types of cells, and the differences in the infiltration of immunocytes in the two muscle layers, may contribute to this phenomena.

The signal transduction pathways for M2 and M3 receptors to contract smooth muscle cells differ (1, 11, 29). The activation of M3 receptors contracts smooth muscle cells directly by the hydrolysis of phosphatidylinositol mediated by Gq protein. On the other hand, the stimulation of M2 receptors produces indirect contraction. M2 receptors are coupled to Gq protein whose activation decreases the stimulation of adenylate cyclase and hence the formation of cAMP. If cAMP is actively relaxing smooth muscle cells at the time of stimulation of M3 receptors, the relaxation is reduced. In other words, M2 receptor stimulation does not actively contract smooth muscle cells, but it decreases their relaxation induced by intracellular cAMP.

Several in vitro studies in muscle strips have indicated that both longitudinal and circular muscle contrac-
tions of the guinea pig and rat ileum are mediated primarily by M3 receptors (3, 11). This is despite the fact that in almost all the species investigated the M2 receptors predominate in the ileum (10, 11). The reasons for this anomaly are unclear. However, our findings indicate that, although the M3 receptors may have only a minor role in stimulating normal canine ileal circular muscle contractions, their role is enhanced during inflammation. We reported previously that the inhibitory dose-response curve of methoatrelin is shifted to the left in the inflamed ileum (39). Our present findings indicate that in normal ileal circular muscle methacholine has no effect on the formation of cAMP by stimulation with forskolin. However, in the inflamed ileum, cAMP stimulation by forskolin is significantly reduced by methacholine. These data suggest that M2 receptors may not be coupled to adenylate cyclase in the normal state, but this coupling is established in the inflamed state.

The synthesis of cAMP by stimulation with 10 µM forskolin was not different in circular muscle cells from that in the normal and inflamed ileum. Inflammation may, therefore, not alter the adenylate cyclase activity in these cells, but it may modulate the coupling of M2 receptors to this enzyme, as discussed above.

The M2 receptors may play a relatively greater role in the mediation of longitudinal muscle contraction than of circular muscle contractions in the normal ileum. The ID50 of methoatrelin was only 10-fold greater than that of 4-DAMP in the longitudinal muscle. In contrast, the ID50 of methoatrelin was over 200-fold greater than that of 4-DAMP in the normal circular muscle (39). Similar differences were noted in the IC50 values in muscle strips. This observation is supported by the finding that, in normal longitudinal muscle, methacholine decreased cAMP formation, whereas it had no effect in normal circular muscle. Inflammation did not alter the basal or the forskolin-stimulated concentrations of cAMP in the longitudinal muscle. Inflammation also did not alter the methacholine-induced decrease in the synthesis of cAMP stimulated by forskolin in the longitudinal muscle. These findings are in agreement with the observation that inflammation has no significant effect on in vivo or in vitro longitudinal muscle contractions.

The in vivo ID50 and in vitro IC50 values of M1 and M4 muscarinic receptor antagonists were, respectively, ~200- and 400-fold greater than those of M3 receptor antagonist. The inhibition of longitudinal and circular muscle contractions by these antagonists at high doses is likely to be a nonspecific effect. The ID50 and IC50 values of these antagonists did not change during inflammation.

Direct stimulation of the longitudinal muscle by cholinergic agonists stimulated contractions of the longitudinal muscle, but stimulation of the enteric neurons with motilin induced only passive elongations of the longitudinal muscle, which were dependent on the concurrent occurrence of circular muscle contractions. This finding suggests that motilin receptors may be localized on selective presynaptic neurons that inner-vate the motor excitatory neurons to the circular but not the longitudinal muscle. We did not investigate the innervation of the nonadrenergic noncholinergic neurons by motilin receptor containing presynaptic neurons in this study. Our previous data indicate, however, that these presynaptic nerves also innervate the inhibitory nitronergic motoneurons (38). This inhibitory innervation to the longitudinal muscle may facilitate its passive elongations when the circular muscle is contracting.

In conclusion, in the ethanol/ acetate-induced inflammation of the ileum, the muscarinic receptor-activated contractions of the longitudinal muscle are not affected in the intact conscious state, in muscle strips, or in single dispersed cells. These contractions are, however, suppressed in the circular muscle. The cholinergic contractions of the longitudinal muscle are mediated primarily by M3 receptors. The M2 receptor is coupled to adenylate cyclase in the longitudinal but not in the circular muscle of the normal ileum. Accordingly, the M2 receptors may play a role in the stimulation of longitudinal muscle contractions but not of circular muscle contractions in the normal ileum. Inflammation establishes the M2 receptor to adenylate cyclase coupling in the circular muscle, and, hence, the M3 receptor may mediate circular muscle contractions in the inflamed state.

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INFLAMMATORY MODULATION OF MUSCARINIC RECEPTORS


