Impairment of Ca\(^{2+}\) mobilization in circular muscle cells of the inflamed colon

Shi, Xuan-Zheng, and Sushil K. Sarna. Impairment of Ca\(^{2+}\) mobilization in circular muscle cells of the inflamed colon. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G234–G242, 2000.—This study investigated whether inflammation modulates the mobilization of Ca\(^{2+}\) in canine colonic circular muscle cells. The contractile response of single cells from the inflamed colon was significantly suppressed in response to ACh, KCl, and BAY K8644. Methoxyverapamil and reduction in extracellular Ca\(^{2+}\) concentration dose-dependently blocked the response in both normal and inflamed cells. The increase in intracellular Ca\(^{2+}\) concentration in response to ACh and KCl was significantly reduced in the inflamed cells. However, Ca\(^{2+}\) efflux from the ryanodine- and inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive stores, as well as the decrease of cell length in response to ryanodine and IP\(_3\), were not affected. Heparin significantly blocked Ca\(^{2+}\) efflux and contraction in response to ACh in both conditions. ACh-stimulated accumulation of IP\(_3\) and the binding of \(^{[3H]}\)ryanodine to its receptors were not altered by inflammation. Ruthenium red partially inhibited the response to ACh in normal and inflamed states. We conclude that the canine colonic circular muscle cells utilize Ca\(^{2+}\) influx through L-type channels as well as Ca\(^{2+}\) release from the ryanodine- and IP\(_3\)-sensitive stores to contract. Inflammation impairs Ca\(^{2+}\) influx through L-type channels, but it may not affect intracellular Ca\(^{2+}\) release. The impairment of Ca\(^{2+}\) influx may contribute to the suppression of circular muscle contractility in the inflamed state.

AN INCREASE IN FREE CYTOSOLIC Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is a critical step in smooth muscle contraction. The force of contraction in these cells is related nonlinearly to [Ca\(^{2+}\)]\(_i\) (15, 30). The two sources from which Ca\(^{2+}\) may be mobilized for this increase in [Ca\(^{2+}\)]\(_i\) are the extracellular medium and the rapidly exchanging intracellular stores in the sarcoplasmic reticulum. It now seems that, in the gut smooth muscle, the utilization of these two sources is organ, species, and agonist dependent. In the guinea pig small intestine, the longitudinal muscle cells utilize Ca\(^{2+}\) influx through L-type dihydropyridine-sensitive channels and intracellular release from ryanodine-sensitive stores to contract in response to CCK and ACh (11, 18, 25). By contrast, the small intestinal circular muscle cells from the same species utilize Ca\(^{2+}\) from the \(\alpha\)-myo-inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive stores (11, 25, 27). Feline esophageal circular muscle cells, on the other hand, utilize Ca\(^{2+}\) influx from the extracellular medium, whereas those from the lower esophageal sphincter and fundus utilize Ca\(^{2+}\) efflux from IP\(_3\)-sensitive stores but not from the extracellular medium (4, 14). CCK-8 mobilizes Ca\(^{2+}\) from the IP\(_3\)-sensitive stores in the feline gallbladder smooth muscle, whereas ACh can mobilize it from both the extracellular and intracellular sources (19). Substance P-induced contraction of the rabbit anal sphincter utilizes intracellular Ca\(^{2+}\) release, whereas that induced by bombesin utilizes extracellular Ca\(^{2+}\) (5). The mobilization of Ca\(^{2+}\) to contract canine colonic circular muscle cells is not fully understood.

Inflammation suppresses the phasic contractions and generation of tone in the circular muscle cells of the colon of several species (12, 22, 28, 40). The molecular mechanisms of this suppression of contractions are not known. However, since an increase in cytosolic Ca\(^{2+}\) is central to smooth muscle contraction, our hypothesis is that Ca\(^{2+}\) mobilization is altered during inflammation.

The aim of this study was to identify the sources of Ca\(^{2+}\) utilized in contracting single circular smooth muscle cells from the canine colon and how this utilization is altered during inflammation. ACh was used as the agonist in this study because it is one of the primary neurotransmitters of spontaneous colonic contractions at the neuroeffector junction (45). Intravenous or close intra-arterial administration of atropine, a nonselective muscarinic receptor antagonist, blocks all spontaneous contractions in the intact conscious state.

EXPERIMENTAL METHODS

Tissue preparation and dispersion of smooth muscle cells. A 7- to 8-cm-long segment of the proximal colon was removed under general pentobarbital sodium anesthesia (30 mg/kg; Abbott Laboratories). The segment was passed over a glass tube and scored along the longitudinal axis with a blunt blade. The longitudinal muscle layer was peeled off and discarded. The remaining tissue was scored deeper. The circular muscle layer was peeled off and collected in ice-cold HEPES buffer (pH 7.4).

Smooth muscle cells were isolated by two consecutive digestions with papain and collagenase, respectively. Briefly, the circular muscle sheet was cut into 0.5 × 0.5 cm\(^2\) pieces and incubated at 37°C in 20 ml of Ca\(^{2+}\)-free Hanks’ solution (pH 7.2) for 15 min. Then they were incubated in 20 ml of Ca\(^{2+}\)-free Hanks’ solution containing 0.38 mg/ml papain and 0.3 mg 1,4-dithiothreitol until the tissue appeared loose and
sticky (~10 min). The tissue was washed with HEPES buffer and further digested at 31°C with 0.2 mg/ml collagenase type II (319 U/mg) and 0.1 mg/ml soybean trypsin inhibitor for 30–40 min. The digested tissue was washed three times with enzyme-free HEPES buffer, and the muscle cells were allowed to disperse spontaneously under gentle to-and-fro motion. Cells were harvested by filtration through a 500-µm Nitex mesh and collected by centrifugation at 350 g for 5 min. Cells were resuspended in HEPES buffer, and volume was adjusted to reach 5 x 10⁶ cells/ml for cell contraction experiments and 10⁶ cells/ml for [Ca²⁺]i measurements.

Permeabilization of smooth muscle cells. The cells were permeabilized with saponin for use in experiments that required intracellular application of large molecules, as previously described (25, 27). The cells were incubated for 10 min in cytosol-like medium containing 35 µg/ml saponin. Saponin-treated cells were centrifuged twice at 350 g for 5 min, washed free of saponin, and resuspended in the same medium containing, in addition, antimycin (10 µM), ATP (1.5 µM), creatine phosphate (5 mM), and creatine phosphokinase (10 U/ml). The volume was adjusted according to different usage of these cells (5 x 10⁶ cells/ml for cell contraction study and 10⁶ cells/ml for [Ca²⁺]i efflux study).

Measurement of free cytosolic Ca²⁺ in intact cells and [Ca²⁺]i efflux in permeabilized cells. [Ca²⁺]i was measured in a 2-ml cell suspension (10⁶ cells/ml) using the Ca²⁺-fluorescent dye fura 2-AM (Molecular Probes, Eugene, OR) as described by Murthy et al. (26). Muscle cells were suspended in a modified HEPES buffer (26) containing (in mM) 10 HEPES, 125 NaCl, 5 KCl, 1 CaCl₂, 0.5 MgSO₄, 5 glucose, 20 taurine, 43 sodium pyruvate, and 5 creatine and were incubated with 2 µM fura 2-AM and 0.02% pluronic acid for 30 min at 31°C. The fura 2-AM-loaded samples were diluted, centrifuged twice, and suspended in 2 ml of solution with the same composition for immediate measurement of [Ca²⁺]i. Fluorescence was measured at 510 nm, with excitation wavelengths alternating between 340 and 380 nm, using an Aminco-Bowman series 2 luminescence spectrometer. Autofluorescence of unloaded cells was subtracted from the fluorescence of fura 2-loaded cells. The [Ca²⁺]i was calculated from the fluorescence ratio as described by Grynkiewicz et al. (13). The dissociation constant (Kd) of fura 2-AM was used to calculate [Ca²⁺]i. The maximum and minimum fluorescence were determined after adding 50 µg/ml digitonin and 4 mM Tris-EGTA (pH 8.7), respectively, in each sample.

Ca²⁺ efflux was measured by a modified method of Poggioli and Putney (32), described by Murthy et al. (18, 25). Briefly, the cells (10⁶ cells/ml) were suspended in cytosol-like solution containing 45Ca²⁺ (10 µCi/ml) and 10 µM antimycin (to prevent mitochondrial uptake of Ca²⁺) at 31°C. 45Ca²⁺ uptake was initiated by the addition of 1.5 mM ATP and ATP regenerating system consisting of 5 mM creatine phosphate and 10 µM creatine phosphokinase. The net uptake of Ca²⁺ was determined from duplicate 100-µl samples removed at intervals for a period of 60 min. Preliminary experiments indicated that 45Ca²⁺ uptake reached a steady state in 60 min (data not shown). Net Ca²⁺ efflux in response to ACh, IP₃, or ryanodine was examined at the end of 60 min of incubation and expressed as percent decrease in the steady-state 45Ca²⁺ cell content.

Measurement of IP₃ by radioreceptor assay. IP₃ was measured in circular muscle tissue using Amersham's assay kit containing 3H-labeled d-myo-IP₃ and bovine adrenal IP₃, as previously described by Murthy and Makhlouf (27). Circular muscle squares (2 x 2 mm²) were incubated in Krebs solution with 10 mM LiCl for 15 min at 37°C. ACh (10⁻⁴ M) was added, and the reaction was terminated with 2 volumes of 6% TCA at 10, 20, 40, and 60 s. The samples were homogenized and centrifuged at 4°C for 15 min at 4,000 rpm. The pellets were collected for protein determination, whereas the supernatant was further extracted three times with 6 volumes of water-saturated ether. The IP₃ content in the aqueous phase was measured by incubating with [3H]P₃ and binding protein on ice for 15 min. After centrifugation at 2,000 g for 15 min, the supernatant was carefully removed and the pellet was dissolved with water. The bound IP₃ in the dissolved pellet was counted with a Packard 1900 CA beta counter.

Measurement of [H]ryanodine binding to single cells. Specific [H]ryanodine binding was measured in isolated single cells, as described previously (18). Aliquots (0.5 ml) containing 5 x 10⁵ cells were incubated at 31°C with [H]ryanodine. In the kinetic and competition studies, 40 nM [H]ryanodine was used. The binding was rapid, attaining a steady state within 10 min, and was reversible with the addition of unlabeled ryanodine (10⁻⁵ M). The Kd and maximal binding (Bmax) were determined by using [H]ryanodine concentrations ranging from 5 to 120 nM. The incubation was stopped at 10 min with 0.75 ml of ice-cold HEPES buffer. Nonspecific binding was determined in the presence of 10⁻⁴ M nonlabeled ryanodine. The cell suspension was centrifuged at 12,000 g for 5 min to separate the bound radioligand from the free radioligand. The wash and spin cycle was repeated two times. Kd and Bmax values were calculated using GraphPad Prism software version 2.0 (GraphPad Software, San Diego, CA).

Measurement of cell length. Cell length was measured by scanning micrometry as described previously (11, 25, 41). An aliquot (0.45 ml) of cells (5 x 10⁵ cells/ml) was exposed to 50 µl of test agent at 31°C for different durations for time-course experiments or 40 s for ACh and 15 s for IP₃ experiments. The reaction in each case was terminated by adding acrolein (final concentration, 1%). In other experiments, the cell samples were incubated with antagonists for 5 min before the addition of agonists. The lengths of 30 consecutive intact healthy cells were measured through a phase-contrast microscope (Nikon), fitted with a video camera (Javelin CCD), and connected to a Macintosh Computer. NIH Image 1.61 was used to measure the length. The contractile response was expressed as percent cell shortening from the vehicle control.

Induction of colonic inflammation and its visual assessment. Colonic inflammation was induced by mucosal exposure to ethanol and acetic acid (22, 40, 41). On day 1, the dogs were anesthetized with Telazol (150 mg im; Elkins-Sinn, Cherry Hill, NJ). The colon was cleansed by inducing defecation with neostigmine (30 µg/kg iv). An intraluminal Silastic tube with side holes in the first 5-cm length was advanced to ~50 cm from the anal margin to flush the colon with 700 ml of Colyte. The dogs were fasted overnight.

On day 2, the dogs were anesthetized again as described above. A Silastic tube with side holes in the first 5-cm length was advanced via rectum to ~50 cm, and another similar tube was advanced to ~15 cm from the anal margin. Ethanol (75 ml, 95%) was perfused through each tube at 5 ml/min. Ten minutes later, 20 ml of 60% acetic acid and 15 ml of 10% acetic acid were perfused through the proximal and the distal tubes, respectively, to induce pancolitis. After 5 min, the colon was flushed with 100 ml of 0.9% saline through each tube. Tissues were harvested 48 h later. We have reported previously that, at this time, the myeloperoxidase activity is increased (22) and the spontaneous phasic contractions and generation of tone in response to ACh are significantly suppressed (22, 40). Lu et al. (22) have also reported that, in tissue samples taken 30 min after exposure to ethanol and acetic acid, the motility changes are absent, indicating that the changes are associ-
ated with the inflammatory response rather than mucosal injury.

The visual scoring system to assess mucosal injury was adapted from Bel et al. (2) as follows: 0 = normal mucosa; 1 = localized hyperemia but no erosions, ulcers, or scars; 2 = linear ulcer or scattered erosion ≤2 mm or ulcer scar with no significant inflammation; 3 = linear ulcer or scattered erosion ≥2 mm or ulcer scar with significant inflammation; 4 = two or more sites of ulceration and/or inflammation, each up to 5 mm; 5 = two or more major sites of inflammation and ulcerations >5 mm each or one major site of inflammation extending >1 cm along the length of the mucosa.

Materials and solutions. Collagenase type II and soybean trypsin inhibitor were obtained from Worthington (Freehold, NJ). Papain, 1,4-dithiothreitol, ACh, IP3, low-molecular-weight heparin, methoxyverapamil (D-600), essential amino acid mixture, saponin, ATP, antimycin, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical (St. Louis, MO), and BAY K8644 was purchased from RBI (Natick, MA). Fura 2-AM, pluronic acid F-127, and digitonin were purchased from Molecular Probes (Eugene, OR). 45Ca2+-CaCl2 and [3H]ryanodine were obtained fromNew England Nuclear (Boston, MA), and IP3 assay kit containing [3H]P2 was purchased from Amersham Life Science (Arlington Heights, IL).

The composition of HEPES buffer (pH 7.4) in mM was 120 NaCl, 2.6 K2HPO4, 4 KCl, 2 CaCl2, 0.6 MgCl2, 25 HEPES, 14 glucose, and 2.1% essential amino acid mixture. The composition of cytosol-like medium in mM was 200 NaCl, 100 KCl, 5 MgSO4, 1 NaH2PO4, 25 NaHCO3, 0.5 CaCl2, 1 EGTA, and 2% BSA. The medium was bubbled with 95% O2 and 5% CO2 to maintain a pH of 7.2. Krebs solution was bubbled with 95% O2 and 5% CO2 and consisted of (in mM) 120 NaCl, 6 KCl, 1.4 NaHCO3, 1.2 MgCl2, 2.5 CaCl2, and 11 glucose. The composition of Hanks’ solution in mM was 135 NaCl, 5.5 KCl, 0.5 KH2PO4, 4 NaHCO3, 0.4 Na2HPO4, 0.5 MgCl2, and 5.5 glucose, pH 7.3. The composition of the Ca2+-measurement solution was the same as that described in Measurement of free cytosolic Ca2+ in intact cells and 45Ca2+ efflux in permeabilized cells.

Statistical analysis. All values are expressed as means ± SE, and n represents the number of animals. Statistical analysis was performed by ANOVA with nonrepeated measures or unpaired t-test. Multiple comparisons were performed by Student-Newman-Keuls test, and P < 0.05 was considered statistically significant.

This study was approved by the Animal Studies Subcommittee of the Zablocki Veterans Affairs Medical Center.

RESULTS

All dogs developed diarrhea within 24 h after the induction of inflammation. The visual morphological score was 4.4 ± 0.3 in the inflamed tissue and 0 ± 0 in the normal tissue. The dispersed cells appeared to be healthy and relaxed. The cell viability tested with trypan blue dye exclusion was 88% ± 4% and 86% ± 3% in cells from the normal and the inflamed colon, respectively (n = 3 for each). Lu et al. (22) have reported previously that, 48 h after the induction of colonic inflammation, the myeloperoxidase activity is increased significantly in the muscularis.

Contractile response to ACh. The resting cell length of circular muscle cells from the normal colon (98 ± 4.2 μm; n = 6) was not different from that of cells obtained from the inflamed colon (97 ± 4.6 μm; n = 5). The cells from both the normal and the inflamed colons con-

tracted in a concentration-dependent manner in response to ACh (10−11–10−5 M). The contractile response in the inflamed cells was significantly suppressed (P < 0.05). The maximum decrease in length in normal cells (25.4 ± 2.3% at 10−5 M ACh) was significantly greater than that in inflamed cells (14.6 ± 3.6%; Fig. 1). The EC50 in the inflamed cells (9.9 ± 0.4 nM) was significantly greater than that in the normal cells (1.9 ± 0.2 nM).

The role of Ca2+ influx in cell contraction. BAY K 8644 (10−12–10−5 M) and KCl (25–100 mM) concentration-dependently decreased the cell length in both normal and inflamed cells (Fig. 2). The maximal effect in the inflamed cells was significantly suppressed in response to both compounds (n = 4 each).

D-600 (10−12–10−5 M) concentration-dependently inhibited the decrease in cell length to 10−5 M ACh in normal and inflamed cells. A significant inhibition to the same dose of ACh was observed at 10−9 M D-600 in normal cells vs. 10−7 M in the inflamed cells (Fig. 3A). However, the residual responses at the maximum concentration of 10−5 M D-600, 80 ± 3.4% and 51 ± 1.2%, were not different in normal and inflamed cells, respectively (n = 4 each, P > 0.05). Similarly, a decrease in extracellular Ca2+ concentration-dependently reduced the response to ACh (Fig. 3B). The residual responses at 0 Ca2+; with 2 mM EGTA were 7.5 ± 1.1% in normal and 5.1 ± 0.4% in the inflamed cells (P > 0.05, n = 4–5). A significant decrease in response was observed first at 0.5 mM external [Ca2+]i in normal cells and at 0.25 mM external [Ca2+]i in inflamed cells.

Cytosolic [Ca2+]i and Ca2+ efflux. The basal [Ca2+]i, was not different between the normal and the inflamed cells. ACh at 10−5 M evoked an increase of 80 ± 10 nM in [Ca2+]i, that reached a peak in 10–15 s (Fig. 4). The increase in [Ca2+]i in the inflamed cells (34 ± 3 nM) was significantly smaller than that in normal cells (Fig. 5A). Similarly, 50 mM KCl increased [Ca2+]i in both types of cells, but the increase in the inflamed cells was smaller than that in the normal cells (Fig. 5B). The increase in [Ca2+]i by 75 mM KCl that induced maximal cell short-
ening in inflamed samples was also less than that in response to 50 mM KCl in normal cells (65 ± 10 nM vs. 103 ± 28 nM; n = 4). The peak [Ca^{2+}], in response to KCl was reached at 15–20 s.

The steady-state 45Ca^{2+} uptake attained at 60 min was not different between the normal and the inflamed cells (0.12 ± 0.02 vs. 0.18 ± 0.04 per 10^6 cells; n = 4; P > 0.05). Both ACh and IP_3 evoked 45Ca^{2+} efflux in normal and inflamed permeabilized cells. The peak response to these agonists occurred at 30 s and 15 s, respectively (data not shown). There was no significant difference between the peak efflux and contraction evoked by IP_3 in normal and inflamed states (Fig. 6). In the presence of 100 µg/ml heparin, an IP_3 receptor antagonist, the evoked efflux in response to ACh was reduced from 31.8 ± 3.7% to 9.7 ± 6.3% in normal cells and from 31.4 ± 4.6% to 17.0 ± 4.8% in inflamed cells (n = 4–6; P < 0.05 each) (Fig. 7A). The ACh-induced contractions in these cells were significantly inhibited also by 100 µg/ml heparin (Fig. 7B).

In another series of experiments, cells from the normal and the inflamed colon were treated with 10^{-6} M thapsigargin for 30 min in Ca^{2+}-free and 2 mM EGTA buffer to deplete intracellular Ca^{2+} stores. The cells were then resuspended in HEPES solution containing 2 mM Ca^{2+}. The thapsigargin-treated normal cells contracted 13.5 ± 1.0% in response to 10^{-5} M ACh (57.7 ± 4.2% of that for nontreated cells, n = 3). However, the thapsigargin-treated inflamed cells contracted only 4.3 ± 0.7% (30.1 ± 1.6% of that for non-thapsigargin-treated cells; n = 4; P < 0.05 vs. normal cells). These data further indicated that the role of Ca^{2+} influx in contracting the cells is diminished in the inflamed state.

IP_3 accumulation in response to ACh. Direct measurement of specific [3H]IP_3 accumulation indicated that the basal level of IP_3 in the inflamed cells (1.2 ± 0.4 pmol/mg protein) was significantly smaller than that in normal cells (3.0 ± 0.5 pmol/mg protein). However, the stimulated levels in response to 10^{-4} M ACh were not different between the two states of the cells (Fig. 8). The peak IP_3 accumulation at 20 s was 9.7 ± 1.4 pmol/mg protein in normal cells and 7.0 ± 1.8 pmol/mg protein in the inflamed cells (n = 4; P > 0.05 vs. normal cells).

Binding of [3H]ryanodine to circular muscle cells and utilization of ryanodine-sensitive stores. In the presence of 10^{-5} M ruthenium red, a ryanodine channel blocker, the ACh-induced 45Ca^{2+} efflux was reduced from 31.5 ± 3.7% to 17.1 ± 1.9% (n = 4; P < 0.05), and contraction in permeabilized cells was reduced from 17.1 ± 2.7% to 5.4 ± 0.7% (n = 4; P < 0.05). These data indicated that ACh also mobilizes Ca^{2+} from the ryanodine-sensitive stores.

Time-course experiments indicated that the binding of [3H]ryanodine attained a steady state in 10 min at 31°C. The binding was inhibited by 63 ± 3% with the addition of 10^{-5} M unlabeled ryanodine and by 58 ± 4% with the addition of unlabeled cADP ribose, indicating...
that both bind to the same receptor. [3H]ryanodine in the range of 5–120 nM exhibited concentration-dependent binding in normal and inflamed cells (Fig. 9). The $K_d$ and $B_{max}$ values of [3H]ryanodine binding were $35.7 \pm 9.8$ nM and $37.8 \pm 4.1$ fmol/5 x $10^5$ cells, respectively, in the normal cells and $32.6 \pm 10.7$ nM and $29.0 \pm 3.6$ fmol/5 x $10^6$ cells in the inflamed cells ($n = 5$; $P > 0.05$).

Ryanodine ($10^{-5}$ M) decreased the length of normal colon circular muscle cells by $12.0 \pm 2.5\%$, evoked $45\text{Ca}^{2+}$ efflux of $23.4 \pm 5.5\%$, and increased cytosolic Ca$^{2+}$ by $61.3 \pm 7.5$ nM. The corresponding values of $10.5 \pm 1.5\%$, $19.1 \pm 4.2\%$, and $50.8 \pm 8.6$ nM in the inflamed cells were not different from those in the normal cells ($n = 4$–$6$; $P > 0.05$ for all comparisons).

**DISCUSSION**

Our findings show that the canine colonic circular muscle cells may utilize Ca$^{2+}$ influx through dihydropyridine-sensitive channels as well as intracellular Ca$^{2+}$ release from both the ryanodine-sensitive and IP$_3$-sensitive stores to contract. Downregulation of Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels may contribute to the suppression of contractility to ACh during inflammation.

Several lines of evidence support the utilization of Ca$^{2+}$ influx to contract canine colonic circular muscle cells. Patch-clamp recordings have demonstrated the presence of dihydropyridine-sensitive L-type Ca$^{2+}$ channels in these cells (21, 35). Close intra-arterial infusion of verapamil, an L-type channel blocker, blocks the contractile response to ACh in intact conscious dogs (23). The contractile response of circular muscle strips to ACh is blocked by nicardipine, another antagonist of L-type channels (39). The blockade of L-type channels in these cells, however, did not completely inhibit the response to ACh. The residual response (~30% of the maximum response) may utilize other pathways, such as intracellular Ca$^{2+}$ release and activation of protein kinase C (1, 25, 43, 47).

Ca$^{2+}$ influx and intracellular Ca$^{2+}$ release interact (6, 16, 24, 33, 34, 44, 46). Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels acts as a cofactor to induce Ca$^{2+}$ release [Ca$^{2+}$-induced Ca$^{2+}$ release (CICR)], whereas the emptying of intracellular Ca$^{2+}$ stores induces capacitive entry of Ca$^{2+}$ from the extracellular medium [Ca$^{2+}$ release-activated current (CRAC)]. The influx by the CRAC mechanism is mostly through nonselective cation channels and is not blocked by L-type Ca$^{2+}$ channel blockers. In the canine colonic circular muscle, the contractile response to ACh was blocked almost completely by D-600, indicating that the influx was primarily through the L-type channels. In addition, when the intracellular stores were depleted by treatment with thapsigargin, the normal cells still exhibited ~60% of the response in nontreated cells, indicating that the Ca$^{2+}$ influx occurred independently of intracellular Ca$^{2+}$ release. Ca$^{2+}$ mobilization by CICR rather...
than by the CRAC mechanism may, therefore, predominate in these cells.

Our findings also demonstrate that Ca$^{2+}$ influx may be impaired during inflammation. The concentration-response curves to both KCl, which depolarizes the membrane, and dihydropyridine BAY K8644, which activates voltage-sensitive L-type channels, were suppressed in the inflamed colon cells. A significant inhibitory effect of verapamil was noted at a concentration that was two log order greater in the inflamed than in normal cells. Similarly, a one-log order smaller concentration of extracellular Ca$^{2+}$ was required to significantly reduce the response in the inflamed than that required in the normal cells. When intracellular Ca$^{2+}$ was depleted by incubation with thapsigargin, the contractile response to ACh was significantly smaller in the inflamed than in normal cells. Together, these findings indicate less dependence of the contractile response on Ca$^{2+}$ influx in the inflamed state. Recently, we have demonstrated also that Ca$^{2+}$ currents recorded by the patch-clamp method are significantly reduced in the inflamed colon cells (21).

The utilization of extracellular Ca$^{2+}$ for smooth muscle contraction may, however, vary among species and in different organs of the gut. Grider and Makhlouf (11), Murthy et al. (25), and Kuemmerle et al. (18) found that single circular muscle cells of the guinea pig small intestine do not utilize Ca$^{2+}$ influx for CCK-8-induced contraction despite the presence of L-type Ca$^{2+}$ channels in these cells, as demonstrated by patch-clamp recordings (7). Biancani et al. (4) reported that the feline esophageal circular muscle cells utilize Ca$^{2+}$ influx for ACh-induced contraction, whereas the cells from the lower esophageal sphincter do not. The feline fundic circular muscle cells also do not seem to utilize Ca$^{2+}$ influx for their contraction (14). ACh-induced, but not CCK-8-induced, contraction of the feline gallbladder smooth muscle utilizes Ca$^{2+}$ influx through the L-type channels (19). Experiments on single human jejunal circular muscle cells show that they invoke Ca$^{2+}$ influx and contraction in response to KCl (11), but the contractile response to ACh is not blocked by D-600...
suggesting that muscarinic receptor activation does not utilize Ca$^{2+}$ influx through L-type channels. By contrast, Farrugia et al. (8) reported that the ACh and erythromycin-induced increase of free cytosolic Ca$^{2+}$ in the human and canine jejunal circular muscle cells utilizes Ca$^{2+}$ influx through L-type channels. Dihydropyridine-sensitive Ca$^{2+}$ channels are present in the human colon circular muscle cells (48).

Canine colonic circular smooth muscle cells also seem to be able to mobilize Ca$^{2+}$ from both the IP$_3$-sensitive and ryanodine-sensitive stores to contract in response to ACh. IP$_3$ accumulation in the cells increased time-dependently and peaked at ~20 s after stimulation with ACh. This peak time is shorter than the peak time for contraction in response to ACh (~40 s), indicating the expected temporal order of events for IP$_3$ to mobilize Ca$^{2+}$ for contraction. Exogenous IP$_3$ contracted permeabilized cells and released Ca$^{2+}$ from the intracellular stores. Furthermore, heparin inhibited the contraction as well as Ca$^{2+}$ efflux in response to ACh. However, at the heparin concentration of 100 µg/ml, there was still a residual response of ~36% of the control, indicating the involvement of other pathways for contraction, independent of IP$_3$ receptors.

The colonic circular muscle cells also mobilized Ca$^{2+}$ from the ryanodine-sensitive stores. Concentration-dependent binding of [H]$^3$ryanodine established the presence of ryanodine receptors in these cells. In addition, ryanodine contracted these cells and the response to ACh was partially but significantly blocked by ruthenium red, an antagonist of ryanodine receptors. Ca$^{2+}$ mobilization from ryanodine-sensitive stores has also been reported in canine circular muscle strips (39) and in the intact conscious state (23).

The availability of Ca$^{2+}$ from both the intracellular sarcoplasmic stores in the canine colonic circular muscle cells seems to be similar to that reported in several other cell types, including vascular smooth muscle, neurons, chromaffin cells, sea urchin eggs, atrial cells, epithelial cells, hepatocytes, and pancreatic acinar cells (3, 29). However, some cells seem to possess only one of the stores, e.g., the skeletal muscle cells have only the ryanodine-sensitive stores and Xenopus oocytes only the IP$_3$-sensitive stores (29). The smooth muscle cells from the longitudinal and circular muscle layers of the guinea pig small intestine also show specialization in the utilization of intracellular stores (11, 18, 25, 27); the circular muscle cells mobilize primarily from the IP$_3$-sensitive stores, whereas the longitudinal muscle cells utilize only the ryanodine-sensitive stores.

In contrast to the impairment in Ca$^{2+}$ influx, Ca$^{2+}$ release from the ryanodine- and IP$_3$-sensitive stores was not affected during inflammation. The basal level of IP$_3$ was reduced in the inflamed cells, but its peak accumulation in response to ACh was not different between the normal and the inflamed cells. Accordingly, Ca$^{2+}$ efflux and decrease in cell length in response to IP$_3$ did not differ between the two states of the cells. Contractile response and $^{45}$Ca$^{2+}$ efflux to ryanodine as well as the binding of [H]$^3$ryanodine to its receptors also did not differ between the normal and the inflamed cells.

The increase in [Ca$^{2+}$]i in response to ACh in normal canine colonic circular muscle cells (80–110 nM) is similar to that reported for other smooth muscle cells in this species, e.g., jejunal (8, 9) and colonic (39) cells. However, this increase is smaller than that reported in the guinea pig small intestinal and tracheal cells (11, 43) but not the gastric smooth muscle cells (42). The decrease in the elevation of [Ca$^{2+}$]i in the inflamed state is likely to be entirely due to the impairment in Ca$^{2+}$ influx. Ca$^{2+}$ efflux from the intracellular stores was not affected by the inflammatory response.

The experiments in single cells demonstrate the availability of specific signal transduction pathways and second messengers in response to the activation of specific receptors. However, the precise contribution or utilization of these pathways to stimulate different types of contractions in the intact conscious state can only be speculated at this time. The colonic circular muscle of several species, including dog, human, and rat, generates three distinct types of contractions: rhythmic phasic contractions, giant migrating contractions (GMCs), and tone (20, 37, 38). The amplitudes, durations, and motility functions of these contractions differ widely. The GMCs are 2–3 times larger in amplitude and 3–4 times longer in duration than the short-duration phasic contractions that occur ~4–6 times per min (17, 36–38). Because Ca$^{2+}$ is a critical second messenger in contracting these cells and the amplitude and duration of cell contraction are nonlinearly correlated with [Ca$^{2+}$], it is likely that the three types of contractions would utilize the two Ca$^{2+}$ sources differently. The availability of both ryanodine-sensitive and IP$_3$-sensitive stores in these cells may help in generating Ca$^{2+}$ oscillations and waves of different characteristics to maintain different amplitudes and duration of contractions. The frequency, amplitude, and duration of Ca$^{2+}$ oscillations provide a dynamic coding system that is specific to cellular function (10, 31). The impairment of Ca$^{2+}$ influx but not intracellular Ca$^{2+}$ release may be one of the means by which these cells exhibit stimulation of GMCs at the same time that the phasic contractions and generation of tone are suppressed during inflammation (22, 40, 45).
In conclusion, the canine colonic circular muscle cells can mobilize Ca\(^{2+}\) from the extracellular medium as well as the IP\(_3\) - and ryanodine-sensitive stores. The contractile response of single dispersed cells from the inflamed colon is suppressed during inflammation. This suppression may in part be due to the decrease in Ca\(^{2+}\) influx through the L-type channels. Ca\(^{2+}\) release from IP\(_3\) - and ryanodine-sensitive intracellular stores may not be affected by the inflammatory response. Selective modulation of Ca\(^{2+}\) utilization may be important in suppressing phasic contractions and tone while concurrently stimulating GMCs in the inflamed colon.

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