Neuronal locus and cellular signaling for stimulation of ileal giant migrating and phasic contractions

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Sarna, Sushil K. Neuronal locus and cellular signaling for stimulation of ileal giant migrating and phasic contractions. Am J Physiol Gastrointest Liver Physiol 284: G789–G797, 2003. First published December 27, 2002; 10.1152/ajpgi.00451.2001.—We investigated the neuronal locus, the role of PKC activation, and utilization of extracellular Ca$^{2+}$ and intracellular Ca$^{2+}$ release in smooth muscle cells for the generation of giant migrating contractions (GMCs) and rhythmic phasic contractions (RPCs) in intact normal and inflamed canine ileum. Calcitonin gene-related peptide (CGRP), administered close intra-arterially, stimulated GMCs at higher doses and RPCs at smaller doses. These effects were blocked by prior close intra-arterial infusions of CGRPα-37, atropine, hexamethonium, and TTX but not by tachykinin, serotonin, and histaminergic receptor subtype antagonists. Both types of contractions were blocked by verapamil in normal and inflamed ileums. Dantrolene and ruthenium red blocked only the RPCs in normal ileum but blocked both GMCs and RPCs in the inflamed ileum. PKC inhibition by chelerythrine blocked GMCs only in inflamed ileum but blocked RPCs in both normal and inflamed ileums. The inhibition of phospholipase C by neomycin blocked both RPCs and GMCs in normal and inflamed ileums. In conclusion, acetylcholine is the common neurotransmitter for the stimulation of both GMCs and RPCs, but the signaling cascades for their stimulation are partially divergent, and they differ also in the normal and inflamed states.

calcitonin gene-related peptide; peristaltic reflux; protein kinase C; calcium influx; L-type calcium channels

SMALL INTESTINAL CIRCULAR smooth muscle cells generate three distinct types of contractions (6, 32, 33): rhythmic phasic contractions (RPCs), ultrapropulsive contractions, and tone. Each type of contraction has a distinct motor function. Phasic contractions cause mixing and slow, orderly distal propulsion of luminal contents in the postprandial and fasting states. The maximum frequency and timing of occurrence of these contractions are regulated by slow waves (34, 46). The duration of RPCs in the canine small intestine varies from ~3 to 5 s, and their amplitude ranges up to ~75 g. The ultrapropulsive contractions are of two types: giant migrating contractions (GMCs) and retrograde giant contractions (RGCs). The occurrence of these contractions is not regulated by slow waves (33). Their duration in dogs is ~18–20 s, whereas the duration of slow wave cycle is only ~3–5 s. The amplitude of ultrapropulsive contractions is 2–4 times larger than the maximum amplitude of phasic contractions. The GMCs and RGCs usually propagate rapidly and uninterruptedly from the point of their origin to the terminal ileum or the gastric antrum, respectively. In doing so, they both produce mass movements; GMCs in the caudal direction and RGCs in the oral direction (22, 37). By contrast, the phasic contractions propagate short distances of a few centimeters at a time (10). The precise role of increase in muscle tone (6) in small intestinal or colonic motor function is not known, but it is thought to enhance the efficacy of RPCs by narrowing the luminal diameter. The generation of tone also is not regulated by slow waves; the increase in tone may last from a few minutes to several hours (6, 44).

It is remarkable that the same circular smooth muscle cells can generate three distinct types of contractions with markedly different physical characteristics and functions. The cellular mechanisms of this versatility in smooth muscle contractions are not known. It may be achieved by utilizing different signaling cascades in the enteric neurons and/or smooth muscle cells and by the release of different neurotransmitters at the neuromuscular junction.

Inflammation affects the above three types of intestinal contractions differently; it suppresses the phasic contractions and tone but increases the spontaneous frequency of GMCs (19). Molecular mechanisms for this selective modulation of the three types of contractions by inflammation are not understood, but it is likely that inflammation remodels the cellular signaling pathways in such a manner that favors the stimulation of GMCs and disfavors the generation of RPCs and tone.

An increase in cytosolic free Ca$^{2+}$, derived from the extracellular medium and endoplasmic reticulum, and PKC activation are two key signaling steps that regulate cell contraction and neurotransmitter release from enteric neurons (1, 3, 4, 18, 27, 29, 40, 42, 43, 47). Our first hypothesis is that these two signaling molecules may be utilized differentially in the stimulation of phasic contractions and GMCs in the normal ileum.

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Additionally, inflammation may alter the relative roles of Ca\(^{2+}\) influx, intracellular Ca\(^{2+}\) release, and PKC activation leading to the suppression of RPCs but enhancement of GMCs. Our second hypothesis is that both RPCs and GMCs are stimulated by the release of the same neurotransmitter (ACh) at the neuroeffector junction.

In dogs and humans, the GMCs have been reported to occur only in the intact conscious state. For this reason, these studies were performed in intact conscious dogs by close intra-arterial infusions of test substances. The doses of test substances in close intra-arterial infusions are so small that they affect the neurons and smooth muscle cells in the infused segment only. By the time these small doses get into general circulation, they are too dilute to have a systemic effect. The close intra-arterial method in conscious animals resembles muscle bath studies in its control over in vivo conditions. The doses of test substances in close intra-arterial infusions are so small that they affect the neurons and smooth muscle cells in the infused segment only.

**Experimental Methods**

**Surgical Procedure**

The following experiments were performed on seven healthy conscious dogs of either sex (mean weight, 22 ± 0.4 kg). This study was approved by the Animal Studies Subcommittee at the Zablocki Veterans Affairs Medical Center.

Access to the abdominal cavity was obtained under general pentobarbital sodium anesthesia (30 mg/kg; Abbott Laboratories). An intraluminal medical grade Silastic catheter (2.6 mm internal diameter, 4.9 mm external diameter) was implanted 180–200 cm proximal to the ileocolonic junction to infuse ethanol and acetic acid for the induction of inflammation, as described below. A stainless steel cannula was implanted 20 cm proximal to the ileocolonic junction to drain excess ethanol and acetic acid and prevent them from reaching the colon.

Two mesenteric arteries were identified between the intraluminal catheter and the fistula. The arteries were freed carefully from the mesentery, preserving the nerves. A Silastic catheter (0.75 mm internal diameter, 1.63 mm external diameter) was inserted in the centripetal direction in a branch artery so that its tip rested 1–2 mm from the junction of the branch artery and the main artery (14, 19, 48). Infusing saline at 15–20 ml/min for 10–15 s identified the boundaries of the perfused segment. The segment refilled with blood within 2–3 s after the end of infusion. Infusion of saline at 1 ml/min for up to 10 min produced no apparent change in color of the segment and did not stimulate any contractions. The length of the infused segment was limited to ~6 cm by ligating the secondary branch arteries, if necessary. Ties to the branch artery and the mesentery secured the catheters.

Three strain-gauge transducers were attached to the submucosal layer in each infused segment to record circular muscle contractions. An additional transducer was implanted 10 cm distal to each infused area to monitor the propagation of GMCs starting in that segment.

Intraluminal and intra-arterial catheters were exteriorized subcutaneously in the subscapular region. The catheters were housed in jackets that the dogs wore at all times. Each intra-arterial catheter was flushed twice daily with 2,000 IU of heparin. The dogs were allowed 5–7 days to recover from surgery.

**Experimental Protocol**

All experiments were performed in the conscious state after an overnight fast. At least one phase III activity was recorded to establish the fasting state. The contractile signals were recorded on a 12-channel recorder (model 7D; Grass Instruments, Quincy, MA), with lower and upper cutoff frequencies set at direct current and 15 Hz, respectively.

All test substances were infused at 1 ml/min during phase I or a quiescent period during phase II activity of the migrating motor complex cycle. The agonists were infused for 1 min and the antagonists for 1-, 5-, or 10-min periods. When an antagonist was infused for 5 or 10 min, the agonist was coinfused during the 3rd or the 5th min of infusion of the antagonist, respectively. When an antagonist was infused for 1 min, it was infused 2–5 min before the infusion of the agonist. A waiting period of at least 30 min was allowed between successive infusions of agonists and antagonists.

Preliminary experiments indicated that the responses to repeated infusions of agonists after this rest period were not different. All experiments were performed first in the normal state and then during inflammation.

The minimum dose of an agonist to stimulate a response, or the minimum dose of an antagonist to inhibit a response, was determined by starting with a higher effective dose and reducing it in steps until the stimulation or inhibition was absent. For antagonists, this minimum dose and one next lower dose were tested again to confirm the absence of the effect at the minimum dose. For agonists, the minimum dose and one next lower dose were tested again to confirm the absence of the response at the lower dose.

**Induction of Inflammation**

Ileal inflammation was induced by mucosal exposure to ethanol and acetic acid as described previously (19, 39). Briefly, 75 ml of 95% ethanol were infused intraluminally on day 1 after an overnight fast. The same amount of ethanol was infused on days 2 and 5, followed 1 h later by infusions of 50 ml of 20% acetic acid. Mucosal exposure to ethanol and acetic acid induced an acute inflammatory response that lasted for ~10 days (19). Myeloperoxidase activity and neutrophil infiltration has been reported to increase in both the mucosa and the muscle layers during this period (19). The experiments in the inflamed state were carried out on days 3–4 and days 6–9 after the first exposure to ethanol. Different antagonists were infused in a random order.

**Tissue Extraction and Radioimmunoaassay of Calcitonin Gene-Related Peptide**

Mucosa was removed from a 3- to 4-cm length of the normal and inflamed ileum. For inflamed ileum, the tissues were taken on the 6th day of inflammation when GMC frequency was increased and phasic contractions were suppressed (19). Of the remaining tissue ~1 g was boiled in 5 volumes of 2N acetic acid for 20 min. The tissue was homogenized on ice and centrifuged at 4°C and 3,800 rpm for 30 min. Supernatant was used for radioimmunoaassay of calcitonin gene-related peptide (CGRP) according to manufacturer's instructions (Peninsula Laboratories, Belmont, CA). A Bio-Rad assay kit determined soluble protein in the supernatant.
Materials

CGRP and CGRP antagonist CGRP8–37 were purchased from Peninsula Laboratories (Belmont, CA); neomycin, verapamil, ruthenium red, N^ω-nitro-l-arginine methyl ester (l-NAME), TTX, and hexamethonium bromide were from Sigma (St. Louis, MO); atropine sulfate was from Lymphomed (Deerfield, IL); dantrolene sodium was from Proctor and Gamble (Cincinnati, OH); and chelerythrine was from RBI (Natick, MA). All substances were dissolved in distilled water except atropine sulfate, which was dissolved in 0.9% saline.

Data Analysis

GMCs were identified visually. The phasic contractile response was quantified as area under contractions (WINDAQ/EX program; DATAQ Instruments, Akron, OH). The area under contractions was measured from the beginning of the first contraction after the start of infusion to the point at which the tracing returned to baseline and contractions ceased to occur. All data are expressed as means ± SE. The n value represents the number of dogs. Statistical analysis was performed by analysis of variance with repeated measures. Student-Newman-Keuls test was used for multiple comparisons when the data were distributed normally, whereas Mann-Whitney’s rank sum test was used when the normality test failed; P < 0.05 was considered to be statistically significant.

RESULTS

We found that close intra-arterial infusions of CGRP, a neurotransmitter of the primary afferent sensory neurons, consistently stimulated GMCs and RPCs at higher doses but stimulated RPCs only at smaller doses in normal and inflamed ileum. The GMCs stimulated by CGRP propagated distally, but the RPCs were localized (Fig. 1A). The minimum dose of CGRP required to stimulate GMCs in the normal ileum, 0.35 ± 0.02 μM/min × 1 min, was significantly greater than that in the inflamed ileum, 0.18 ± 0.01 μM/min × 1 min (n = 7 each, P < 0.05). In all further experiments, 1 μM/min × 1 min CGRP was used to stimulate GMCs and 0.1 μM/min × 1 min to stimulate only the RPCs.

Stimulation of GMCs and accompanying RPCs by 1 μM/min × 1 min dose of CGRP was blocked completely by 3.4 ± 1.5 μM/min × 5 min infusion of CGRP antagonist CGRP8–37 (n = 7) (Fig. 1B). The same dose of CGRP8–37 also blocked the RPCs stimulated by 0.1 μM/min × 1 min infusion of CGRP. CGRP was infused for 1 min during the 3rd min of infusion of its antagonist.

Locus of Action of CGRP to Stimulate GMCs and RPCs

Stimulation of GMCs by CGRP was blocked completely by the blockade of muscarinic receptors with 30 μM/min × 1 min infusion of atropine, nicotinic receptors with 70 mM/min × 1 min hexamethonium, or inhibition of sodium-channel enteric neural conduction with 70 μM/min × 1 min infusion of TTX (Fig. 2). All of the above antagonists were infused ~2–3 min before the infusion of CGRP. Previous studies (14, 19, 39, 48) have shown that these antagonists block their respective targets for 20–30 min.

Inhibition of nitric oxide (NO) synthase by 10 mM/min × 1 min close intra-arterial infusion of l-NAME significantly reduced the minimum dose of CGRP required to stimulate a GMC (0.35 ± 0.03 μM/min × 1 min control vs. 0.16 ± 0.05 μM/min × 1 min after l-NAME; n = 4, P < 0.05). The same effect was seen in the inflamed ileum where the corresponding doses of CGRP were 0.18 ± 0.01 μM/min × 1 min and 0.05 ± 0.00 μM/min × 1 min (n = 4, P < 0.05), respectively.

RPCs in response to the smaller dose of CGRP were also blocked by atropine, hexamethonium, and TTX in normal and inflamed ileum at the same concentrations that blocked the GMCs (n = 4). By contrast, the RPCs accompanying the stimulation of GMCs at higher doses of CGRP were not blocked by hexamethonium or TTX. The neuromuscular mechanisms of stimulation of RPCs accompanying GMCs are likely to be different from those that cause the RPCs in response to smaller doses of CGRP.

Receptor Subtypes Involved in the Stimulation of GMCs and RPCs

Stimulation of GMCs by CGRP was inhibited by the block of M1 receptors with 2 μM/min × 5 min infusion.
of pirenzepine and of M3 receptors with the infusion of 2 μM/min × 5 min 4-diphenylacetoxy-N-methyl-piperidine methiodide but not by the block of M2 or M4 receptors with 2 μM/min × 5 min infusions of methoctramine or tropicamide, respectively. The block of none of the following receptors inhibited the CGRP-induced stimulation of GMCs: neurokinin (NK)1 receptors with 1.6 μM/min × 5 min infusion of L-703606; NK2 receptors with 1.6 μM/min × 5 min infusion with L-659877; NK3 receptors with 1.6 μM/min × 5 min infusion of [Trp7,β-Ala8]NKA4–10; serotonin (5-HT)1A receptors with 1.2 μM/min × 1 min infusion of NAN-190Br; 5-HT2/5-HT1C receptors with 2 μM/min × 5 min infusion of LY-53857; 5-HT3 receptors with 2 μM/min × 5 min infusion of ondansetron; 5-HT4 receptors with 2 μM/min × 5 min infusion of SDZ-20555 HCl; histamine H1 receptors with 15 μM/min × 5 min infusion of mepyramine; and H2 receptors with 150 μM/min × 5 min infusion of ranitidine. These doses of antagonists have been established previously to effectively block their respective receptor subtypes (14, 19, 39.48, 49).

Roles of Ca2+ Mobilization and PKC Activation in Stimulation of GMCs and RPCs in Normal and Inflamed Ileum

Ca2+ influx through L-type calcium channels and intracellular Ca2+ release. The inhibition of L-type Ca2+ channels by verapamil blocked the stimulation of GMCs by CGRP in normal and inflamed ileum (Fig. 3). There was no difference between the minimum doses of verapamil required to block the GMCs in the two states (817 ± 101 μM/min × 5 min and 867 ± 67 μM/min × 5 min, respectively; n = 5, P > 0.05). The same doses of verapamil also completely blocked the RPCs stimulated by the smaller doses of CGRP.

Inhibition of intracellular Ca2+ release by 400 μM/min × 5 min dantrolene had no effect on the stimulation of GMCs in the normal ileum (Fig. 4; n = 5). By contrast, a smaller dose of 283 ± 72 μM/min × 5 min dantrolene blocked CGRP-stimulated GMCs in the inflamed ileum of all dogs (n = 5), indicating that release of Ca2+ from the intracellular stores is important for the stimulation of GMCs in the inflamed but

Fig. 2. Prior close intra-arterial infusions of atropine, hexamethonium, and TTX in the infused segment blocked the stimulation of GMCs by CGRP. Stimulation of GMCs by high doses of CGRP is accompanied by phasic contractions. These phasic contractions were blocked by atropine but not hexamethonium and TTX. By contrast, the phasic contractions stimulated by low doses of CGRP were blocked by hexamethonium and TTX (see Locus of Action of CGRP to Stimulate GMCs and RPCs).

Fig. 3. Close intra-arterial infusion of verapamil blocked the stimulation of GMCs by CGRP in both normal (A) and inflamed (B) ileum.
not in the normal ileum. The 400 μM/min × 5 min infusion of dantrolene partially but significantly blocked the phasic contractions in the normal ileum (47.0 ± 14.0% residual response). In the inflamed ileum, the infusion of 283 ± 72 μM/min × 5 min almost completely blocked phasic contractions (6.2 ± 4.8% residual response). Thus the stimulation of phasic contractions is more dependent on intracellular Ca^{2+} release in the inflamed ileum than in the normal ileum.

The effect of ruthenium red, an inhibitor of ryanodine-sensitive intracellular Ca^{2+} release channels, was similar to that of dantrolene in normal and inflamed ileum (Fig. 4). Ruthenium red (2.0 ± 0 μM/min × 5 min) had no effect on the stimulation of GMCs in the normal ileum, whereas a smaller dose of 1.3 ± 0.3 μM/min × 5 min blocked them in the inflamed ileum. Ruthenium red also blocked the RPCs, partially but significantly, in the normal ileum (68 ± 12% residual response) as well as in the inflamed ileum (50 ± 14% residual response).

**PKC and phosphatidylinositol-phospholipase C.** Inhibition of PKC activation by 400 μM/min × 5 min infusion of chelerythrine had no effect on the stimulation of GMCs in the normal ileum (Fig. 5; n = 6). However, in the inflamed ileum, a smaller dose of chelerythrine (167 ± 116 μM/min × 5 min) completely blocked the CGRP-stimulated GMCs in all dogs (n = 6). By contrast, chelerythrine at the same doses almost

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**Fig. 4.** Close intra-arterial infusions of dantrolene and ruthenium red did not block the CGRP-induced GMCs in the normal ileum (A, 2nd and 3rd traces), but both blocked them in the inflamed ileum (B) at smaller doses of infusion.

**Fig. 5.** Close intra-arterial infusion of chelerythrine, which inhibits the activation of PKC, had no effect on the stimulation of GMCs by CGRP in normal ileum (A), but at a smaller dose, it blocked this effect of CGRP in the inflamed ileum (B).
completely blocked the RPCs in response to CGRP in both normal and inflamed ileum (4.4 ± 4.4 and 9.8 ± 4.5% residual responses).

Inhibition of phosphatidylinositol-phospholipase C (PI-PLC) by 11.6 ± 4.2 mM/min × 10 min and 8.0 ± 2.0 mM/min × 10 min infusions of neomycin completely blocked the stimulation of GMCs in normal and inflamed ileum, respectively (Fig. 6; n = 5, P > 0.05). The same concentrations of neomycin also blocked almost completely the stimulation of RPCs in both normal and inflamed ileum (2.8 ± 1.7 and 11.0 ± 4.5% residual responses, respectively). The roles of Ca²⁺ and PKC activation in the stimulation of GMCs and RPCs are summarized in Table 1.

Immunoreactive CGRP in normal and inflamed ileum. Immunoreactive CGRP content of the inflamed ileum devoid of mucosa (0.55 ± 0.04 ng/g protein) was significantly less than that of the normal ileum (1.1 ± 0.27 ng/g protein; n = 5, P < 0.05).

DISCUSSION

Locus of Action of CGRP to Stimulate GMCs and RPCs

Our findings show that close intra-arterial administration of CGRP, a neurotransmitter of the primary afferent sensory neurons in the gut, consistently and reliably stimulates GMCs in the normal and inflamed ileum of intact conscious dogs. Among several agonists (erythromycin, serotonin, substance P, NKA, histamine, motilin, and ACh) that have been tried (14, 36, 48), only CGRP and NKB (48) have thus far been found to stimulate ileal GMCs. All other substances stimulate primarily the phasic contractions. In this study, we used CGRP as the agonist to investigate its locus of action and the cellular mechanisms for the stimulation of GMCs and RPCs.

Role of Enteric Neurons in the Stimulation of GMCs and RPCs

Stimulation of GMCs by CGRP was inhibited by blockade of nicotine receptors with hexamethonium or of sodium channel enteric neural conduction with TTX, indicating that CGRP acted at a presynaptic neural site. The neurotransmitter at the neuroeffector junction was ACh, because the stimulation of GMCs was blocked also by atropine. The RPCs stimulated by smaller doses of CGRP were blocked similarly by atropine, hexamethonium, and TTX, suggesting the same neurotransmitter and locus of action for the stimulation of both types of contractions.

The minimum dose of CGRP required to stimulate GMCs in normal and inflamed ileum decreased significantly when NO synthase was blocked by L-NAME. The presynaptic neurons on which CGRP acts may, therefore, synapse on both the cholinergic excitatory and nitric inhibitory motor neurons. On stimulation by CGRP, the excitatory and inhibitory inputs to smooth muscle cells (ACh and NO, respectively) compete, but the excitatory input dominates to stimulate GMCs. The excitatory effect of ACh is unopposed when NO synthesis is blocked by L-NAME, resulting in a smaller dose of ACh effectively stimulating GMCs.

Sarna et al. (36) found that putative prokinetics agents, such as erythromycin, motilin, and ABT-229, also act on presynaptic enteric neurons in the small ileum.

Table 1 Roles of Ca²⁺ and PKC in the stimulation of GMCs and RPCs in normal and inflamed ileum

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GMC, giant migrating contractions; RPC, rhythmic phasic contraction. *Indicates difference between RPCs and GMCs; **indicates difference between normal and inflamed ileum.

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Fig. 6. The inhibition of phosphatidylinositol-phospholipase C by neomycin blocked the stimulation of CGRP-induced GMCs in both normal (A) and inflamed (B) ileum.
intestine, but they stimulate only RPCs at the highest doses used. These agonists utilize a cascade of presynaptic neurons and a subset of serotoninergic, tachykininergic, and histaminergic receptors, as well as M3 and nicotinic receptors, to stimulate RPCs. By contrast, CGRP utilized only the M1, M3, and nicotinic receptors to stimulate GMCs. It seems, therefore, that the presynaptic locus of action of CGRP is different from that of other agonists that stimulate primarily the RPCs. Both nicotinic and M1 receptors are thought to be located on the cell bodies of the motor neurons (13). The absence of a role of other receptor subtypes in the action of CGRP suggests that the presynaptic neurons containing CGRP receptors may synapse directly on the motor neurons or that they utilize only nicotinic synapse in the neuronal cascade.

AH-type neurons are the intrinsic primary afferent neurons in the gut (9, 11, 12, 17, 23, 50). They synapse on S-type motor neurons (15, 16, 51), which ultimately release excitatory (ACh) and inhibitory (NO) neurotransmitters at the neuromuscular junction. CGRP has been reported to enhance the excitability of AH neurons, which would increase the number of neural action potentials during membrane depolarization, resulting in increase of neurotransmitter release. Slow excitatory postsynaptic potentials (EPSPs) can last from 1 to 2 s to >20 s, which covers the range of duration of phasic contractions (3–5 s) and GMCs (18–20 s). In our study, smaller doses of CGRP stimulated smaller-amplitude and shorter-duration RPCs. On the other hand, larger doses of CGRP stimulated large-amplitude and long-duration GMCs. Several other excitatory neurotransmitters, such as serotonin, also induce EPSPs (8). However, Cornelissen et al. (8) found that microinjections of serotonin cause a fast nicotine-like depolarization that lasts only 3 ± 1.3 s, which is typically the duration of a phasic contraction. On the other hand, the microinjection of CGRP produces a slow EPSP that lasts for 56 ± 27.5 s, which resembles the duration of GMCs. Taken together, these findings suggest that enhanced and sustained release of ACh by long-duration EPSPs may be one of the factors in the stimulation of GMCs.

Relative Roles of Ca2+ Influx, Intracellular Ca2+ Release, PKC Activation, and Hydrolysis of PI in Stimulation of GMCs and RPCs

It appears from the above that GMCs are stimulated by large, prolonged release of ACh from excitatory motor neurons and RPCs by its small, short-duration release. Previous studies (2, 52, 53) have reported that small and large doses of neurotransmitters, such as ACh or cholecystokinin, utilize different signaling pathways in cell contraction. The physiological significance of differential cell signaling by large and small doses of the same agonist is not known. Our findings suggest that such differential signaling may lead to the generation of different types of contractions, which have markedly different motility effects. We investigated, therefore, whether the utilization of Ca2+ influx, intracellular Ca2+ release, and PKC activation differs for the stimulation of GMCs and RPCs by the large and small doses of CGRP, respectively.

It is clear from the above that the stimulation of both types of contractions requires presynaptic neurons, postsynaptic motor neurons, and smooth muscle cells working together. Therefore, the following discussion on the role of Ca2+ influx, intracellular Ca2+ release, PKC activation, and hydrolysis of PI by PLC refers to the enteric neurons and smooth muscle cells as one unit. This unit cannot be dissected any further in vivo, because the blockade of any single element of it, e.g., neurons with TTX or smooth muscle cells with atropine or verapamil, would totally inhibit the GMCs and RPCs, which are the end points of measurement.

Our findings show that Ca2+ influx through L-type channels is essential for the stimulation of both RPCs and GMCs in normal and inflamed ileum. Because L-type Ca2+ channels are present primarily on smooth muscle cells (21), both GMCs and phasic contractions require this source of Ca2+ for circular smooth muscle cells to contract.

Intracellular Ca2+ release seems to play a differential role in the stimulation of GMCs and RPCs in the normal ileum. The inhibition of intracellular Ca2+ release by dantrolene or ruthenium red had no effect on the stimulation of GMCs but significantly inhibited RPCs. A previous study (35) has shown that ryanodine does not contract the smooth muscle cells directly, but it releases ACh from the enteric neurons to contract them. Taken together, these data suggest that the release of Ca2+ from intracellular stores is utilized by the enteric neurons to release ACh, which then stimulates RPCs. However, this source of Ca2+ in neurons may not be essential for the stimulation of GMCs in the normal ileum.

PI hydrolysis by PLC produces diacylglycerol (DAG) and inositol-1,4,5-trisphosphate [Ins(1,4,5)P3]. DAG, in turn, activates PKC, whereas Ins(1,4,5)P3 releases Ca2+ from the Ins(1,4,5)P3-sensitive stores. The stimulation of GMCs was not affected by blocking the activation of PKC by chelerythrine in the normal ileum. However, inhibiting the hydrolysis of PI-PLC by neomycin blocked the GMCs. It seems, therefore, that Ins(1,4,5)P3-induced Ca2+ release, but not PKC activation, is required for the stimulation of GMCs in the normal ileum. By contrast, the stimulation of RPCs requires PKC activation, because they were blocked by chelerythrine.

Enteric neural as well as smooth muscle function is altered in small intestinal inflammation (7, 24, 49), but the molecular mechanisms of these alterations are not fully understood. Our findings show that, whereas the intracellular release of Ca2+ from the ryanodine-sensitive stores is not essential for the stimulation of GMCs in the normal ileum, this source of Ca2+ becomes essential in the inflamed ileum. Both dantrolene and ruthenium red blocked the GMCs stimulated by CGRP in the inflamed ileum at doses smaller than those that had no effect on them in the normal ileum. Shi and Sarna (40) found that, whereas Ca2+ influx through
L-type Ca\textsuperscript{2+} channels is impaired in inflammation, the intracellular Ca\textsuperscript{2+} release from the ryanodine or Ins(1,4,5)P\textsubscript{3}-sensitive stores is not affected, providing support for the use of this source of Ca\textsuperscript{2+} for the stimulation of GMCs in the inflamed state.

The role of PKC activation in the stimulation of GMCs also seems to be different between normal and inflamed ileum. PKC inhibition by chelerythrine did not block the stimulation of GMCs by CGRP in the normal ileum but did so in the inflamed ileum. By contrast, chelerythrine blocked the phasic contractions in both normal and inflamed ileum. Ali and Sarna (1) found that PKC activation in response to ACh in dissociated single circular muscle cells is impaired in inflammation (1). Therefore, PKC activation that occurs for the stimulation of GMCs in the inflamed ileum is likely to be that in the neurons to regulate the release of ACh. A switch in the utilization of PKC has been reported also in gallbladder smooth muscle cells whose contraction is not blocked by chelerythrine in cells from the normal gallbladder but is blocked in cells from the gallbladder with cholesterol stones (5).

**Modulation of Tissue Levels of CGRP by Inflammation**

CGRP is released from the primary afferent neuronal endings in response to noxious stimuli, such as inflammation (25, 31, 51). Our findings suggest that CGRP thus released is a candidate for the stimulation of ileal GMCs whose frequency is increased in inflammation (19). The increased frequency of GMCs in inflammation is, however, due to a remodeling of signaling pathways by inflammatory mediators and possibly due to the upregulation of CGRP receptors (26) rather than due to an increase in the release of CGRP. Our data show that the tissue content of CGRP is actually reduced even as the affinity and/or the potency of CGRP to stimulate contractions is increased in the inflamed ileum. It is noteworthy that, whereas both CGRP and substance P are colocalized in the primary afferent sensory neurons (9, 41, 45) and coreleased by noxious stimuli, only CGRP, but not substance P, stimulates GMCs in the ileum (49). CGRP is a well-established vasodilator and secretagogue, but our findings show that it may also play a major role in stimulating ileal GMCs associated with diarrhea and abdominal cramping (19, 20).

In conclusion, CGRP, a neurotransmitter of the primary afferent sensory neurons, may be a potent agonist for the stimulation of small intestinal GMCs. CGRP acts at a presynaptic site in the ileum to release ACh, which stimulates GMCs, utilizing M\textsubscript{1}, M\textsubscript{3}, and nicotinic receptors but not the serotoninergic, tachykininergic, or histaminergic receptor subtypes. Concurrent release of NO by CGRP causes a partial inhibitory effect on the stimulation of GMCs. ACh is the excitatory neurotransmitter for the stimulation of both GMCs and phasic contractions. However, the utilization of intracellular Ca\textsuperscript{2+} release and PKC activation in the enteric neurons and circular smooth muscle cells differs for the stimulation of the two types of contractions. Also, inflammation modulates the mobilization of calcium and activation of PKC, which may account for an increase in the frequency of GMCs and concurrent suppression of phasic contractions in the inflamed ileum. Enteric neural and smooth muscle signalopathies may partially account for abnormal motility patterns in ileal inflammation.

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**REFERENCES**


