Regulation of slow wave frequency by IP$_3$-sensitive calcium release in the murine small intestine

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Malysz, John, Graeme Donnelly, and Jan D. Huizinga. Regulation of slow wave frequency by IP$_3$-sensitive calcium release in the murine small intestine. Am J Physiol Gastrointest Liver Physiol 280: G439–G448, 2001.—Slow waves determine frequency and propagation characteristics of contractions in the small intestine, yet little is known about mechanisms of slow wave regulation. We propose a role for contractions in the small intestine, yet little is known about mechanisms of slow wave regulation. We propose a role for intracellular Ca$^{2+}$, inositol 1,4,5-trisphosphate (IP$_3$)-sensitive Ca$^{2+}$ release, and sarcoplasmic reticulum (SR) Ca$^{2+}$ content in the regulation of slow wave frequency because 1) 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM), an intracellular Ca$^{2+}$ chelator, reduced the frequency or abolished the slow waves; 2) thapsigargin and cyclopiazonic acid (CPA), inhibitors of SR Ca$^{2+}$-ATPase, decreased slow wave frequency; 3) xestospongin C, a reversible, membrane-permeable blocker of IP$_3$-induced Ca$^{2+}$ release, abolished slow wave activity; 4) caffeine and phospholipase C inhibitors (U-73122, neomycin, and 2-nitro-4-carboxyphenyl-N,N-diarylaminium) inhibited slow wave frequency; 5) in the presence of CPA or thapsigargin, stimulation of IP$_3$ synthesis with carbachol, norepinephrine, or phenylephrine acting on $\alpha_1$-adrenoceptors initially increased slow wave frequency but thereafter increased the rate of frequency decline, 6) thimerosal, a sensitizing agent of IP$_3$ receptors increased slow wave frequency, and 7) ryanodine, a selective modulator of Ca$^{2+}$-induced Ca$^{2+}$ release, had no effect on slow wave frequency. In summary, these data are consistent with a role of IP$_3$-sensitive Ca$^{2+}$ release and the rate of SR Ca$^{2+}$ refilling in regulation of intestinal slow wave frequency.

pacemaker activity; intestinal motility; cation channel; smooth muscle; interstitial cell of Cajal

PERISTALTIC CONTRACTIONS ARE generated through interplay between smooth muscle cells, the enteric nervous system, and interstitial cells of Cajal (ICC). ICC are critical in the generation of slow wave activity, which in turn sets the maximum frequency of contractions and determines propagation characteristics of the rhythmic contractions in the intestine (7, 14, 33, 38). The ionic and regulatory mechanisms responsible for slow wave generation still remain obscure. In the canine colon, slow waves were found to be relatively insensitive to changes in the membrane potential (11), insensitive to L-type Ca$^{2+}$ channel blockers (12), and highly sensitive to the removal of extracellular Ca$^{2+}$ and to cytosolic levels of cAMP (11). Furthermore, the canine colonic slow wave frequency could be reduced with either cyclopiazonic acid (CPA), a selective inhibitor of the sarcoplasmic reticulum (SR) Ca$^{2+}$-ATPase, and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-AM (BAPTA-AM), an intracellular Ca$^{2+}$ chelator (21). Hence, it was suggested that an intracellular metabolic process involving Ca$^{2+}$ release from the SR and sensitive to cAMP triggers the canine colonic slow waves. Much less is known about the regulatory mechanisms for the small intestinal slow waves. The temperature sensitivity of the slow waves, as observed in the small intestines of cats (5) and mice (19), suggests involvement of a metabolic process in the regulation of the slow wave. The sensitivity of the slow waves to removal of extracellular Ca$^{2+}$ and their insensitivity to L-type Ca$^{2+}$ channel blockers in the murine (17, 24, 25) and feline (5) small intestines point to participation of Ca$^{2+}$ conductances other than those mediated by L-type Ca$^{2+}$ channels. The intracellular regulatory mechanisms appear to be different in the colon and small intestine, because activation of the protein kinase A (PKA) pathway decreases slow wave activity in the former (11) and increases it in the latter (current study). It still remains to be established whether intracellular Ca$^{2+}$ also plays a critical role in the regulation of the slow waves in the small intestine. Data in this study provide such evidence in the murine small intestine.

Intracellularly, the major sites for Ca$^{2+}$ release are inositol 1,4,5-trisphosphate (IP$_3$) and ryanodine-sensitive Ca$^{2+}$ stores residing in sections of the SR. Release of Ca$^{2+}$ from these stores is highly regulated and involves Ca$^{2+}$ channels in the SR membrane. Unique IP$_3$-regulated Ca$^{2+}$ channels have been purified from bovine aortic microsomes (3) and from membranes of rat vas deferens smooth muscle (29). In intestinal smooth muscle, existence of the IP$_3$- and ryanodine-sensitive Ca$^{2+}$ stores has been demonstrated (15, 16, 18, 44). The roles of these stores in regulation of intestinal slow waves remain undetermined.
The present study set out to determine whether intracellular Ca\(^{2+}\) and intracellular Ca\(^{2+}\) stores, particularly of the IP\(_3\) type, are involved in regulating slow wave frequency in the murine small intestine. A major obstacle that this study had to overcome was the lack of selective pharmacological agents acting on the IP\(_3\) receptor. Two of the most commonly utilized pharmacological agents known to act on IP\(_3\)-induced Ca\(^{2+}\) release are IP\(_3\), an activator of the receptor, and heparin, a blocker of the receptor or channel. Because of their plasma membrane impermeability, neither agent could be used in this study. Instead, this study relied on a novel, membrane-permeable blocker of IP\(_3\)-induced Ca\(^{2+}\) release, xestospongin C (8), and other agents known to act by different mechanisms on the IP\(_3\) signaling pathway, leading to the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores. The approach was to test a number of agents involved in IP\(_3\) metabolism and Ca\(^{2+}\) release and construct a hypothesis based on the experimental findings.

METHODS

Preparation and recording of electrical activities. A standard microelectrode technique, as previously described in detail elsewhere (13, 24), was used to record slow waves from the murine small intestine. Adult mice used in this study were of either sex and supplied by Charles River Laboratories (CD1, St. Constant, QC, Canada). The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Throughout the experiments, L-type Ca\(^{2+}\) blockers (1–5 \(\mu\)M nifedipine or verapamil) were present. As reported in detail in our (13, 24) previous studies on the murine small intestinal musculature, a major effect of the blockade is to attenuate generation of spike-like action potentials superimposed onto the plateau phases of the slow waves. In contrast, the intrinsic pacemaker activity (41) is insensitive to L-type Ca\(^{2+}\) channel blockade and is reflected in tissue as the nifedipine-insensitive part of the slow wave (24).

Consequently, mechanisms of the regulation and generation of slow waves can be studied in the presence of the L-type Ca\(^{2+}\) blockers (verapamil or nifedipine).

Solutions and drugs. The composition of Krebs solution was (in mM) 120.3 NaCl, 5.9 KCl, 2.5 CaCl\(_2\), 1.2 MgCl\(_2\), 20 NaHCO\(_3\), 1.2 NaH\(_2\)PO\(_4\), and 11.5 glucose. The Krebs solution (7.30–7.35 pH) was continuously gassed with a mixture of 95% O\(_2\)-5% CO\(_2\). Verapamil, nifedipine, CPA, neomycin, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC), caffeine, forskolin, sodium nitroprusside (SNP), norepinephrine, phenylephrine, prazosin, carbachol, thimerosal, and U-73122 were all obtained from Sigma Chemical (St. Louis, MO). BAPTA-AM was obtained from Molecular Probes (Eugene, OR). Xestospongin C was from Calbiochem (La Jolla, CA) and thapsigargin from Alomone Labs (Jerusalem, Israel).

Data analysis. Data were obtained usually from the same cells, before and in the presence of a drug. A difference was considered to be significant at \(P < 0.05\). Data are expressed as means \(\pm SE\), with \(n\) representing the number of different animals used. When the effects of a specific drug or experimental condition are described, only one such experiment was carried out on a single tissue. Any given animal tissue on a single day was used to perform two or more separate pharmacological experiments. In experiments reporting percent increase or decrease, the percent change was obtained first for each individual experiment, and then mean and SE values were calculated based on these numbers. Paired or unpaired Student’s \(t\)-tests were employed whenever appropriate for comparison of the electrical activities determining the effects of drugs and increased extracellular K\(^+\) solutions.

RESULTS

Pharmacological modulation of intracellular Ca\(^{2+}\) homeostasis. The murine proximal small intestine displayed slow wave activity at 45.8 ± 1.9 cycles/min, with a duration of 0.75 ± 0.03 s, an amplitude of 20.3 ± 2.1 mV, and an average rate of rise of the upstroke of 249 ± 57 mV/s from a resting membrane potential of −62.3 ± 1.8 mV (\(n\) = 42). The membrane permeable Ca\(^{2+}\) chelator BAPTA-AM (25–50 \(\mu\)M) led to a decline in frequency and amplitude of the slow waves and in three of five experiments complete abolishment of the activity (\(n\) = 5; Fig. 1A). CPA (1–3 \(\mu\)M), an inhibitor of the SR Ca\(^{2+}\)-ATPase (SERCA) pump (9), decreased the slow wave frequency by 46.1 ± 7.3% (\(n\) = 11, \(P < 0.001\)) from 39.8 ± 2.1 to 20.9 ± 1.5 cycles/min (Fig. 1B) after at least 20 min of perfusion. During the initial 10–20 min of superfusion, CPA transiently depolarized the cells by up to 20 mV. At this time, it also became technically difficult to maintain stable impalements. After this initial period of recording instability, stable impalements could be obtained. In addition to the reduction in frequency, CPA also depolarized the tissues by 6.2 ± 1.7 mV (\(P < 0.006\)) at the ~30- to 40-min perfusion recording mark. At this point, CPA (1–3 \(\mu\)M) significantly affected the slow wave amplitude, rate of upstroke increase, and duration. To establish the effect of depolarization per se, extracellular K\(^+\) was increased from 5.9 to 11.8 mM, causing a depolarization of 6.0 ± 1.5 mV (\(n\) = 7, \(P < 0.05\)). Similar reductions in the slow wave amplitude and the average rate of upstroke increase were observed with CPA and K\(^+\)-induced depolarization. In contrast, reductions in the slow wave frequency and duration with CPA were significantly different from those identified with 11.8 mM K\(^+\). CPA decreased the slow wave frequency by 46.1 ± 7.3% (\(n\) = 11), whereas the decrease with 11.8 mM K\(^+\) was 15.1 ± 3.6% (\(n\) = 7, \(P < 0.02\)). The reduction in the slow wave duration by CPA was 26.0 ± 5.1% (\(n\) = 11), significantly different from that by 11.8 mM K\(^+\), which was 6.2 ± 0.4% (\(n\) = 7, \(P < 0.05\)). Hence, the effects of CPA on the slow wave frequency and duration were for the most part independent from the effects on the membrane potential. Depolarization per se with CPA likely produced the observed reductions in the slow wave amplitude and in the average rate of increase of the upstroke. This conclusion found confirmation in results with thapsigargin, another SERCA inhibitor (40). Thapsigargin (1 \(\mu\)M) decreased the slow wave frequency at a rate of 0.12 cycles/min (\(n\) = 15; regression coefficient (\(R^2\)) = 0.96) (Figs. 1C and 2) until elimination of the slow wave at ~400 min of perfusion. No depolarization was observed, and the membrane potentials at 0, 2, and 4 h of perfusion were not significantly different, being −57.8 ± 2.7, −61.7 ± 3.4, and −62.1 ± 3.4 mV, respec-
Effect of blockade of IP$_3$-induced Ca$^{2+}$ release. Xestospongin C has been shown to be a blocker of IP$_3$-induced Ca$^{2+}$ release (8, 22), which, unlike heparin, is membrane permeable, although it has been suggested that it can also block the SERCA pump (6). On addition of xestospongin C (0.5 μM; n = 5), normal slow wave activity began to “wax and wane” (n = 3), and at a higher concentration of 1 μM, slow wave activity was abolished (n = 3) (Fig. 3).

Effect of α$_1$-adrenoceptor activation. Stimulation of α$_1$-adrenoceptors activates phospholipase C (PLC) to generate IP$_3$ via a G protein-dependent mechanism (36). Norepinephrine (3 and 10 μM) and phenylephrine (1 and 10 μM) increased the slow wave frequency by 5.7 ± 1.8% (n = 19, P < 0.005), 7.0 ± 1.8% (n = 12, P < 0.005), 4.1 ± 1.6% (n = 11, P < 0.04), and 5.1 ± 1.7% (n = 6, P < 0.05), respectively (Fig. 4A). These effects of phenylephrine and norepinephrine (and those described below) were inhibited by pretreatment of tissues with prazosin (5 μM). Prazosin on its own had no effects on the slow wave (n = 8, Fig. 4B).

In the presence of CPA (1–3 μM) with a reduction in frequency of at ~40%, norepinephrine (2 μM) and phenylephrine (3 μM) increased the slow wave frequency by 69.5 ± 28.3% (n = 6, P < 0.002) and 78.8 ± 32.4% (n = 7, P < 0.04), respectively, within the first 20 min of perfusion (Fig. 4, C and D). No other slow wave characteristics were affected. Long-term effects of α$_1$-adrenoceptor agonist perfusion in CPA were not determined due to the developing depolarization by CPA.
In the presence of thapsigargin (1 μM) with the slow wave frequency reduced by at least 20%, phenylephrine (3 μM) either increased or decreased the slow wave frequency in the first few minutes. Thereafter phenylephrine caused an increase in the rate of decline in slow wave frequency to 1.3 cycles/min (n = 5, R²=0.68) compared with thapsigargin alone (Fig. 2). The slow wave activity was completely abolished within ~30 min of perfusion with phenylephrine and thapsigargin.

In addition to an effect on frequency, when examined in the absence of SERCA blockade, phenylephrine and norepinephrine affected the slow wave amplitude, average rate of increase, and duration. To describe the effects of the adrenoceptor agonists on the amplitude, it became necessary to make a distinction between the upstroke amplitude and plateau amplitude. In other experiments described here, the plateau amplitude was affected to the same degree as the upstroke amplitude, and only the upstroke amplitude was reported. Phenylephrine (10 μM) decreased plateau amplitude but increased upstroke amplitude. The plateau amplitude was decreased from 17.0 ± 1.3 to 14.6 ± 1.5 mV (n = 19, P < 0.02) with 3 μM phenylephrine and from 17.3 ± 0.9 to 11.8 ± 0.9 mV (n = 12, P < 0.0001) with 10 μM phenylephrine. In comparison, the upstroke amplitude increased from 19.3 ± 1.0 to 20.5 ± 1.0 mV (n = 19, P < 0.05) with 3 μM phenylephrine and from 20.3 ± 1.3 to 22.1 ± 1.3 mV (n = 12, P < 0.01) with 10 μM phenylephrine. Compared with the effects of phenylephrine, norepinephrine did not increase upstroke amplitude but also decreased plateau amplitude. At 1 μM norepinephrine, plateau amplitude decreased from 17.4 ± 2.2 to 14.6 ± 2.4 mV (n = 11, P < 0.05), and at 10 μM norepinephrine, from 16.6 ± 0.7 to 11.1 ± 0.8 mV (n = 6, P < 0.01). A concentration of 1 μM norepinephrine had no effect on the upstroke amplitude, whereas 10 μM norepinephrine decreased the upstroke amplitude from 21.1 ± 1.5 to 15.2 ± 1.7 mV (n = 5, P < 0.03). Hence, the slow wave upstroke amplitude could be increased by phenylephrine but not by norepinephrine. In addition, phenylephrine decreased the slow wave duration and increased the average rate of increase of the upstroke, and both phenylephrine (3 and 10 μM) and norepinephrine (10 μM) produced significant hyperpolarizations. The respective hyperpolarizations were 2.5 ± 0.7 (n = 19, P < 0.002), 3.4 ± 0.8 (n = 12, P < 0.003), and 8.8 ± 1.0 mV (n = 6, P < 0.0006). It was unlikely that any of the observed effects, especially those of phenylephrine, were due to hyperpolarization because 1 μM cromakalim, a KATP channel opener, produced a hyperpolarization of 8.6 ± 1.4 mV (n = 11, P < 0.0001) without significantly affecting any slow wave characteristics (data not shown).

Effect of carbachol. Carbachol induces IP₃ synthesis via activation of M₃ receptors in intestinal smooth muscle (2). On addition of carbachol in the presence of CPA, a consistent transient increase in slow wave frequency was observed of 99.0 ± 35.0% (n = 7, P < 0.05) (Fig. 5A) followed by a progressive reduction in frequency. In the presence of thapsigargin, an initial increase in slow wave frequency also occurred, although less consistently. Thereafter, a progressive decline in frequency occurred at a rate of 1.04 cycles/min (n = 15, R²=0.91), an approximate 10-fold more rapid decline than in the presence of thapsigargin alone (Figs. 1C and 2).

![Fig. 2. Slow wave frequency over time during SR Ca²⁺-ATPase inhibition. Thapsigargin (1 μM) progressively decreases the slow wave frequency. Perfusion with phenylephrine (3 μM) or carbachol (0.5 μM), in the presence of thapsigargin (1 μM), enhanced the rate of decline in the slow wave frequency. Experiments were performed in the presence of L-type Ca²⁺ channel blockade (1 μM nifedipine).](image)

![Fig. 3. Disruption of slow wave activity after blockade of inositol 1,4,5-trisphosphate (IP₃)-induced Ca²⁺ release. Xestospongin C (0.5 μM) progressively disrupts the slow wave pattern. Compared with the control tracing at top, observe the “waxing and waning” of the middle trace. With higher concentrations of xestospongin C (1 μM), the slow wave was abolished (bottom trace). Experiments were performed in the presence of L-type Ca²⁺ channel blockade (1 μM nifedipine).](image)
Effect of PLC inhibition with U-73122, neomycin, and NCDC. To further characterize a possible role of IP$_3$-induced Ca$^{2+}$ release, we carried out experiments with known inhibitors of the phosphatidylinositol-specific PLC, thus limiting the generation of IP$_3$ (10, 39). U-73122 (1 mM), although not significantly affecting the resting membrane potential or the slow wave duration, decreased slow wave frequency from 51.4 ± 2.1 to 41.4 ± 1.5 cycles/min ($n = 5$, $P < 0.05$; Fig. 5B). The amplitude was also reduced from 20.6 ± 1.0 to 13.5 ± 2.3 mV ($P < 0.05$). When U-73122 (1 mM) was added in the presence of thapsigargin (1 μM), when the slow wave frequency was already reduced, complete abolishment of the slow waves occurred within ~40 min of perfusion ($n = 5$). Neomycin (4 mM) decreased the slow wave frequency by 27.2 ± 8.8% and in the presence of CPA completely abolished slow wave activity ($n = 7$; Fig. 5C). NCDC at 0.3 mM abolished the slow wave activity in four of six experiments (Fig. 5C). In the remaining two experiments, marked reductions in frequency and amplitude were observed. Effects of NCDC and neomycin were not associated with significant effects on the resting membrane potential.

Effects of thimerosal. Thimerosal reportedly sensitizes the IP$_3$ receptors, leading to an enhancement of IP$_3$-induced Ca$^{2+}$ release (1). The addition of thimerosal (50 μM) caused increased slow wave frequency by 16.4 ± 4.2%, ($n = 5$, $P < 0.03$) with a depolarization...
(7.0 ± 1.2 mV, n = 5, P < 0.03) within 2–5 min of perfusion. In addition, thimerosal reduced both the amplitude (by 24.6–40.4%) and the rate of increase of slow waves (by 31.8–38.2%). These effects were similar to those obtained with 11.8 mM extracellular K⁺, thus suggesting their dependence on depolarization.

**Effect of caffeine.** Caffeine is known to block IP₃-induced Ca²⁺ release at high concentrations (10–20 mM) (30). At 10 mM, caffeine led to a transient hyperpolarization lasting for 1–3 min followed by recovery to near original levels. At this time, the slow waves were either completely abolished or marked reductions in the slow wave frequency and amplitude were observed. Prolonged perfusions with caffeine of 10 or more minutes produced a marked depolarization of 14.0 ± 3.4 mV (n = 11, P < 0.01) and usually complete abolition of the slow wave. The effects of prolonged perfusion with caffeine on the slow wave were likely independent of depolarization because 17.7 mM extracellular K⁺ failed to abolish the slow waves.

**Effects of forskolin and SNP.** One of the known effects of caffeine is to inhibit cytosolic nucleotide phosphodiesterases (4). This leads to increases in cytosolic levels of cAMP and cGMP and subsequent activation of the PKA and PKG signaling pathways. Neither forskolin, which increases intracellular levels of cAMP, nor
SNP, which increases cGMP (34, 45), mimicked the effects of caffeine on the slow wave, except for the hyperpolarization. Concentrations of 1 μM forskolin and 1 μM SNP hyperpolarized the tissues by 13.8 ± 2.9 (n = 7, P < 0.004) and 6.3 ± 1.2 mV (n = 7, P < 0.003), respectively. SNP also significantly increased slow wave amplitude (11.0 ± 3.4%, n = 7, P < 0.003) and the average rate of increase of the upstroke (24.9 ± 0.3%, n = 7, P < 0.002). Forskolin (1 μM) affected slow wave amplitude (decrease of 4.8 ± 1.9%, n = 7, P < 0.04) and frequency (increase of 10.2 ± 2.2%, n = 7, P < 0.002).

Effect of Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Both caffeine and ryanodine act on the Ca\(^{2+}\)-induced Ca\(^{2+}\)-release mechanism (15, 16, 18). To examine the role of this Ca\(^{2+}\) store in the regulation of slow wave activity, we tested the effects of caffeine (5 mM) and ryanodine (10 and 50 μM, Fig. 6). Both are expected to stimulate Ca\(^{2+}\)-induced Ca\(^{2+}\) release in intestinal smooth muscle (15, 16, 18). The effects of caffeine at 5 mM, a concentration exhibiting greater selectivity for Ca\(^{2+}\)-induced Ca\(^{2+}\) release over IP\(_3\)-induced Ca\(^{2+}\) release, were similar to those of the 10 mM concentration described above, albeit with a lower magnitude of response. An initial transient hyperpolarization was observed, and after 10 min of perfusion, the tissues were depolarized by 8.8 ± 2.9 mV (n = 8, P < 0.02). Ryanodine (10 μM) did not have any effect on the electrical activity during 30 min of perfusion. The addition of higher concentrations of ryanodine (50 μM, n = 6) caused a 10.0 ± 2.4 mV (P < 0.01) depolarization, a 55.0 ± 7.7% (P < 0.005) reduction in slow wave amplitude, and a 13.1 ± 0.2% (P < 0.005) reduction in slow wave frequency after ~20 min of perfusion. However, a similar depolarization caused by 17.7 mM K\(^+\) produced similar changes in slow wave activity, suggesting that rya-
dine had negligible effects on slow wave activity that were not related to depolarization.

DISCUSSION

The data presented in this study are consistent with the hypothesis that the regulation of the slow wave frequency in the murine small intestine involves the release of Ca\(^{2+}\) from IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores. Intracellular Ca\(^{2+}\) is critical for the generation of slow waves, as the experiments with BAPTA showed. When Ca\(^{2+}\) release induced by IP\(_3\) was blocked by xestospongin C, slow wave production was abolished, suggesting a critical role of IP\(_3\)-induced Ca\(^{2+}\) release in slow wave initiation. Stimulators of IP\(_3\) synthesis or the IP\(_3\) receptor caused an increase in frequency (premature slow waves), suggesting that IP\(_3\)-induced Ca\(^{2+}\) release may trigger the initiation of the slow wave. This may be mediated by activation of a nonselective cation channel (24, 41). Both CPA and thapsigargin, at submaximal concentrations that inhibit the SR Ca\(^{2+}\) pump, produced a progressively declining slow wave frequency. This is consistent with the rate of Ca\(^{2+}\) store refilling being an important factor in determining the frequency of the slow wave, because IP\(_3\)-induced Ca\(^{2+}\) release is contingent on a critical level of luminal SR Ca\(^{2+}\). Missiaen and co-workers (26, 28) showed that in smooth muscle cells release of Ca\(^{2+}\) from IP\(_3\)-sensitive stores is dependent on SR Ca\(^{2+}\) levels. As luminal SR Ca\(^{2+}\) concentration increases, so too does the IP\(_3\) receptor sensitivity to endogenous IP\(_3\). In the presence of CPA, increasing IP\(_3\) can increase the slow wave frequency as higher concentrations of IP\(_3\) can cause the activation of the IP\(_3\) receptor to occur at lower SR Ca\(^{2+}\) concentrations (27, 28, 31). Although increased levels of IP\(_3\) may cause more Ca\(^{2+}\) to be released from the stores, initially the SERCA pump can still compensate for the increase in release (35). As the SR pump is inhibited more strongly, the store will take longer and the slow wave frequency will further decrease. This proposed role of IP\(_3\)-sensitive Ca\(^{2+}\) release in the regulation of the slow wave finds strong support in the recent observation (37) that gastric smooth muscles that lack the IP\(_3\) receptor do not generate slow waves.

The effects of drugs with a more or less specific action on IP\(_3\) synthesis or signaling pathway were consistent with the decreased or increased generation of IP\(_3\) causing a decrease or increase, respectively, in slow wave frequency. Activation of M\(_3\) muscarinic receptors by carbachol and stimulation of \(\alpha_1\)-adrenoceptors by phenylephrine or norepinephrine increase IP\(_3\) synthesis and release of Ca\(^{2+}\) from IP\(_3\)-sensitive Ca\(^{2+}\) stores in a variety of cells, including smooth muscle cell types (2, 36). These agents, in the presence of SERCA blockade, could substantially increase the slow wave frequency. Perfusion with carbachol or phenylephrine in the presence of SERCA blockade, however, reduced the slow wave frequency or abolished the electrical activity consistent with depletion of the IP\(_3\)-sensitive Ca\(^{2+}\) stores. U-73122, neomycin, and NCDC are inhibitors of phosphatidylinositol-specific PLC, and they inhibit agonist-stimulated IP\(_3\) generation as seen in both ileal longitudinal smooth muscle cells (43) and coronary arterial smooth muscle cells (39). In this study, these PLC blockers reduced the slow wave frequency and amplitude or abolished the electrical activity altogether. Thimerosal sensitizes the IP\(_3\) receptor, causing greater Ca\(^{2+}\) release from SR and elevating cytosolic Ca\(^{2+}\) levels (1), although other mechanisms by which thimerosal activates intracellular Ca\(^{2+}\) release have been proposed (32). Perfusion with thimerosal (for up to 5 min) increased the slow wave frequency. Additional effects of thimerosal on the slow wave amplitude and the rate of increase of the upstroke were likely due to the depolarization that also developed.

Caffeine at high concentrations is known to block IP\(_3\)-induced Ca\(^{2+}\) release (30). In the present study, 10 mM caffeine inhibited the slow wave, apparently independent of the effect of depolarization. In addition to the change in frequency, caffeine caused a transient hyperpolarization followed by depolarization. The hyperpolarization was likely due to increase in cytosolic cAMP or cGMP levels similar to the action of forskolin or SNP. Effects of high concentrations of caffeine (5–10 mM) were previously studied on antral (42) and colonic slow waves (21). In these tissues, caffeine inhibited the slow waves. However, the effect of caffeine on the antral and colonic slow waves may not only be explained by the inhibition of the IP\(_3\)-induced Ca\(^{2+}\) release. Increases in cytosolic cAMP levels may also play a role as activation of the PKA signaling pathway led to the abolition of the slow waves or marked reductions in the slow wave amplitude or frequency in the stomach and colonic musculature (11, 42). In this respect, the murine small intestine offers an advantage in interpreting the effects of caffeine. Activation of the PKA and PKG signaling pathways, judged in this study by respectively identifying responses to forskolin and SNP, did not mimic the effects of caffeine on the slow wave except for the hyperpolarization.

Another well-established effect of caffeine is on the stimulation of the Ca\(^{2+}\)-induced Ca\(^{2+}\) release via ryanodine receptors (15). To determine the role of these stores in the regulation of the electrical activity in the murine small intestine, we tested the effects of ryanodine as well as a lower concentration of caffeine. Both of these agents produced a depolarization, suggesting that activation of this release mechanism is linked to the regulation of the membrane potential. Further study is needed to determine the intracellular mechanisms responsible for this effect. Other observed effects of ryanodine and caffeine on the slow wave, apart from alterations in frequency (by caffeine), can be explained by depolarization, consistent with only a minor role of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in regulation of the slow wave. As explained above, reduction of frequency by caffeine can be attributed to inhibition of IP\(_3\)-induced Ca\(^{2+}\) release.

Because the initiation of slow waves occurs in ICC (17, 41), it is likely that IP\(_3\)-induced Ca\(^{2+}\) release in ICC influences the slow wave frequency. The hypothe-
sis we would like to put forward is that cyclic release of Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores initiates the rhythmic slow wave activity. The rate of refilling of the SR Ca\textsuperscript{2+} stores would determine the slow wave frequency. Basal levels of IP\textsubscript{3} would be sufficient to release Ca\textsuperscript{2+} when a certain level of luminal SR Ca\textsuperscript{2+} is reached. When Ca\textsuperscript{2+} is released from the SR, luminal IP\textsubscript{3} concentration is decreased, thus desensitizing the IP\textsubscript{3} receptor (26) and halting Ca\textsuperscript{2+} release until SR Ca\textsuperscript{2+} levels are restored via the SERCA pump. Indeed, we showed that interference with any of these components affects the slow wave frequency. IP\textsubscript{3} receptor-associated cyclic Ca\textsuperscript{2+} release is common in nonexcitable cells (28). The above-described hypothesis will have to be proven in isolated ICC, which have recently been shown (17, 41) to generate rhythmic slow wave activity. Slow waves actively propagate into the smooth muscle layers (20), hence smooth muscle cells possess most of the ionic mechanisms to generate slow waves, apart from the triggering mechanism. This means that drug action on the IP\textsubscript{3} signaling pathway in smooth muscle cells can be responsible for the observed changes in the slow wave parameters and the resting membrane potential. For example, U-73122 and NCDC, PLC inhibitors, reduced the slow wave amplitude, and adrenoceptor agonists affected the resting membrane potential, the slow wave amplitude (upstroke and plateau), and the duration.

In summary, we provide evidence for a role of intracellular Ca\textsuperscript{2+} stores, IP\textsubscript{3}-induced Ca\textsuperscript{2+} release, and cytosolic Ca\textsuperscript{2+} in the regulation of slow wave frequency and amplitude in the murine small intestine. The proposed hypothesis is that cyclic release of Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores in ICC underlies the rhythmic activation of the slow waves in the intestinal musculature. The main factors in the regulation of the slow wave frequency may be the rate of refilling of the SR stores together with the regulation of the sensitivity of the IP\textsubscript{3} receptor.

A portion of the current work was presented at the Ninth American Motility Society Meeting (Traverse City, MI, 1996) and has been published previously in abstract form (23).

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