Caffeine inhibits nonselective cationic currents in interstitial cells of Cajal from the murine jejunum

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Jin NG, Koh SD, Sanders KM. Caffeine inhibits nonselective cationic currents in interstitial cells of Cajal from the murine jejunum. Am J Physiol Cell Physiol 297: C971–C978, 2009. First published July 22, 2009; doi:10.1152/ajpcell.00155.2009.—Interstitial cells of Cajal (ICC) discharge unitary potentials in gastrointestinal muscles that constitute the basis for pacemaker activity. Caffeine has been used to block unitary potentials, but the ionic conductance responsible for unitary potentials is controversial. We investigated currents in cultured ICC from murine jejunum that may underlie unitary potentials and studied the effects of caffeine. Networks of ICC generated slow wave events under current clamp, and these events were blocked by caffeine in a concentration-dependent manner. Single ICC generated spontaneous transient inward currents (STICs) under voltage clamp at −60 mV and noisy voltage fluctuations in current clamp. STICs were unaffected when the equilibrium potential for Cl (EC1) was set to −60 mV (excluding Cl− currents) and reversed at 0 mV, demonstrating that a nonselective cationic conductance, and not a Cl− conductance, is responsible for STICs in ICC. Caffeine inhibited STICs in a concentration-dependent manner. Reduced intracellular Ca2+ and calmidazolium (CMZ; 1 μM) activated persistent inward, nonselective cation currents in ICC. Currents activated by CMZ and by dialysis of cells with 10 mM BPAT were also inhibited by caffeine. Excised inside-out patches contained channels that exhibited spontaneous openings, and resulting currents reversed at 0 mV. Channel openings were increased by reducing Ca2+ concentration from 10−6 M to 10−8 M. CMZ (1 μM) also increased openings of nonselective cation channels. Spontaneous currents and channels activated by CMZ were inhibited by caffeine (5 mM). The findings demonstrate that the Ca2+-inhibited nonselective cation channels that generate STICs in ICC are blocked directly by caffeine. STICs are responsible for unitary potentials in intact muscles, and the block of these events by caffeine is consistent with the idea that a nonselective cation conductance underlies unitary potentials in ICC.

pacemaker; visceral smooth muscle; gastrointestinal motility; slow wave; electrical rhythmicity; spontaneous transient inward currents; nonselective cationic currents

THERE ARE SPECIALIZED PACEMAKER cells, known as interstitial cells of Cajal (ICC), within the tunica muscularis that generate the spontaneous electrical activity of the gastrointestinal (GI) tract known as slow waves. ICC are electrically coupled via gap junctions, forming networks of cells within specific planes of the muscularis. ICC also form gap junctions with smooth muscle cells, providing low-resistance electrical coupling and creating a syncytium of ICC and smooth muscle cells. ICC contain an abundance of mitochondria and endoplasmic reticulum, and these organelles are closely associated with each other and with areas of the plasma membrane, forming structures with specialized Ca2+ handling properties, known as “pacemaker units.” ICC also express specialized Ca2+-permeable and Ca2+-regulated conductances that provide the means to generate and propagate electrical slow waves. Loss of ICC from pacemaker regions results in defects in pacemaker generation, either loss of the ability to generate electrical slow waves (total loss of pacemaker cells), or compromised ability to actively propagate slow waves without decrement over distances needed to organize motor behavior [partial or patchy loss of ICC resulting in functional uncoupling between regions (15)].

Slow wave activity is important physiologically because it imposes a periodic depolarization/repolarization cycle on membrane potentials of smooth muscle cells, ranging from negative values (−85 to −55 mV) between slow waves, where voltage-dependent Ca2+ channels have a low probability of opening, to potentials of −40 to −30 mV at the peaks of slow waves, where the open probability of Ca2+ channels increases markedly. The plateau phase of slow waves, which is characterized by sustained depolarization lasting up to several seconds, can result in activation of Ca2+ channels and sustained Ca2+ entry in smooth muscle cells. In some regions of the GI tract, slow wave depolarization reaches the threshold for action potentials in smooth muscle cells, and these events result in further enhancement in Ca2+ channel openings, Ca2+ influx, and excitation-contraction coupling.

The plateau phase of slow waves is critical for excitation-contraction coupling and is thought to be due to the summation of unitary potentials that are due to the basic pacemaker mechanism in ICC. Caffeine inhibits unitary potentials and reduces the plateau phase in recordings from ICC in situ. The mechanism for the effects of caffeine on unitary potentials, however, has not been described. We have reported a nonselective cation conductance that is activated by Ca2+ handling mechanisms in ICC. These channels are inhibited by cytoplasmic Ca2+ and therefore require a mechanism to decrease subplasmalemmal Ca2+ for activation. Others have suggested that a Ca2+-activated Cl− conductance underlies unitary potentials and the plateau phase of slow waves in ICC. Understanding the effects of caffeine on unitary potentials may help clarify the conductance underlying unitary potentials and the plateau phase of slow waves. In a previous study we noted that isolated ICC generate spontaneous transient inward currents (STICs) that might be the basis for unitary potentials in intact muscles. In the present study we studied the effects of caffeine on STICs and the conductance that appears to be responsible for these events in ICC.

MATERIALS AND METHODS

Culture of ICC. BALB/C mice (7–14 days old) of either sex were anesthetized with chloroform and killed by cervical dislocation. The jejunum, 2 cm in length from 1 cm below the pyloric ring, was
removed and opened along the myenteric border, and the luminal contents were washed with Ca$^{2+}$-free Hanks’ solution (see Solutions and drugs). Tissues were pinned to the base of a dish lined with Sylgard, and the mucosa was removed by sharp dissection. The experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996), and all protocols were approved by the Institutional Animal Care and Use Committee at the University of Nevada, Reno.

Small strips of intestinal muscle were equilibrated in Ca$^{2+}$-free Hanks’ solution consisting of (in mM) 125 NaCl, 5.6 KCl, 15 NaHCO$_3$, 0.36 Na$_2$HPO$_4$, 0.4 KH$_2$PO$_4$, 10 glucose, 2.0 sucrose, and 10 N-2-hydroxyethylpiperazine-2’-ethanesulfonic acid (HEPES) adjusted to pH 7.2 with NaOH, for 30 min. The buffer was replaced with an enzyme solution containing collagenase (Worthington Type II), 1.3 mg ml$^{-1}$, bovine serum albumin (Sigma), 2.0 mg/ml; trypsin inhibitor (Sigma), 2.0 mg/ml; and ATP, 0.27 mg/ml. The tissues were placed in a 37°C water bath for 25 min without agitation. After five washes with Ca$^{2+}$-free Hanks’ solution to remove the enzyme, the tissues were triturated through a blunt glass pipette. The resulting cell suspension was plated onto murine collagen-coated (2.0% w/v) sterile glass coverslips, in 35-mm culture dishes. The cells were allowed to settle for 15 min before addition of culture medium. The cultures were made from single ICC that were isolated from other cells.

For whole cell patch-clamp experiments, sterile glass coverslips were coated (2.0 µg/ml) with a 1322A, Axon Instruments. Data were recorded from networks of cultured jejunal ICC under current clamp. The resting membrane potential of ICC in networks was $-50 \pm 2.5$ mV. The amplitude of spontaneous oscillation was $12 \pm 2.8$ mV, and frequency averaged $16 \pm 1.6$ cycles/min ($n = 6$) at 30–32°C. Caffeine (0.1–0.5 mM) had no significant effect on slow waves (Fig. 1, A–D). However, higher concentrations of caffeine (i.e., 1–5 mM) hyperpolarized membrane potential (Fig. 1B) and inhibited the amplitude and frequency of the membrane potential oscillations (Fig. 1, C and D) in a concentration-dependent manner.

**Solutions and drugs.** For whole cell recording, the bath solution (Ca2-containing physiological saline solution (CaPSS)) was composed of (in mM) 5 KCl, 135 NaCl, 2 CaCl$_2$, 10 glucose, 1 MgCl$_2$, and 10 HEPES, adjusted to pH 7.4 with tris (hydroxymethyl) aminomethane (Tris). Several pipette solutions were used (see Table 1). Calculated junction potentials for each solution are also provided in Table 1. Data from each experiment and data presented in all figures were corrected for the appropriate junction potentials.

For single-channel recordings, bath solution contained (in mM) 135 potassium gluconate, 15 KCl, 1 EGTA, and 10 HEPES, adjusted to pH 7.3 with Tris. The pipette solution in single-channel recordings was CaPSS (see above). Caffeine and calmidazolium (CMZ) was purchased from Sigma. CMZ was dissolved in DMSO. The final concentration of DMSO was <0.1%, and DMSO had no effects at this concentration.

**Statistical analyses.** Results were analyzed using pCLAMP 9 (version 9.2.0.09, Axon Instruments) and GraphPad Prism software (version 3.02, San Diego, CA). All electrophysiological data are expressed as means ± SE. Differences in the data were evaluated by Student’s t-test. $P$ values < 0.05 were taken as a statistically significant difference. The $n$ values reported in the text refer to the number of cells used in patch-clamp experiments or the number of membrane patches used in studies of single-channel activity.

**RESULTS**

Caffeine inhibits slow waves in ICC. As previously reported (10, 14, 25), Kit-positive cells from dispersion of the tunica muscularis grow in culture and can be observed either as single cells or as networks of cells with morphological features similar to ICC in situ (e.g., fusiform cell bodies, large, prominent nuclei with sparse perinuclear cytoplasm, and multiple, thin processes extending from the nuclear region). Processes of the Kit-positive cells interconnect to form a network.

Single ICC generated spontaneous noisy activity (described below) under current clamp ($I = 0$) but did not generate large-amplitude, constant frequency oscillations, as seen in intact jejunal muscles. Spontaneous oscillations of membrane potential of high amplitude and regular frequency were recorded from networks of cultured jejunal ICC under current clamp. The resting membrane potential of ICC in networks was $-50 \pm 2.5$ mV. The amplitude of spontaneous oscillation was $12 \pm 2.8$ mV, and frequency averaged $16 \pm 1.6$ cycles/min ($n = 6$) at 30–32°C. Caffeine (0.1–0.5 mM) had no significant effect on slow waves (Fig. 1, A–D). However, higher concentrations of caffeine (i.e., 1–5 mM) hyperpolarized membrane potential (Fig. 1B) and inhibited the amplitude and frequency of the membrane potential oscillations (Fig. 1, C and D) in a concentration-dependent manner.

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**Table 1. Pipette solutions for whole cell patch-clamp experiments**

Under physiological extracellular ionic conditions and using pipette solution I [equilibrium potential for Cl$^-$ ($E_{Cl}$) = 0 mV], single ICC displayed spontaneous transient inward currents (STICs); $-28 \pm 5.7$ pA in amplitude; Fig. 2A, top). STICs were irregular in frequency and amplitude. Under current clamp, STICs were associated with irregular depolarizations from an average resting potential of $-50 \pm 5.0$ mV ($n = 6$; current clamp with $I = 0$; Fig. 2A, bottom). These spontaneous depo-
Inhibition of NSCC and STICs in ICC by caffeine

A: effects of caffeine on spontaneous slow wave depolarization in networks of ICC. Caffeine caused dose-dependent inhibition of slow wave activity and hyperpolarization. B–D: summary data from 6 experiments showing the effects of caffeine on resting membrane potential [RMP; most negative potential between slow waves (B)], slow wave amplitude (Amp; C), and slow wave frequency (Freq; D). Slow waves were blocked completely above 1 mM caffeine. *P < 0.05, **P < 0.01.

Fig. 1. Caffeine inhibited slow wave oscillations in networks of cultured interstitial cells of Cajal (ICC). A: effects of caffeine on spontaneous slow wave depolarization in networks of ICC. Caffeine caused dose-dependent inhibition of slow wave activity and hyperpolarization. B–D: summary data from 6 experiments showing the effects of caffeine on resting membrane potential [RMP; most negative potential (B)], slow wave amplitude (Amp; C), and slow wave frequency (Freq; D). Slow waves were blocked completely above 1 mM caffeine. *P < 0.05, **P < 0.01.

To exclude contamination from K⁺ and Cl⁻ currents, a Cs-tetraethylammonium (TEA)-containing pipette solution was used (see Materials and Methods) to set the equilibrium potential for Cl⁻ (E_Cl) at −60 mV (pipette solution II). When cells were dialyzed with this solution, STICs were not resolved (Fig. 2B). To exclude contamination from K⁺ and Cl⁻ currents, cells were dialyzed with pipette solution II (Cs and TEA included, E_Cl = −60 mV) and held at −60 mV. Application of caffeine (5 mM) inhibited STICs and decreased basal inward current from −41 ± 11 pA to −17 ± 5 pA (n = 8; Fig. 2E). We observed considerable variability in the kinetics of STICs from cell to cell, even under the same conditions of recording (see Figs. 2, A–C, and 3, A and E). In current clamp (I = 0, using pipette solution IV) the irregular spontaneous depolarizations that correlated with STIC activity were also inhibited by caffeine (5 mM; Fig. 2F). These data suggest that inhibition of unitary potentials by caffeine is due to inhibition of STICs.

We have shown previously that a nonselective cationic conductance responsible for pacemaker activity (6). This activity is inhibited by caffeine, but the mechanism for the effect of caffeine is unknown. We tested caffeine on STICs in single ICC. Caffeine (5 mM) completely inhibited STICs and reduced the “basal” holding current in ICC (Fig. 3A). It should be noted that in contrast to the effects of caffeine on spontaneous transient outward currents, which are typically due to activation of Ca²⁺-activated K⁺ conductances in smooth muscles (1, 3), inhibition of STICs by caffeine in ICC was not preceded by a burst of activity that might indicate unloading of stores and activation of a Ca²⁺-dependent conductance. The inhibitory effects of caffeine on STICs were concentration dependent and are summarized in Fig. 3, B–D. Again, to exclude the contamination of Cl⁻ and K⁺ currents, cells were dialyzed with pipette solution II (Cs and TEA included, E_Cl = −60 mV) and held at −60 mV. Application of caffeine (5 mM) inhibited STICs and decreased basal inward current from −41 ± 11 pA to −17 ± 5 pA (n = 8; Fig. 3E). We observed considerable variability in the kinetics of STICs from cell to cell, even under the same conditions of recording (see Figs. 2, A–C, and 3, A and E). In current clamp (I = 0, using pipette solution IV) the irregular spontaneous depolarizations that correlated with STIC activity were also inhibited by caffeine (5 mM; Fig. 3F). These data suggest that inhibition of unitary potentials by caffeine is due to inhibition of STICs.

Effect of caffeine on STICs in single ICC. ICC in situ generate unitary potentials that are thought to be the basis for pacemaker activity (6). This activity is inhibited by caffeine, but the mechanism for the effect of caffeine is unknown. We tested caffeine on STICs in single ICC. Caffeine (5 mM) completely inhibited STICs and reduced the “basal” holding current in ICC (Fig. 3A). It should be noted that in contrast to the effects of caffeine on spontaneous transient outward currents, which are typically due to activation of Ca²⁺-activated K⁺ conductances in smooth muscles (1, 3), inhibition of STICs by caffeine in ICC was not preceded by a burst of activity that might indicate unloading of stores and activation of a Ca²⁺-dependent conductance. The inhibitory effects of caffeine on STICs were concentration dependent and are summarized in Fig. 3, B–D. Again, to exclude the contamination of Cl⁻ and K⁺ currents, cells were dialyzed with pipette solution II (Cs and TEA included, E_Cl = −60 mV) and held at −60 mV. Application of caffeine (5 mM) inhibited STICs and decreased basal inward current from −41 ± 11 pA to −17 ± 5 pA (n = 8; Fig. 3E). We observed considerable variability in the kinetics of STICs from cell to cell, even under the same conditions of recording (see Figs. 2, A–C, and 3, A and E). In current clamp (I = 0, using pipette solution IV) the irregular spontaneous depolarizations that correlated with STIC activity were also inhibited by caffeine (5 mM; Fig. 3F). These data suggest that inhibition of unitary potentials by caffeine is due to inhibition of STICs.

We have shown previously that a nonselective cationic conductance activated during pacemaker activity is potentiated by calmodulin inhibitors, CMZ and W7, in ICC (13). To explore whether caffeine affects the nonselective conductance, which are potentiated by calmodulin inhibitors in single ICC, we applied CMZ (1 μM) under permeabilized whole cell conditions with pipette solution V (E_Cl = −60 mV). CMZ (1 μM) induced a persistent basal inward current from −74 ± 20 pA to −702 ± 260 pA (n = 4; P < 0.05; Fig. 4A) and increased STICs in two of six cells (see inset in Fig. 4A). Currents activated by CMZ in single ICC reversed at −0 mV (not shown). Application of caffeine (5 mM) in the presence of CMZ (1 μM) reduced the amplitude of the inward current (270 ± 102 pA; P < 0.05) induced by CMZ at the holding potential of −60 mV (Fig. 4, A and B). In current clamp (I = 0), CMZ (1 μM) induced depolarization, and continuous application of caffeine (5 mM) reversed the effect of CMZ (Fig. 4C). Under the permeabilized whole cell conditions, caffeine (5 mM) reduced basal inward current from −53 ± 6 pA to −32 ± 5 pA (n = 6) and decreased STICs. The inhibitory
effects of caffeine (5 mM) on inward currents were not different in permeabilized whole cell conditions and in cells studied with the dialyzed, whole cell configuration (Fig. 4D). When caffeine (5 mM) was present, CMZ (1 μM) failed to increase inward holding current or restore STIC activity (Fig. 4D).

We reported previously that dialysis of ICC with 10 mM BAPTA caused development of a large inward current (13). The effects of caffeine on the BAPTA-activated current in single ICC were determined in cells held at −60 mV (to exclude K+ and Cl− currents). A sustained inward current developed during BAPTA dialysis and reached an average of −158 ± 29 pA (n = 4, Fig. 5, A and B). Application of caffeine decreased the BAPTA-activated current to −76 ± 21 pA (P < 0.01 when compared with maximal BAPTA-activated current; Fig. 5B). BAPTA-activated and the portion of the current; Fig. 5B). BAPTA-activated current reversed near 0 mV (not shown). In contrast to ICC, dialysis of smooth muscle cells from murine jejunum with BAPTA (10 mM) failed to activate inward current (n = 8, data not shown).

Inhibitory effect of caffeine on nonselective cation conductance from inside-out patch recording. Whole cell experiments suggested that caffeine inhibited a nonselective cation conductance that was activated when cells were dialyzed with 10 mM BAPTA. To test the effects of caffeine on nonselective cation channels, patches were excised from spontaneously active cells. The pipette solution contained CaPSS [calculated equilibrium potential for K+(Ek) and ECl were −87 and −60 mV, respectively]. Inside-out patches exhibited channel openings that reversed at −0 mV (Fig. 6A). Under these conditions, we tested the intracellular Ca2+ sensitivity by lowering Ca2+ concentration ([Ca2+]i) in the bathing solution from 10−6 M to 10−8 M (ECI = −60 mV). At the holding potential of −60 mV, exposure of the cytoplasmic surface of the patches to 10−6 M Ca2+ resulted in sporadic activation of inward currents. The kinetics of the openings and the presence of multiple channels made it difficult to clearly resolve the single channel conductance from our records. When the cytosolic surface was exposed to 10−7 or 10−8 M Ca2+, channels openings increased, creating very noisy current records (see Fig. 6B). Stepping potentials from −100 to +60 mV showed that the currents reversed at −0 mV, suggesting that nonselective cation channels were responsible for the currents activated in inside-out patches by reducing intracellular Ca2+.

With intracellular Ca2+ (10−8 M) to maximize channel openings, caffeine (5 mM) inhibited the nonselective cation conductance (Fig. 7A). We measured the averaged amplitude of the current every 50 s to compare the difference of average amplitude before and after caffeine from Fig. 7A. Application of CMZ (1 μM) to inside-out patches increased the openings of nonselective cation bands at a holding potential of −60 mV ([Ca2+]i = 10−6 M). Application of caffeine (5 mM) inhibited the CMZ-induced activation of nonselective cation bands (Fig. 7C). CMZ-activated currents were not resolved when potential was stepped from −60 to 0 mV, confirming that CMZ activated a nonselective cation conductance (see Fig. 7C, arrow).

DISCUSSION

In gastrointestinal muscles, “unitary potentials” arise from ICC because these events are not recorded from muscles of mutant animals that lack ICC (4). Unitary potentials are likely to be the fundamental mechanism underlying pacemaker activity in ICC, because these spontaneous depolarizations initiate voltage-dependent currents [possibly a voltage-dependent Ca2+ current (10) that entrains pacemaker activity and provides active propagation of slow waves within an ICC network (17)]. Previous studies have shown that caffeine inhibits unitary potentials (and the “regenerative potentials” or slow waves that arise from summation of unitary potentials) (6, 8, 21), but the mechanism for this inhibitory effect is unknown. In past studies we have used cultured ICC from murine intestinal and gastric muscles as a model of ICC pacemaker activity (13, 14, 25). In the present study we found that cultured ICC generate STICs, and these events cause transient depolarizations under current clamp. The STICs we recorded may be the basis for unitary potentials recorded from intact GI muscles with intracellular recording techniques. There has been speculation that STICs (and unitary potentials) are due to activation of Ca2+-activated Cl− currents (7, 12), but the STICs recorded from...
cultured ICC reversed at 0 mV with the Cl\textsuperscript{−} equilibrium potential set at −60 mV, suggesting that these events were due to activation of a nonselective cation conductance. Caffeine inhibited STICs in isolated ICC in a concentration-dependent manner. The nonselective cation current, thought to underlie pacemaker activity in ICC, is inhibited by intracellular Ca\textsuperscript{2+} and can be activated by dialysis of cells with BAPTA or treatment with CMZ (13). We found that, similar to STICs, currents activated in ICC by BAPTA and CMZ were also inhibited by caffeine.

Noisy nonselective cation currents in excised membrane patches from ICC were inhibited by raising [Ca\textsuperscript{2+}] at the cytoplasmic surface. Currents were also activated in excised patches by CMZ. The Ca\textsuperscript{2+}-sensitive currents and the conductance activated by CMZ were inhibited by caffeine, suggesting that these could be the same conductance activated during STICs. While several mechanisms might theoretically contribute to the inhibition of nonselective cation currents by caffeine, it is likely that our experiments on excised patches exclude possible mechanisms such as inhibition of phosphodiesterase or interactions with ryanoide receptors. Our data suggest that at least part of the inhibitory effect of caffeine is due to direct inhibition of channels or to interference with channel gating by binding to a subunit. We also found that caffeine inhibits slow wave activity recorded from networks of ICC. This observation suggests that STICs may underlie the generation of pacemaker activity in ICC networks and that summation of STICs may be responsible for the slow wave events in networks of cells (8). Our data strengthen the idea that activation of a nonselective cation conductance contributes to the generation of unitary potentials and pacemaker activity in ICC.

Spontaneous transient depolarizations (referred to as unitary potentials) have been recorded from strips of GI muscles (4), from small bundles of muscle (6, 21), and during direct impalements of ICC (12). Unitary potentials result from the stochastic discharge of STICs. We tested the effects of caffeine on STICs since this has been a major drug used to investigate the nature and kinetics of unitary potentials in studies of intact muscles (7, 12). The fact that caffeine inhibited STICs in cultured cells is consistent with the idea that these events could be due to the same conductance that generates unitary potentials in intact muscles.

Several authors have suggested that activation of Ca\textsuperscript{2+} activated Cl\textsuperscript{−} currents is responsible for unitary potentials in GI muscles (7, 12). This idea is based on the fact that Cl\textsuperscript{−} channel-blocking drugs and alterations in extracellular chloride concentration ([Cl\textsuperscript{−}]\textsubscript{o}) inhibit unitary potentials (and summation of unitary potentials into slow waves) in some GI muscles.
channel-blocking drugs are nonspecific, and we have shown, for example, that these agents are also capable of blocking nonselective currents in ICC (13). Changing $[\text{Cl}^-]_o$ can produce complex effects on ionic homeostasis, and neither $E_{\text{Cl}}$ nor the effects of altering $[\text{Cl}^-]_o$ have been determined in ICC. Thus, it is hard to predict the consequences of changing $[\text{Cl}^-]_o$ on the conductance underlying unitary potentials. Interestingly, examples of unitary potentials not being blocked by Cl$^-$-blocking drugs have also been reported, and this has led to the suggestion that different conductances might be responsible for unitary potentials in different regions of the GI tract (2). In the present study we investigated STICs in ICC from the small intestine, where there have been few direct studies of unitary potentials, but others have shown that the plateau phase of slow waves, which was assumed to be summation of unitary potentials, was inhibited by caffeine (11). Because of the possibility that unitary potentials and STICs could be due to different conductances in different regions of the gut, we cannot be certain that the conductance we observed or the apparent direct block of STICs (and an underlying nonselective cation conductance and slow waves) by caffeine is universally responsible for the effects.

**Fig. 4.** Effect of caffeine on calmidazolium (CMZ)-activated inward current in single ICC. A: under perforated whole cell conditions (pipette solution V; $E_{\text{Cl}} = -60$ mV), CMZ (1 mM added to bath) induced a large-amplitude inward current at a holding potential of $-60$ mV ($A_a$). In a different cell, CMZ-activated current was inhibited by caffeine ($A_b$). B: summary of the effects of caffeine on CMZ-activated current ($n = 4$). $*P < 0.05$ compared with control. $#P < 0.05$ compared with CMZ. C: when V-C mode switched to current clamp ($I = 0$), CMZ (1 mM) induced depolarization, and caffeine reversed this effect. D: CMZ failed to induce the inward current after pretreatment with caffeine.

**Fig. 5.** Effect of caffeine on inward current activated by BAPTA in single ICC. A sustained inward current developed during BAPTA (10 mM) dialysis at a holding potential of $-60$ mV. A: caffeine decreased the current activated by BAPTA. B: summarized data from 4 experiments. $**P < 0.01$. 
of cells with BAPTA turned on a persistent, large-amplitude nonselective current that may result from the same conductance activated during STICs. Caffeine blocked both conductances. Our findings would suggest that if unitary potentials are due to the conductance responsible for STICs, then BAPTA would tend to activate, not inhibit, this conductance. There is evidence for activation of an inward current by BAPTA, since treatment with BAPTA caused depolarization in some muscles (12). Reduced extracellular Ca\(^{2+}\) also produces this effect in many GI muscles (26). Thus, treatment of muscles with BAPTA, while potentially interfering with intracellular Ca\(^{2+}\) signaling, may interfere with unitary potentials by tonically activating the conductance responsible for these events. We also found that STICs were inhibited by BAPTA during the persistent inward current that developed; tonic activation of the channels responsible STICs would, in effect, reduce the availability of these channels for periodic activation by the mechanism responsible for STICs. Our data demonstrate how tonic activation of the conductance responsible for STICs can inhibit these events. BAPTA dialysis activated a sustained inward current and inhibited STICs in ICC.

We utilized CMZ as another means of activating nonselective cation channels in ICC. We have previously shown that

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\text{Fig. 6. Ca}\^{2+}\text{-inhibited nonselective cation conductance from inside-out patch recordings. A: channel openings were observed in excised inside-out patches. Channel activity at } -60 \text{ mV is shown in inset below main trace. Patch was stepped from } -100 \text{ to } +60 \text{ mV, and currents were reversed at } 0 \text{ mV. Representative traces at various holding potentials } (E_C = -60 \text{ mV}) \text{ show that the channel activity reversed around } 0 \text{ mV. B: effects of Ca}\^{2+}\text{ on single channel currents (holding potential } = -60 \text{ mV). Low concentrations of Ca}\^{2+}\text{ (10}^{-7} \text{ or } 10^{-8} \text{ M} \text{ increased channel activity, whereas a higher concentration (10}^{-6} \text{ M) reduced currents.}
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STICs are likely to be activated by periodic changes in intracellular Ca\(^{2+}\), most likely in excluded volumes between the endoplasmic reticulum and the plasma membrane, as in other cells (18, 20, 23, 28). A complication to the standard mechanism proposed for STICs is the involvement of mitochondrial Ca\(^{2+}\) handling, which may participate in the pacemaker mechanism in ICC (17). Release of Ca\(^{2+}\) from inositol 1,4,5-trisphosphate (IP\(_3\)) receptor-operated stores and sequestration by mitochondria has been suggested as a means for transient reduction in [Ca\(^{2+}\)] within intracellular organelle complexes referred to as pacemaker units in ICC. This mechanism could be responsible for the activation of a Ca\(^{2+}\)-inhibited conductance in ICC, as observed in our experiments (13).

One apparent difference between unitary potentials recorded from intact muscle bundles and STICs recorded from isolated ICC is the response to BAPTA. The frequency of unitary potentials is greatly decreased when muscles are treated with membrane-permeable forms of BAPTA, and this technique was used to help analyze the kinetics of STICs. These studies also led to the conclusion that BAPTA (i.e., Ca\(^{2+}\) chelation) inhibited or reduced the occurrence of unitary potentials, so these events were likely to be due to a Ca\(^{2+}\)-activated conductance (6, 7). Our data suggest that STICs recorded from isolated ICC are due to a Ca\(^{2+}\)-inhibited current, and dialysis

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\text{Fig. 7. Caffeine blocked the Ca}\^{2+}\text{-inhibited nonselective cation currents. A: caffeine (5 mM) decreased inward currents in excised inside-out patches (holding potential } = -60 \text{ mV; } A_a \text{ and } A_b, \text{ expanded traces from } A). B: \text{ averaged amplitudes (50-s bins) before and after caffeine. C: CMZ (1 } \mu\text{M) increased activity of nonselective cation channels at the holding potential of } -60 \text{ mV. Caffeine (5 mM) inhibited the CMZ-activated nonselective cation channels. The arrow above the trace shows that these currents were not resolved when holding potential was switched briefly from } -60 \text{ mV to } 0 \text{ mV to test the reversal potential of the inward currents } (E_C = -60 \text{ mV).}
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CMZ activates the conductance responsible for the slow wave like events in ICC networks (13). In the present experiments, CMZ activated nonselective cation currents in excised patches that were blocked by caffeine, as were the currents due to the Ca\textsuperscript{2+}-inhibited nonselective cation conductance. We have suggested previously that a canonical transient receptor potential (TRPC)-like conductance might be responsible for the Ca\textsuperscript{2+}-inhibited, CMZ-activated nonselective cation conductance in ICC (13, 22) because TRPC channels have binding sites for calmodulin and gating of these channels can be negatively regulated by binding of Ca\textsuperscript{2+}/calmodulin (27). Demonstration that STICs in ICC are related to TRPC channels will require extensive studies of TRPC-knockout models and potentially knockout of multiple isoforms of these channels. At present, we cannot sustain the phenotype of ICC in culture conditions regulated by binding of Ca\textsuperscript{2+}/calmodulin (27). Demonstration that STICs in ICC are related to TRPC channels will require extensive studies of TRPC-knockout models and potentially knockout of multiple isoforms of these channels.

In summary, caffeine inhibits slow wave activity in networks of ICC and STICs in isolated ICC. This may be the mechanism behind the inhibitory effects of caffeine on unitary potentials and slow waves in intact GI muscles. STICs in cultured intestinal ICC were due to periodic activation of nonselective cation channels, not a Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} conductance. STICs appear to result from activation of nonselective cation channels that are inhibited by Ca\textsuperscript{2+} and activated by CMZ. Caffeine blocks inward currents activated in cells or in excised membrane patches by reduced [Ca\textsuperscript{2+}] or CMZ. Our data, and the similarity of the effects of caffeine, suggest that nonselective cation channels are responsible for unitary potentials in the small intestine and may be the basis of spontaneous pacemaker activity in GI muscles.

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GRANTS

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