Voltage-gated Ca\textsuperscript{2+} currents are necessary for slow-wave propagation in the canine gastric antrum

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Bayguinov O, Ward SM, Kenyon JL, Sanders KM. Voltage-gated Ca\textsuperscript{2+} currents are necessary for slow-wave propagation in the canine gastric antrum. Am J Physiol Heart Circ Physiol 293: C1645–C1659, 2007. First published September 13, 2007; doi:10.1152/ajpheart.00165.2007.—Electrical slow waves determine the timing and force of peristaltic contractions in the stomach. Slow waves originate from a dominant pacemaker in the orad corpus and propagate actively around and down the stomach to the pylorus. The mechanism of slow-wave propagation is controversial. We tested whether Ca\textsuperscript{2+} entry via a voltage-dependent, dihydropyridine-resistant Ca\textsuperscript{2+} conductance is necessary for active propagation in canine gastric antral muscles. Muscle strips cut parallel to the circular muscle were studied with intracellular electrophysiological techniques using a partitioned-chamber apparatus. Slow-wave upstroke velocity and plateau amplitude decreased from the greater to the lesser curvature, and this corresponded to a decrease in the density of interstitial cells of Cajal in the lesser curvature. Slow-wave propagation velocity between electrodes impaling cells in two regions of muscle and slow-wave upstroke and plateau were measured in response to experimental conditions that reduce the driving force for Ca\textsuperscript{2+} entry or block voltage-dependent Ca\textsuperscript{2+} currents. Nicardipine (0.1–1 μM) did not affect slow-wave upstroke or propagation velocities. Upstroke velocity, amplitude, and propagation velocity were reduced in a concentration-dependent manner by Ni\textsuperscript{2+} (1–100 μM), mibefradil (10–30 μM), and reduced extracellular Ca\textsuperscript{2+} (0.5–1.5 mM). Depolarization (by 10–15 mM K\textsuperscript{+}) or hypoperfusion (10 μM pinacidil) also reduced upstroke and propagation velocities. The higher concentrations (or lowest Ca\textsuperscript{2+}) of these drugs and ionic conditions tested blocked slow-wave propagation. Treatment with cyclopiazonic acid to empty Ca\textsuperscript{2+} stores did not affect propagation. These experiments show that voltage-dependent Ca\textsuperscript{2+} entry is obligatory for the upstroke phase of slow waves and active propagation. Interstitial cells of Cajal; gastric motility; calcium channels; pacemaker activity

The timing and force of phasic and peristaltic contractions in gastric muscles are determined by electrical slow waves (37). Gastric slow waves are generated from a dominant pacemaker in the orad corpus, and these events propagate rapidly around the stomach and then down the stomach, from the corpus to the pylorus, organizing the contractions into gastric peristalsis (16). Slow waves are initiated by interstitial cells of Cajal (ICC-MY) that lie in the myenteric region throughout the phasically active regions of the gastrointestinal tract (GI), and slow waves spread actively through ICC networks (8, 14, 28). Simultaneous intracellular recordings, from ICC-MY and circular smooth muscle cells, show that slow waves conduct with decrement from ICC-MY to smooth muscle cells through low-resistance electrical connections (6, 8).

Electrical slow waves in GI muscle cells have two components. The first is a relatively rapid upstroke depolarization with rates of rise of up to 1 V/s. The upstroke is transient and partially repolarizes before a sustained plateau phase develops that lasts for several seconds before repolarization to the intra-slow-wave (resting) potential (see Ref. 35). The upstroke is due to activation of voltage-dependent, dihydropyridine-resistant Ca\textsuperscript{2+} channels, whereas the plateau phase results from summation of unitary potentials that result from the discharge of cellular pacemaker units (10, 34) and activation of voltage-dependent Ca\textsuperscript{2+} channels in smooth muscle cells (e.g., 29). We have previously identified Ca\textsuperscript{2+} conductances in ICC of the dog and mouse that are resistant to dihydropyridines and blocked by Ni\textsuperscript{2+} and mibebradil (17, 22). Reduction of this conductance inhibits the upstroke component of slow waves in ICC networks (19, 21) and slow-wave propagation in ICC networks (30, 40). We have suggested that voltage-dependent, dihydropyridine-resistant Ca\textsuperscript{2+} entry is responsible for active slow-wave propagation in ICC networks (17, 34). The mechanism of slow-wave propagation is an important topic because these events must actively propagate over many centimeters of GI muscles to accomplish coordination of gastric peristalsis and other motility behaviors.

Most investigators agree that active propagation of slow waves, which leads to coordination of the intrinsic pacemaker activity of thousands of ICC, depends on a voltage-dependent mechanism (e.g., see Refs. 10, 17, 34, 38), and recent mathematical models of slow-wave propagation demonstrate the requirement of a voltage-dependent mechanism to accomplish regenerative propagation at appropriate rates (10, 15). Despite this agreement, the nature of the voltage sensor and how changes in membrane potential couple to release of Ca\textsuperscript{2+} from intracellular stores and slow-wave propagation are under dispute. Some authors have suggested that voltage-dependent enhancement of inositol 1,4,5 triphosphate (IP\textsubscript{3}) production or voltage-dependent “sensitization” of IP\textsubscript{3} receptor-dependent Ca\textsuperscript{2+} release causes cell-to-cell entrainment of pacemaker discharge (e.g., 10, 38), but voltage-dependent enhancement of IP\textsubscript{3} in ICC has not been demonstrated. We have previously reported expression of a voltage-dependent, dihydropyridine-resistant Ca\textsuperscript{2+} conductance in ICC (17, 22) and suggested that voltage-dependent Ca\textsuperscript{2+} entry could be responsible for entrainment of pacemaker activity (see Ref. 34). Here we tested this idea by measuring the amplitudes, rate of rise, and propagation velocities of slow waves in strips of canine gastric antral muscle.
smooth muscles under conditions where the amplitude of dihydroxypyridine-resistant, voltage-dependent Ca\(^{2+}\) currents was reduced or when Ca\(^{2+}\) stores were depeleted. The results are consistent with a model in which voltage-dependent Ca\(^{2+}\) entry is required for active propagation of slow waves.

**METHODS**

Preparation of muscle strips from the gastric antrum. Mongrel dogs of either sex (total of 24 animals; mean weight, 15 kg) were obtained from vendors licensed by the United States Department of Agriculture. The use of dogs for these experiments was approved by the Institutional Animal Care and Use Committee. The animals were euthanized with an overdose of pentobarbital sodium (100 mg/kg). The abdomen was opened and the entire stomach was removed. A portion of the ventral tunica muscularis, about 3 cm from pyloric sphincter, was dissected from underlying submucosal tissues and pinned to the bottom of a dissecting dish containing oxygenated Krebs-Ringer bicarbonate solution (KRB). Strips of smooth muscle (3 mm \(\times\) 40 mm \(\times\) 1 mm) were cut along the axis of the circular muscle fibers from the greater to the lesser curvature of the antrum.

Electrophysiological recordings. The muscle strips were transferred to an electrophysiological recording chamber that was partitioned into two chambers by a thin strip of Plexiglas with a 6-mm hole at the level of the Sylgard elastomer floor (Fig. 1A). Sheets of latex rubber (Armkel) were glued to one side of the Plexiglas partition, and small holes were made in the latex. The muscle strips were pulled through the latex diaphragms and pinned on either side to the Sylgard elastomer floor. The latex around the muscle strip formed an effective seal between the two chambers, and the two chambers were perfused independently with KRB or test solutions maintained at 37.5 \(^\circ\)C. Cells in regions of muscle in both chambers were impaled with glass microelectrodes having resistances of 50–90 M\(\Omega\). Transmembrane potentials were recorded with a dual-channel, high-input impedance electrometer (Duvo 773; World Precision Instruments, Sarasota, FL). Data were digitized and recorded by a computerized data acquisition and analysis system (MP 100, BIOPAC Systems, Santa Barbara, CA).

In all experiments the end of the muscle strip from the greater curvature of the stomach was secured in chamber A, and the end from the lesser curvature was pulled through the diaphragm and secured in chamber B (Fig. 1A). Chamber B was perfused with test solutions after control recordings of slow-wave propagation velocity were obtained. Slow waves were evoked in the region of muscle in chamber A by electrical field stimulation (EFS; 130 V; 5 ms pulse duration) applied via platinum wires attached through a stimulus isolation unit to an electronic stimulator Grass S48 (Warwick, RI). Canine muscles were used for these studies because they were of sufficient length to make accurate measurements of distances between recording sites and intervals between slow waves.

The partitioned chamber made it possible to evoke slow waves in chamber A that were unaffected by experimental solutions added to chamber B. The integrity of the seal between chambers A and B was demonstrated by the fact that drugs and ionic conditions that had dramatic effects on resting membrane potential and slow-wave activity in chamber B did not affect resting membrane potential and slow-wave activity in chamber A. Thus the slow waves evoked in chamber A, and most importantly, the events entering chamber B were of constant amplitude and rate of rise during control conditions and during application of drugs and experimental ionic solutions in chamber B.

Solutions and drugs. The standard KRB used in this study contained (in mM) 137.4 Na\(^{+}\), 5.9 K\(^{+}\), 2.5 Ca\(^{2+}\), 1.2 Mg\(^{2+}\), 134, Cl\(^-\), 15.5 HCO\(_3^-\), 1.2 H\(_2\)PO\(_4^-\), and 11.5 dextrose. This solution had a final pH of 7.3–7.4 after equilibration with 97% O\(_2\)-3% CO\(_2\). In some experiments, external K\(^+\) was increased in chamber B by equimolar replacement of Na\(^+\) with K\(^+\). Nicardipine, nickel chloride, mibefradil dihydrochloride, cyclopiazonic acid, and pinacidil were all obtained from Sigma (St. Louis, MO). These compounds were diluted in the KRB to the desired concentrations and perfused into chamber B of the recording apparatus (Fig. 1).

Immunohistochemistry. Muscle strips from the greater to the lesser curvatures of the gastric antrum were also prepared for immunohistochemical studies. The muscle strips were placed in a dissecting dish and stretched to 110% of the slack length and width in situ. The muscles were then fixed in ice-cold acetone (4 \(^\circ\)C) for 20–30 min. After fixation was completed, tissues were washed for 30 min in phosphate-buffered saline (PBS, 0.05 M, pH 7.4). Immunohistochemical studies were performed on whole mount tissues. After fixation was completed, the muscles were preincubated in bovine serum albumin for 1 h (1% in 0.1 M PBS) before incubation with a mouse monoclonal antibody raised against human Kit protein (2 \(\mu\)g/min in 0.01 M PBS with 0.5% Triton-X; CD117; Lab Vision, Freemont, CA) at 4 \(^\circ\)C overnight. Immunoreactivity was detected with Alexa Fluor 488 goat anti-mouse IgG-conjugated secondary antibody (1:500, 1 h, room temperature; Molecular Probes, Eugene, OR). Control tissues were prepared in a similar manner, omitting either primary or secondary antibody from the incubation solution. The muscles were examined with a Zeiss LSM 510 Meta (Zeiss, Germany) with an excitation wavelength appropriate for Fluor 488. Confocal micrographs are digital composites of Z-series scans of 10 optical sections through a depth of 10–15 \(\mu\)m (see Fig. 1). Final images were constructed with Zeiss LSM 5 Image Examiner software.

Measurements and statistical analysis of intracellular microelectrode data. We tabulated slow-wave parameters, including: 1) resting membrane potential; 2) upstroke and plateau amplitudes; 3) rate of rise of the upstroke; and 4) propagation velocity determined from the distance between the sites of recording divided by the latency between the times of 10% of maximal slow-wave depolarization at each recording site.

Data are reported as means \(\pm\) SE and tested for significance using Student’s \(t\)-test. Data were considered significantly different from control values when \(P < 0.05\). The “\(n\) values” in the text corresponds to the number of tissue strips from which recordings were performed. Muscle strips from three to five dogs were used for each experimental protocol. In experiments on each muscle strip, 10 slow waves were averaged before and during experimental manipulations to calculate upstroke and propagation velocities.

**RESULTS**

Differences in slow waves recorded from greater and lesser curvatures of the stomach. We made simultaneous recordings from smooth muscle cells in chambers A and B, as depicted in Fig. 1. As described in METHODS, cells in chamber A were from the region of muscle close to the greater curvature in situ, whereas cells at the terminus of muscle strips in chamber B were from the region near the lesser curvature. In 29 of 32 antral muscles from 19 animals, spontaneous slow waves were initiated in the region near the greater curvature and propagated toward the lesser curvature. The latencies were not constant between spontaneous slow waves, suggesting a cycle-to-cycle shift in the site of pacemaker initiation, as we have observed previously (31). Slow-wave shape and characteristics depended on where the activity was recorded. In the greater curvature, the upstroke depolarization of slow waves averaged 44 \(\pm\) 0.7 mV in amplitude, and the rate of rise averaged 508 \(\pm\) 15.6 mV/s. The plateau phase of slow waves averaged 35 \(\pm\) 0.4 mV in amplitude. Upstroke velocity and plateau amplitude decreased as a function of distance from the greater curvature. For example, slow waves recorded in chamber B, in cells 23–25 mm from the greater curvature electrode, had slow waves with an average upstroke velocity of 408 \(\pm\) 14.9 mV/s.
Fig. 1. A: schematic of the recording chamber (see METHODS), which was partitioned into two compartments (A and B) by a latex membrane (LM). Muscle strips, cut parallel to the circular muscle layer, were pulled through a 1-mm hole in the latex membrane, and this formed a seal such that perfusion of one compartment did not influence the components of the adjacent compartment. In all experiments, the muscle strips were positioned such that the portion from the greater curvature lay in compartment A and the portion from the lesser curvature was in compartment B. Slow waves were evoked by two parallel platinum electrodes positioned on either side of the muscle strip in chamber A and recorded by intracellular electrodes in cells in each chamber. Experimental protocols were performed by changing the solution perfusing chamber B from Krebs-Ringer bicarbonate (KRB) solution (used to obtain control values) to a test solution (TS) containing drugs or ion concentrations as described in the RESULTS. Cells were impaled at various points (B1–B3) in chamber B to illustrate the latency between slow waves at various points along the muscle strip and to show the changes in the waveforms of slow wave as a function of distance from the greater curvature. B: slow-wave pairs (recorded by electrodes in chambers A and B at distances noted). The scale bars apply to the recordings in each panel; the x-axis is 5 s and the y-axis is 10 mV. These recordings demonstrate that evoked slow waves in chamber A were of constant waveform, but the shape of slow waves varied as a function of position and the latency between slow waves changed as a function of the distance between electrodes A and B. In all subsequent experiments, recordings were taken from approximately the positions depicted by electrodes A and B3. C: immunohistochemical labeling of interstitial cells of Cajal in myenteric region (ICC-MY) using antibodies for kit in whole mount preparations from the greater to the lesser curvature of the canine antrum. These images were selected from approximately the same distances from which the recordings were made in B. There was a distance-dependent decrease in the density of ICC-MY from the greater to the lesser curvature. Slow waves are generated by ICC-MY and conduct to the circular muscle cells (see Ref. 9). Thus, the decreasing density of ICC-MY may explain why slow waves showed slower upstroke velocities and smaller plateau potentials as a function of distance from the greater curvature.
were 23–25 mm from the greater curvature electrode). Cells in chamber A significantly decreased from the activity in cells near the greater curvature. The amplitudes of the upstroke potentials, however, averaged 42 ± 1.5 mV in chamber B, which was not significantly different from the events in the greater curvature (P = 0.352, n = 29; Fig. 1B).

In a minority of muscles (3 of 32) dominant slow-wave pacemaker activity occurred in cells closer to the lesser curvature recording electrode, and these events propagated toward the greater curvature. This was apparent because slow waves were recorded first by the lesser curvature electrode and then by the greater curvature electrode (i.e., reversed latency). The electrical characteristics of slow waves initiated in the region of the lesser curvature were similar to the events generated when the greater curvature was the dominant pacemaker region. In one of the three muscles with lesser curvature pacing, the position of the dominant pacemaker was markedly unstable, and the site of origin of slow waves changed periodically from the lesser to the greater curvature.

As described above, latencies between slow waves varied from cycle to cycle. This variability would have complicated our analysis of propagation velocity, therefore, in all subsequent experiments we used electrical pacing at a rate slightly above the spontaneous rate to fix the site of slow-wave generation at the greater curvature end of the muscle strips (i.e., in chamber A; see Fig. 1). EFS (1 pulse; 130 V; 5 ms duration) evoked highly consistent slow waves in chamber A. These events were similar in properties to the spontaneous events [e.g., upstroke depolarization averaged 42 ± 0.5 mV; upstroke velocity averaged 575 ± 19 mV/s (upstroke velocity was significantly greater than spontaneous slow waves; P = 0.007), and plateau amplitude averaged 34 ± 0.7; P = 0.709; n = 29]. Under control conditions, every evoked slow wave evoked in chamber A propagated along the muscle strip and was recorded in the cell impaled in chamber B (impalment sites in chamber B were 23–25 mm from the greater curvature electrode). Cells in chamber B had average upstroke amplitude of 42 ± 0.3 mV, average upstroke velocity of 478 ± 9.3 mV/s, and average plateau amplitude of 26 ± 6. These values were not significantly different to spontaneous slow waves recorded in chamber B (all P > 0.05; n = 29), and, as with spontaneous slow waves, the upstroke velocities and plateau amplitudes of slow waves were decreased in cells of the lesser curvature in comparison to cells in chamber A. Slow waves propagated between the cells in chambers A and B at an average rate of 64.6 ± 1.6 mm/s (n = 29). Slow waves, like action potentials in other excitable cells, display refractory properties, as described previously (18, 33). Thus we used interstimulus intervals of sufficient length to allow full recovery of slow waves between events.

Slow waves also decrease in amplitude as they propagate from the greater to lesser curvature in the murine stomach (27), and this was associated with a reduction in the density of ICC-MY. Thus we performed immunohistochemical analysis on the ICC-MY of four antral muscles to test whether a gradient in the ICC-MY density also occurs in a nonrodent mammal. Images from these studies support the conclusion that ICC-MY decrease in density from the greater to the lesser curvatures in the gastric antrum in the dog (Fig. 1). Reduction in the density of ICC-MY might explain the tendency for the greater curvature to be the dominant pacemaker and the reductions in the slow-wave upstroke velocity and plateau amplitude in the lesser curvature cells.

Dihydropyridines do not affect the upstroke and propagation velocity of slow waves. After control slow-wave parameters and the latencies between propagated slow waves were recorded, nicardipine (100 nM to 1 μM), a CaV1.2 (L-type) Ca^{2+} channel blocker, was added to the solution perfusing the portion of the muscle strip in chamber B. Nicardipine did not affect the propagation velocity of slow waves at any concentration tested (e.g., 71 ± 3.8 mm/s in control to 73 ± 4.3 mm/s in presence of 1 μM nicardipine, P > 0.5 n = 5). The average upstroke velocity slightly increased in response to nicardipine (i.e., from 552 ± 12.6 mV/s in control to 588 ± 12.8 mV/s in presence of 1 μM nicardipine, P < 0.05, n = 5). Nicardipine did not affect the upstroke amplitude of slow waves but decreased the plateau potentials as described previously (29) (see Fig. 2F). These data show that dihydropyridine-sensitive Ca^{2+} channels are not required for slow-wave propagation. Currents via these channels provide local inward currents in smooth muscle cells that summate with the currents during plateau potentials of slow waves and result in excitation-contraction coupling (see Ref. 29).

Nondihydropyridine Ca^{2+} channel blockers reduce the upstroke and propagation velocity of slow waves. Previous studies have shown that low concentrations of Ni^{2+} (<100 μM) block a dihydropyridine-resistant Ca^{2+} current in ICC (17, 22). In the present study, Ni^{2+} (1 to 100 μM) reduced propagation and upstroke velocity in a concentration-dependent manner (Fig. 3). At the lowest concentration tested, Ni^{2+} (1 μM) decreased propagation velocity (from 94 ± 3.1 to 85 ± 3.4 mm/s, P < 0.05, n = 5), but at this concentration we could not resolve a significant reduction in upstroke velocity (i.e., from 399 ± 14.8 to 380 ± 18.7 mV/s, P = 0.433, n = 5). Ni^{2+} reduced upstroke velocity when muscles were exposed to 10 μM (P = 0.009). The effects of Ni^{2+} were augmented at 50 μM, which slowed propagation velocity to 54 ± 0.5 mm/s (P < 0.001, n = 5) and reduced the upstroke velocity to 227 ± 4.4 mV/s (P < 0.001, n = 5). Slow-wave propagation was blocked by 100 μM Ni^{2+}. The effects of Ni^{2+} are summarized in Fig. 3, G–I.

Mibebradil (10 and 30 μM), a T-type Ca^{2+} channel blocker (26), had effects similar to Ni^{2+}. As shown in Fig. 4, 10 μM mibebradil significantly reduced slow-wave propagation velocity (from 49 ± 3.4 mm/s in control to 38 ± 2.5 mm/s, P < 0.001, n = 6), and the upstroke velocity was reduced from 356 ± 12.9 to 289 ± 16.8 mV/s (P < 0.001, n = 6). Slow-wave propagation was blocked with 25–30 μM mibebradil.

In most of our experiments the effects of the drugs tested were independent of refractory period effects at the pacing frequencies used (i.e., the period between slow waves was sufficient for complete recovery of the slow-wave mechanism; see Ref. 33 and see Fig. 5A). However, significant effects on the slow-wave refractory period were observed with mibebradil. Thus it was necessary to control for refractoriness with this compound. To study the effects of mibebradil on refractory period, we designed a stimulus protocol in which the duration between pulses was decreased with each successive stimulus and the minimum duration for recovery between slow waves was determined. As seen in Fig. 5B, shortening the interval between

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stimuli reduced the upstroke and propagation velocities (e.g., compare latencies between stimuli and responses with slow-wave pairs 4–6 in Fig. 5B). With mibefradil (10 μM), slow-wave propagation was blocked when the period between stimuli was reduced to ~9 s, but at an interstimulus interval of 12 s, this concentration of mibefradil failed to block slow-wave propagation. Refractoriness due to mibefradil increased with concentrations of 15–20 μM in Fig. 5, D–E. Propagation of slow waves evoked by any interval between stimuli was at 25–30 μM mibefradil (see Fig. 4E).

Upstroke and propagation velocities of slow waves are dependent on external Ca^{2+}. We also investigated the effects of reducing external Ca^{2+} concentration ([Ca^{2+}]_o) on slow-wave propagation and shape. Replacement of the control KRB solution (containing 2.5 mM [Ca^{2+}]_o), with a solution containing 1.5 mM [Ca^{2+}]_o, significantly reduced propagation velocity from 66 ± 3.1 to 53 ± 2.5 mm/s (P < 0.05, n = 5; Fig. 6, A and B). The upstroke velocity was reduced from 434 ± 20.1 to 330 ± 21.2 mV/s (P < 0.001, n = 5). Decrease in [Ca^{2+}]_o to 1 mM slowed propagation velocity and upstroke velocity of slow waves by 48% and 50% of control values, respectively (Fig. 6C). Slow-wave propagation was completely blocked when [Ca^{2+}]_o was reduced to 0.5 mM Ca^{2+} (Fig. 6D).

Figure 6E shows superimposed upstroke potentials in the presence of different [Ca^{2+}]_o, demonstrating the dependence of the upstroke on the driving force for Ca^{2+}. The effects of
reduced \([\text{Ca}^{2+}]_o\) on slow-wave parameters and propagation are summarized in Fig. 6, F–H.

Changes in resting membrane potential affects propagation and shapes of slow waves. Previous experiments in this study show that \([\text{Ca}^{2+}]_o\) entry via a dihydrophyridine-resistant \([\text{Ca}^{2+}]_o\) entry pathway is required for active propagation of slow waves. We investigated the effects of membrane potential on the \([\text{Ca}^{2+}]_o\) entry pathway by causing depolarization and hyperpolarization of cells in chamber B. Increasing external \([\text{K}^+]_o\) concentration \((|\text{K}^+]_o|\) to 10 mM, a concentration that does not block slow-wave generation (see Ref. 3) but would be expected to increase the inactivation of the voltage-dependent \([\text{Ca}^{2+}]_o\) channels in ICC; (see Ref. 17), depolarized cells from \(-75 \pm 0.8\) to \(-65 \pm 1.2\) mV \((P < 0.001, n = 5)\). This degree of depolarization substantially reduced propagation velocity from 46.9 \pm 2.5 to 27.5 \pm 1.6 mm/s \((P < 0.001, n = 5)\) (Fig. 7, A and B). The upstroke velocity was reduced from 486 \pm 14.5 to 299 \pm 15.9 mV/s \((P < 0.001, n = 5)\) by this depolarization. Increasing \([\text{K}^+]_o\) to 15 mM depolarized cells in chamber B to \(-54 \pm 1.9\) mV \((P < 0.001, n = 5)\) and blocked slow-wave propagation (Fig. 7C). The
effects of increased $[K^+]_o$ on slow-wave parameters and propagation are summarized in Fig. 7, E–G.

Hyperpolarization was accomplished by treating the region of muscle in chamber B with the ATP-sensitive K ($K_{ATP}$) channel opener pinacidil. Application of pinacidil (1 μM) hyperpolarized cells from $-78 \pm 2.3$ mV to $-83 \pm 2.9$ mV. Hyperpolarization caused a reduction in the propagation velocity from $83.2 \pm 2.1$ to $60.8 \pm 1.2$ mm/s ($P < 0.001$, $n = 5$; Fig. 8, A and B). The upstroke velocity of slow waves was reduced from $607 \pm 20.2$ to $521 \pm 16.5$ mV/s ($P < 0.001$, $n = 5$). In some cases, as shown in Fig. 8B, multiple upstroke potentials occurred in the presence of pinacidil. Increasing the concentration of pinacidil to 5–10 μM further hyperpolarized membrane potential (e.g., to $-91 \pm 1.6$ mV at 10 μM) and reduced upstroke and propagation velocities of slow waves in a concentration-dependent manner. E: pinacidil at 25–30 μM reduced slow-wave propagation. F: superimposed propagated slow waves recorded by electrode B during exposure to the concentrations of mibefradil noted. Summarized data from 6 experiments show the effects of pinacidil on propagation velocity (G), upstroke velocity (H), and slow-wave amplitude (I; upstroke is solid squares; plateau is solid circles) as a function of pinacidil concentration. Averaged control values (C) for each parameter are shown, and a dotted line extends horizontally from the control mean as a reference in each panel. The points show means ± SE.

Effects of blocking Ca$^{2+}$ uptake into stores on propagation of slow waves. It has been suggested that the voltage dependence of propagation is due to a voltage-sensor linked to the
Fig. 5. Mibefradil increased the slow-wave refractory period. It was important to isolate the effects of test agents from the long refractory periods experienced by slow waves (see Ref. 33). A: with stimulus intervals <10 s, mibefradil caused an alternating pattern of slow-wave propagation between electrodes A and B, which is a signature for a pacing frequency that does not allow for full recovery from refractoriness between stimuli (see Ref. 33). B: a stimulus protocol in which the interval between stimuli was progressively decreased was used to determine the minimum interstimulus intervals needed for full recovery from refractory period. C–E: recorded pairs of slow waves from chambers A and B during exposure to various concentrations of mibefradil. Note that in C and D, slow-wave propagation was blocked by 10–15 μM when stimulus intervals less than 10 s were used. The same problem occurred with 20 μM mibefradil with even longer interstimulus intervals (E). Thus, for tests of the effects of mibefradil, interstimulus intervals greater than 15 s were always used (as in Fig. 4).
production of IP3 as seems to occur in some nonexcitable cells (10, 38). We tested the importance of Ca2+/H11001 stores in active propagation of slow waves using the SERCA pump inhibitor cyclopiazonic acid (CPA; Fig. 9). In these experiments, CPA (50 μM) was added to chamber B for 10 min along with caffeine (10 mM) and acetylcholine (1 μM) to stimulate release of Ca2+ from internal stores. At the end of 10 min, the solution in chamber B was switched to one containing 50 μM CPA. After the protocol to empty Ca2+ stores in cells within chamber B, normal slow waves were evoked in chamber A, and these events propagated through chamber B (Fig. 9B). The plateau phase of slow waves in Fig. 9B was completely inhibited as expected, because this phase involves release of Ca2+ from intracellular stores (see Ref. 34). Upstroke potentials were slightly reduced by the CPA protocol (i.e., 47 ± 0.7 mV in control and 42 ± 0.9 mV after the procedure to unload Ca2+ stores; P < 0.001), but propagation velocity was not significantly affected (i.e., from 34.9 ± 1.4 to 35.5 ± 1.3 mm/s after store unloading, P > 0.05, n = 5; Fig. 9, A and B, D). After the effects of CPA in chamber B were recorded, 50 μM

Fig. 6. Effects of reducing extracellular Ca2+ ([Ca2+]o) on slow-wave propagation. A–D: slow-wave pairs recorded from intracellular electrodes in cells in chambers A and B (as depicted in Fig. 1A). Reducing [Ca2+]o (2.5–0.5 mM) reduced the upstroke and propagation velocities of slow waves in a concentration-dependent manner. D: at 0.5 mM [Ca2+]o, slow-wave propagation was blocked. E: superimposed propagated slow waves recorded by electrode B during exposure to the concentrations of Ca2+ noted. Summarized data from 5 experiments show the effects of [Ca2+]o on propagation velocity (F), upstroke velocity (G), and slow-wave amplitude (H; upstroke is solid squares; plateau is solid circles) as a function of [Ca2+]o. The points show means ± SE.
CPA was also added to chamber A. With CPA in both chambers, slow waves were evoked that consisted of an upstroke depolarization, 43 ± 0.2 mV, and these events propagated at 39.2 ± 2.4 mm/s (n = 5; Fig. 9D). These values were not significantly different from control values (P = 0.152).

**DISCUSSION**

Past evaluations of the factors affecting slow-wave propagation have suggested that pacemaker unit currents, which discharge in a stochastic manner in gastrointestinal networks of ICC (11), rely on a voltage-dependent mechanism for pacemaker entrainment and active propagation of slow waves (see review in Ref. 34). A previous study showed that drugs that inhibit voltage-dependent Ca\(^{2+}\) entry can block slow-wave propagation and block the ability of a dominant pacemaker region to drive a distant region of muscle (40). In the present study we have supplemented earlier findings and shown that drugs affecting voltage-dependent Ca\(^{2+}\) entry have significant effects on slow-wave upstroke depolarizations and propagation velocities of slow waves. Slow-wave propagation was retarded in a concentration-dependent manner by treatments that reduce the availability of dihydropyridine-resistant Ca\(^{2+}\) channels and/or modulate the driving force for Ca\(^{2+}\) entry. Others have suggested that the voltage-sensor necessary for entrainment of pacemakers and active propagation is linked to production of IP\(_3\) (10, 38). We cannot exclude all possible actions of IP\(_3\) or actions of other metabolites of phospholipase C, but our data show that after treatments to deplete Ca\(^{2+}\) stores, the upstroke

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**Fig. 7. Effects of depolarization by increasing extracellular K\(^+\) ([K\(^+\)]\text{o}) on slow-wave propagation.** A–C: slow-wave pairs recorded from intracellular electrodes in cells in chambers A and B (as depicted in Fig. 1A). Increasing [K\(^+\)]\text{o}, (5.9–15 mM) reduced the upstroke and propagation velocities of slow waves in a concentration-dependent manner. C: at 15 mM [K\(^+\)]\text{o}, slow-wave propagation was blocked. D: superimposed propagated slow waves recorded by electrode B during exposure to the concentration of K\(^+\) noted. Differences in resting membrane potential were normalized to superimpose these traces. Summarized data from 5 experiments show the effects of depolarization on propagation velocity (E), upstroke velocity (F), and slow-wave amplitude (G; upstroke is solid squares; plateau is solid circles) as a function of [K\(^+\)]\text{o}. The points show means ± SE.
phase of slow waves continues to propagate normally. These findings add further strength to the conclusion that voltage-dependent Ca\(^{2+}\)/H\(^{+}\) entry is the fundamental mechanism that facilitates active slow-wave propagation.

ICC contain intracellular clusters of organelles near the plasma membrane, known as pacemaker units. At a minimum, the main functional elements of pacemaker units are an IP\(_3\) receptor-operated Ca\(^{2+}\)/H\(^{+}\) store and mitochondria that lie in close proximity to the plasma membrane (see Ref. 34). Ultrastructural studies have shown that Ca\(^{2+}\) stores and mitochondria are closely associated, such that Ca\(^{2+}\) release from IP\(_3\) receptors initiates Ca\(^{2+}\) uptake by mitochondria (41). The close proximity of the mitochondria and Ca\(^{2+}\) stores to the plasma membrane creates a restricted volume, such that Ca\(^{2+}\) movements into and out of these organelles and across the plasma membrane might result in moment-to-moment fluctuations in the Ca\(^{2+}\) concentration in the limited cytoplasmic volume of the pacemaker unit. Ca\(^{2+}\) handling via this mechanism leads to periodic activation of Ca\(^{2+}\)-sensitive nonselective cation channels in the plasma membrane and initiation of spontaneous electrical rhythmicity. Currents generated by pacemaker units cause transient depolarizations, referred to as unitary potentials (9, 18), which initiate the voltage-dependent mechanism that entrains pacemaker unit currents. These processes lead to the development of slow waves and active propagation of slow waves through ICC networks.

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**Fig. 8. Effect of hyperpolarization caused by pinacidil on slow-wave propagation.** A–D: slow-wave pairs recorded from intracellular electrodes in cells in chambers A and B (as depicted in Fig. 1A). Pinacidil (1–10 \(\mu\)M) reduced the upstroke and propagation velocities of slow waves in a concentration-dependent manner. E: superimposed propagated slow waves recorded by electrode B during exposure to the concentrations of pinacidil noted. Differences in resting membrane potential were normalized to superimpose these traces. Summarized data from five experiments show the effects of hyperpolarization on propagation velocity (F), upstroke velocity (G), and slow-wave amplitude (H; upstroke is solid squares; plateau is solid circles) as a function of pinacidil concentration. Averaged control values for each parameter are shown (C), and a dotted line extends horizontally from the control mean as a reference in each panel. The points show means ± SE.
Several published observations also support the idea that the upstroke component of slow waves (also sometimes referred to as the “primary component”; see Ref. 19) is due to a voltage-dependent, dihydropyridine-resistant Ca$^{2+}$/H11001 conductance: 1) the slow-wave upstroke is largely unaffected by concentrations of dihydropyridines that block L-type Ca$^{2+}$/H11001 currents in smooth muscle cells and block contractures to elevated K$^{+}$/H11001 (this study and see Refs. 29 and 42); 2) slow waves recorded directly from ICC were unaffected by micromolar concentrations of nifedipine (19); 3) nifedipine does not block generation or propaga-

![Diagram of slow-wave propagation](https://example.com/diagram.png)

**Fig. 9.** Effect of cyclopiazonic acid (CPA) on slow-wave propagation. A–C: pairs of slow waves recorded from in cells in chambers A and B (top and bottom traces in each panel, respectively). B: effects of adding CPA to chamber B for 10 min along with caffeine (10 mM) and acetylcholine (1 μM). After 10 min the solution in chamber B was changed to one containing CPA (10 nM). The CPA protocol completely abolished the plateau phase of the slow waves, but the upstroke continued to propagate with the same velocity as before CPA. C: effects of adding CPA to both chambers. Upstroke potentials were evoked by stimulating the muscle in chamber A and the slow waves propagated with normal velocities. D–F: summaries of the propagation velocities, upstroke velocities, and upstroke amplitudes, respectively, under control conditions, after the area of muscle in chamber B was treated with CPA (KRB/CPA in each panel) and after areas of muscle in both chambers A and B had been treated with CPA (CPA/CPA in each panel) from 5 experiments. The bar graphs show means ± SE.
tion of slow waves (29, 40, 42); and 4) relatively low concentrations of Ni2+ or mibebradil reduce the upstroke velocity of slow waves (this study), reduce the upstrokes of slow waves recorded directly from ICC (19, 21), and reduce slow-wave upstroke velocity in intact muscles (42).

Despite the general acceptance of the mechanism of the upstroke depolarization, the nature of the voltage-dependent mechanism that recruits pacemaker unit currents to form slow waves and leads to active propagation is controversial. Some authors have suggested that ICC possess a voltage-sensor linked to either production of IP3 (e.g., a voltage-dependent phospholipase C) or to sensitization of agonist-dependent Ca2+ release from IP3 receptor-operated stores (see Refs. 10 and 15). Although this mechanism has been demonstrated in megakaryocytes (e.g., 25), voltage-dependent regulation of IP3 production or agonist-dependent, IP3 receptor-operated Ca2+ release has not been shown to occur in ICC. Furthermore, our data show that conditions of Ca2+ store depletion do not block propagation of slow waves (Fig. 9). Several undefined factors might contribute to voltage-dependent modulation of IP3 receptor-operated Ca2+ release in ICC, but our results show that, at a minimum, voltage-dependent Ca2+ entry is required for slow-wave propagation. This conclusion is consistent with the demonstration of a suitable conductance that might mediate voltage-dependent Ca2+ entry in ICC of two species (17, 22). Thus, based on a growing list of experimental evidence, we suggest that Ca2+ entry via dihydropyridine-resistant Ca2+ channels increases Ca2+ concentration in the tiny cytoplasmic volumes of pacemaker units at the wavefront of propagating slow waves. The open probability of IP3 receptors is regulated by both IP3 concentration and cytoplasmic Ca2+. Thus IP3 receptors display properties of Ca2+-induced Ca2+ release (5, 24). Therefore, voltage-dependent Ca2+ entry and increased local Ca2+ concentration in pacemaker units appears to be the primary mechanism that synchronizes Ca2+ release from IP3 receptors. The plateau phase of slow waves is a summation of unitary pacemaker currents produced by synchronized activation of pacemaker units (9, 34).

The increase in the open probability of IP3 receptors by Ca2+ entry into pacemaker units would, by our concept, be a function of the degree of depolarization and the degree of Ca2+ entry just ahead of the wavefronts of propagating slow waves (i.e., during the late diastolic phase). The degree of depolarization in the active zone of a wavefront would decrease exponentially as a function of distance, such that regions closest to the active zone would experience the largest depolarization, the largest activation of voltage-dependent Ca2+ entry, the most rapid accumulation of Ca2+ in pacemaker units, and the largest increase in the open probability of IP3 receptors. We suggest that this mechanism could provide the sequential recruitment of pacemaker units that occurs in propagating slow waves (and has been referred to by some authors as “entainment” of a network of spontaneous oscillators giving an “apparent” conduction velocity to slow-wave activity; e.g., 38). Our data show that constraining dihydropyridine-resistant Ca2+ entry reduces the amount of current that can be recruited to support the upstroke depolarization, and this leads to a reduction in the rate of propagation of slow waves.

It is interesting to compare the propagation of the gastric slow wave with the propagation of action potentials in nerve and striated muscle. In these cells, activation of voltage-gated Na+ channels and the resulting Na+ current are necessary and sufficient to generate and propagate action potentials. Reduction of the Na+ current by replacement of external Na+ or by specific block of Na+ channels reduces the amplitude, rate of rise, and the conduction velocity of the action potential (e.g., 13, 39, 43). In our work, we examined the effects of reducing Ca2+ current on these parameters in gastric smooth muscle and found qualitatively similar effects (see also Refs. 21 and 42). Specifically, block of dihydropyridine-insensitive Ca2+ channels by Ni2+ or mibebradil or reduction of extracellular Ca2+ reduced the amplitude, the rate of rise, and the propagation velocity of slow waves. Our data show that these channels and the currents that they mediate are necessary for these processes and that the electrical activities of smooth muscle and the classical preparations are qualitatively similar.

The effects of mibebradil on slow-wave refractoriness we noted were intriguing. Previously it has been shown that slow waves are followed by refractory periods lasting several seconds (e.g., 20, 33). The durations of the refractory period are difficult to explain simply on the basis of recovery of a voltage-dependent ionic conductance from inactivation. In the present study we used pacing frequencies that were meant to allow full recovery of slow waves between events to avoid contamination of our propagation velocity and upstroke velocity measurements from refractoriness. Most of the experimental treatments we used caused no problematic lengthening of the refractory period. However, mibebradil increased the slow-wave refractory period, and we found it was necessary to decrease pacing frequency to avoid overlap with the refractory period. Previous studies have demonstrated use-dependent block of Ca2+ channels by mibebradil (2, 4, 23). Recovery from use-dependent block was shown to be voltage dependent and slower at depolarized membrane potentials (e.g., τ = 75 s at −70 mV; see Ref. 2). These authors concluded that use-dependent block by mibebradil was due to slow recovery from open channel block and not because the compound binds to inactivated channels. Others have reported that mibebradil also significantly delays the onset of channel recovery from inactivation (4). As described above, we have previously suggested that mibebradil-sensitive Ca2+ channels in ICC are largely responsible for the upstroke depolarization of slow waves, and Ca2+ entry entrains Ca2+ release from IP3 receptors and organizes activation of a nonselective cation conductance (see Refs. 17 and 34). The features of Ca2+ channel block by mibebradil could affect the availability of channels and contribute to the interval-dependent effects on refractoriness that we observed.

We have recently shown, by using gap junction uncouplers, that virtually all cells in ICC networks are capable of independent spontaneous rhythmicity (30). This has been the speculation of investigators for many years, and some investigators have suggested that the concept of active propagation is not applicable to the spread of slow waves in GI muscles. These tissues perform instead as a matrix of coupled relaxation oscillators (e.g., 7). This concept has been revived of late and used to create models of the spread of slow waves (15, 38). Unfortunately, most formalizations of coupled oscillator models do not include realistic parameters, such as passive cable properties, cell-to-cell electrical resistance, and voltage-dependent ionic conductances known to be expressed by ICC. Thus the predictive capabilities of these models are limited. Data in
the present study demonstrate properties of the slow-wave mechanism that are analogous to other excitable cells that have been modeled by cable analysis (i.e., voltage-dependent activation of Ca\(^{2+}\) channels). The leading edge of the electrical wavefront in GI muscles (e.g., the upstroke or primary component) behaves in a similar manner to action potentials in a variety of excitable cells. Active propagation between regions (i.e., coupling of oscillators in the coupled oscillator lexicon) can be rapidly turned off and on by reducing the driving force for Ca\(^{2+}\) entry or by blockers of dihydropyridine-resistant, voltage-dependent Ca\(^{2+}\) entry. Such rapid changes in propagation are consistent with cable models of propagation but not consistent with coupled oscillators that require time to establish and re-establish coupled oscillation. Thus we would suggest future models of the slow-wave mechanism and propagation should employ parameters that, at a minimum, account for active cable-like properties and voltage-dependent Ca\(^{2+}\) entry.

It should be noted that the changes in propagation velocity we measured in response to test solutions used to perfuse the region of muscle in chamber B (see Fig. 1) are an underestimation of the total reduction in propagation velocity. This is because part of the propagation pathway (i.e., up to 3–5 mm) between the recording electrodes was in chamber A that was not exposed to the test solution. The length of the propagation pathway between the recording electrode in chamber A and the partition never exceeded 20% of the total distance between the recording electrodes. Thus the underestimation in measured propagation velocities during perfusion of test solutions was <20%, and these errors did adversely affect any of the analyses or conclusions of this study.

The conduction velocity of slow waves in gastrointestinal muscles is considerably slower than conduction velocities of action potentials in nerve axons and skeletal and cardiac muscles. This is likely to be due to the cable properties of the ICC network, the load imposed upon the ICC network by the action potentials in nerve axons and skeletal and cardiac muscles is considerably slower than conduction velocities of ICC-IM possess regenerative properties that might amplify and/or accelerate slow-wave propagation, as recently described (12). Excitability mechanisms in ICC-IM, which are abundant in the circular layer, but either absent or far less common in the longitudinal layer (see Ref. 36), may result in anisotropic slow-wave propagation (i.e., higher slow-wave propagation in the circular axis than in the longitudinal axis; e.g., 32). Hirst and coworkers (12) have recently provided data supporting this explanation for anisotropic propagation.

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