ERG $K^+$ currents regulate pacemaker activity in ICC

Yaohui Zhu, Catherine M. Golden, Jing Ye, Xuan-Yu Wang, Hamid I. Akbarali, and Jan D. Huizinga

ERG $K^+$ currents regulate pacemaker activity in ICC. Am J Physiol Gastrointest Liver Physiol 285: G1249–G1258, 2003. First published September 4, 2003; 10.1152/ajpgi.00149.2003.—Ether-à-go-go-related gene (ERG) K channels have been implicated in the generation of pacemaker activities in the heart. To study the presence and function of ERG K channels in the pacemaker cells of the small intestine (the interstitial cells of Cajal (ICC)), a combination of patch-clamp techniques, tissue and live cell immunohistochemistry, RT-PCR, and in vitro functional studies were performed. Nonenzymatically isolated ICC in culture were identified by vital staining and presence of rhythmic inward currents. RT-PCR showed the presence of ERG mRNA in the intestinal musculature, and immunohistochemistry on tissue and cultured cells demonstrated that protein similar to human ERG was concentrated on ICC in the Auerbach’s plexus region. Whole cell ERG $K^+$ currents were evoked on hyperpolarization from 0 mV (but not from −70 mV) up to −120 mV and showed strong inward rectification. The currents were inhibited by E-4031, cisapride, La$^{3+}$, and Gd$^{3+}$ but not by 50 μM Ba$^{2+}$. The ERG $K^+$ inward current had a typical transient component with fast activation and inactivation kinetics followed by significant steady-state current. E-4031 also inhibited tetraethylammonium (TEA)-insensitive outward current indicating that the ERG $K^+$ current is operating at depolarizing potentials. In contrast to TEA, blockers of the ERG $K^+$ currents caused marked increase in tissue excitability as reflected by an increase in slow-wave duration and an increase in superimposed action potential activity. In summary, ERG K channels in ICC contribute to the membrane potential and play a role in regulation of pacemaker activity of the small intestine.

Address for reprint requests and other correspondence: J. D. Huizinga, McMaster Univ., HSC-3N5C, 1200 Main St. West, Hamilton, ON, Canada L8N 3Z5 (E-mail: huizinga@mcmaster.ca).

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ERG K⁺ CURRENTS IN ICC

Fig. 1. Isolation and identification of interstitial cells of Cajal (ICC). A: cross-section of frozen whole mount jejunum from a 2-day-old mouse. ICC associated with Auerbach’s plexus (ICC-AP) and with deep muscular plexus (ICC-DMP) (arrow) were identified as c-kit immunopositive. B: after separating muscle from submucosa by cutting through the deep muscular plexus area, most ICC-DMP were lost. Preparation B is contracted compared with preparation in A. The scale bars of A and B represent 20 μm. C: smooth muscle cells and ICC growing from a small piece of tissue cut from a preparation as in B (explant; see MATERIALS AND METHODS). D: c-kit-positive ICC were labeled in live cell culture (C) with ACK2-Alexa 488 conjugate and observed through a fluorescence microscope. E: single ICC connected to a smooth muscle cell was patched with a pipette in the whole cell configuration. F: this cell (but never smooth muscle cells) displayed rhythmic inward currents. Rhythmic inward currents were observed at negative membrane potentials (0 to −120 mV) with 140 mM K in pipette and 140 mM K in the bath solution, i.e., the same conditions in which the inwardly rectifying currents were observed. G: inward currents at different time scale. H: relationship between amplitude of spontaneous current oscillations and voltage.

per vial and stored at −70°C. A rabbit anti-mouse human ERG (HERG) antibody with additional NH₂-terminal cysteine and tyrosine (APC-016; Alomone Labs, Jerusalem, Israel) was reconstituted with deionized water into a concentration of 0.6 μg/μl, divided into units of 5 μl per vial, and stored at −70°C. For labeling single cells around explant on the day of experiments, the culture was washed with culture medium 3–5 times for 1 min and subsequently with culture medium containing 1.5% BSA. The prediluted ACK2 dye was then added to the 3–6 days’ culture with a final concentration of 0.75–1 μg/ml. After 1 h of incubation at 37°C with 95% O₂-5% CO₂ for 1 h, Texas Red anti-rabbit IgG (cat. no. TI-1000; Vector Laboratories), at a dilution of 1:100, was added to the mixture for 1 h after three washes with medium-199. Stained cultures were examined by using a Zeiss laser scanning confocal microscope (Germany) equipped with an Argon/HeNe laser and attached to a Zeiss Axiovert microscope with excitation wavelengths appropriate for FITC and Texas Red.

Total RNA isolation and RT-PCR. Total RNA was extracted from jejunal muscle layer tissue by using TRIzol reagent following manufacturer’s instructions (Life Technologies). For reverse transcription, the RNA was first mixed with DNase I reaction buffer and DNase I Amp Grade (GIBCO-BRL) for 15 min at room temperature and was subsequently mixed with EDTA at 65°C for 10 min. Thereafter, the DNase-treated RNA was incubated with oligo(dT) at 65°C for 5 min and kept on ice for 2 min. The following were added to the superscript cocktail: 10 mM DTT, 0.55 mM dNTP, 40 units RNasein, 150 units Superscript II, 4 μl first-strand buffer, and 0.25 μl DEPC H₂O. This oligo(dT) sample was processed at 4°C for 10 min, 42°C for 80 min, and 70°C for 15 min. The PCR reaction was undertaken in a 25-μl volume containing the PCR mixture with the following cDNA sample: 0.5 μM of each primer, 200 μM of each dNTP, 1.5 mM MgCl₂, 2 μl PCR buffer, and 0.15 μl Taq polymerase (MBI Fermentas, Flamborough, ON, Canada). An initial reaction was performed at 94°C for 2 min, followed by 35 cycles (94°C, 30 s; 55°C, 40 s; and 72°C, 50 s) and followed by 10 min of final extension at 72°C. Electrophoresis was applied to the PCR product (10 μl) with size markers on a 1.5% agarose gel stained with ethidium bromide. The primer region was designed to traverse an intronic region (forward: 5’TCT GTG TCA TGC TC-3’; reverse: 5’ACC AAC AGG CAT GCT GGA AGT AC-3’) specific for the transmembrane region of the ERG-coded voltage-gated K channel (identical–HERG) (16) and corresponds to nucleotide positions 2252–2449, generating a 198-nucleotide amplification product.

Standard whole cell patch-clamp recording. The whole cell configuration was employed to record whole cell membrane currents from cultured ICC. Data acquisition and analysis were performed with the pClamp suite with standard patch-clamp amplifiers and digitizers (Axon Instruments). Data were filtered at 2 kHz by using an eight-pole Bessel filter (four-pole Bessel filter, 3 dB at 2 kHz). ACK2-stained single ICC were visualized as c-kit positive under an inverted fluorescence microscope. Cultured cells were continuously superfused with Tyrode solution containing (in mM): 135 NaCl, 5.4 KCl, 2 CaCl₂, 0.33 NaH₂PO₄, 5 HEPES, 0.8 MgCl₂, and 5.5 glucose (pH 7.35 with NaOH). High-K solution exchanges were completed within 2 min and contained (in mM) 140 KCl, 0.1 CaCl₂, 1 MgCl₂, 5 HEPES, 5 tetraethylammonium (TEA), 3 4-aminopyridine (4-AP), and 5.5 glucose (pH 7.35 with KOH). The pipette solution contained (in mM) 100 K-aspartate, 30 KCl, 5 HEPES, 5 ATP-γ-S, 1 MgCl₂, 0.1 GTP, and 5 EGTA. Modifications to the solutions are indicated in the text. Some experiments were conducted by using current clamp (I = 0) with Tyrode solution as extracellular solution and as intracellular solution (in mM) of 70 K-aspartate, 70 KCl, 5 NaCl, 10 HEPES, 2 ATP-Mg, 1 MgCl₂, and 0.1 GTP. Extracellular recording and chemicals used. Extracellular recordings were made in an organ bath by using segments of small intestine. Activity was evoked by distension as described previously (9). All experiments were conducted in Krebs solution, which contained 1 μM atropine at a temperature of 37°C. E-4031 was purchased from Alomone Labs; cisapride was a gift from Dr. Jan Schuurkes at Janssen...
RESULTS

Identification of ICC in culture. Explants in culture showed consistent rhythmic contractions with a frequency of 20 ± 4 contractions/min (n = 35). Different types of cells gradually grew from the explants. Smooth muscle cells formed bundles, whereas ICC formed networks (Fig. 1) that contracted rhythmically. At the light microscopy level, the morphological characteristics of ICC were similar to those in situ. Their characteristics included a triangular- or stellate-shaped cell body, large prominent nuclei with little perinuclear cytoplasm, and multiple, thin processes (Fig. 1E). ICC grown from the explants were seen to contract either in synchrony with smooth muscle cells or apparently independently. Smooth muscle cells contracted in a simple way, i.e., whole body contraction, whereas rhythmic ICC contractions could occur in a similar manner or only locally in one or more branches or the cell body. Under a fluorescence microscope, ACK2 coupled to Alexa 488 stained ICC bright green (Fig. 1D). Staining with ACK2 did not affect contractility when antibody dilution was <1:500. In the presence of the ACK2 antibody, the membrane potential was −58 ± 8 mV (n = 6) compared with −61 ± 11 mV (n = 15) in control. The contraction frequency was 17 ± 4 contractions/min (n = 6), not statistically different from the control value of 20 ± 4 contractions/min. However, with dilutions of 1:100, the contractility and membrane potentials were decreased and difficulty in giga seal formation was observed. Most experiments in the present study were performed with 140 mM K⁺ in both the intra- and extracellular solution, and under these conditions, rhythmic inward currents could be observed with hyperpolarizing holding potentials, thus further identifying the ICC (Fig. 1F).

Molecular expression of the ERG K channel in mouse intestine. An ERG-specific RT-PCR product generated from RNA derived from the muscle layer of mouse jejunum tissue was detected (Fig. 2; n = 3). The RT-PCR reaction used total RNA with specific primers designed to amplify a 198-nucleotide sequence of ERG (nucleotide positions 2252–2449, see MATERIALS AND METHODS) identified as the region of ERG transcriptional expression. As positive control, heart muscle was taken from the same mouse (Fig. 2). No PCR products were visible in a negative control experiment without templates. The amplified sequence was screened by using the BLAST program from the National Center for Biotechnology, and no overlap with other channels was identified.

Immunohistochemical identification of a HERG-like channel in ICC. Isolated ICC showed immunoreactivity by using the HERG antibody (Fig. 3). Double labeling with c-kit and HERG antibodies showed that 66 ± 4% of ICC were HERG positive and that smooth muscle cells were mainly negative. Some scattered smooth muscle cells were weakly positive. Immunohistochemical examination of tissue confirmed the colocalization of HERG and c-kit in the ICC in the Auerbach’s plexus area (Fig. 4). Some smooth muscle cells showed weak staining. Neurons and mast cells did not show positive HERG protein immunoreactions. Negative controls omitting primary antibodies revealed no immunoreactive cells.

Properties of the inwardly rectifying K⁺ current. In symmetrical high-K⁺ concentrations, the voltage protocol consisting of a holding potential of 0 mV and step voltage pulses to −120 up to +30 mV resulted in inwardly rectifying currents. These currents demonstrated a transient and sustained component (Fig. 5). Pharmacological blockade of most K channels was achieved by the addition of TEA (5 mM), 4-AP (3 mM), and low Ca²⁺ (0.1 mM), as well as Mg²⁺ (1–3 mM) in all extracellular solutions and ATP-Na⁺ (2.5 mM) in pipette solutions. Furthermore, the hyperpolarizing voltage protocols would inactivate most outward K⁺ currents by stepping from a holding potential of 0 mV to −120 mV. Under these conditions, hyperpolarizing voltage steps evoked fast-activating inward current displaying strong inward rectification (Fig. 5, A and B) in 50 of 100 cells. Peak currents of −564 ± 148 pA occurred after a voltage step from 0 to −100 mV (n = 11) with steady-state currents of −150 ± 10 pA at −30 mV and −40 ± 8 pA at −100 mV measured 100 ms after the start of the voltage pulse (n = 11). Voltage steps from a holding potential of −70 mV evoked relatively little current (Fig. 6). When 140 mM K⁺ in the extracellular solution was replaced by Na⁺, no notable inwardly rectifying currents were observed (n = 10). The inwardly rectifying current was not affected by the TTX (1 μM) or SITS (10 μM) (n = 8). The reversal potential of the current-voltage relationship was related to the equilibrium potential of the K⁺ ion, providing further evidence for...
high selectivity for K⁺. With 140 mM K⁺ at both sides of the cell membrane, the reversal potential was 0 ± 5 mV, consistent with K⁺ being the main current carrier. A change in the reversal potential was observed after a change in the equilibrium potential for K⁺ ions (Fig. 7). The inward current could also be evoked when both the extra- and intracellular concentration of K⁺ was set at 5 mM with intracellular K⁺ replaced by Cs⁺. The peak

Fig. 3. Confocal images of dual labeling with ACK2 and anti-human ERG (HERG) on isolated ICC. The HERG-like protein is identified by using the anti-HERG antibody coupled to Texas Red and shown in red (a). The c-kit protein is identified by using ACK2 coupled to FITC and shown in green (b). The arrowhead identifies a cell that is ACK2 positive and HERG positive; the open arrow identifies a cell that is ACK2 negative and HERG positive; the closed arrow identifies a cell that is ACK2 positive but HERG negative. Dual labeling is shown as orange (c). ICC shown were obtained from a 6-day culture. Most ICC reacted positively to the anti-HERG antibody. The scale bar indicates 50 µm.

Fig. 4. Whole tissue dual labeling with ACK2 and anti-HERG immunoreactivity was obtained by using ACK2 anti-c-kit antibodies and anti-HERG antibodies on serial sections of the mouse small intestine. A: c-kit-positive cells were mainly concentrated at the level of Auerbach’s plexus between the circular (CM) and longitudinal (LM) muscle layer. B: in the adjacent section, HERG-positive cells were concentrated in the same area. However, scattered smooth muscle cells in both muscle layers were also positive, primarily in the longitudinal muscle layer (small arrows). Arrows indicate colocalization of HERG and c-kit immunoreactivity. Insets are enlarged pictures of the boxed areas. G, ganglion; M, mucosa. Scale bars refer to both A and B.

ERG K⁺ CURRENTS IN ICC
The current amplitude under these conditions was \(-500 \pm 80\) pA (n = 8).

The transient part of the current showed fast activation with a time constant of \(5.7 \pm 0.6\) ms after a voltage step from 0 to \(-120\) mV (Fig. 5). The inwardly rectifying currents showed fast inactivation, which exhibited voltage dependence (Fig. 5). When fitted by single exponentials, the time constant decreased from \(14 \pm 3\) ms at \(-60\) mV to \(6 \pm 3\) ms at \(-110\) mV (n = 10).

Although the current showed fast inactivation, the current profile always showed a sustained steady-state component amounting to \(23 \pm 1\)% of the peak current.

Inwardly rectifying currents were sensitive to E-4031. Both the peak currents and the steady-state currents were markedly reduced in the presence of 1 \(\mu\)M E-4031 (Fig. 8). The peak current was inhibited (64 \(\pm\) 6\%), and the steady-state current (58 \(\pm\) 9\%) was measured with voltage steps from 0 to \(-100\) mV (n = 5). Cisapride (1 \(\mu\)M) inhibited both the transient and steady-state component of the ERG K\(^+\) currents (Fig. 9). The peak current was inhibited (45 \(\pm\) 5\%), and the sustained current was inhibited from 10 to 80\% (60 \(\pm\) 4\%) as measured with voltage steps from 0 to \(-100\) mV (n = 5).

Inwardly rectifying currents were rather insensitive to Ba\(^2+\). Concentrations \(\leq 50\) \(\mu\)M had little or no effect (n = 15); a concentration of 100 \(\mu\)M inhibited the

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**Fig. 5.** Hyperpolarization-evoked, inwardly rectifying inward current, assessed by using the standard whole cell patch-clamp technique with 140 mM intra- and extracellular K\(^+\). Whole cell currents were measured in isotonic (140 mM) K\(^+\) solution containing 5 mM tetraethylammonium (TEA), 3 mM 4-aminopyridine (4-AP), and low Ca\(^2+\) (0.1 mM). A: cell was held at 0 mV, and voltage steps were applied from \(-120\) up to \(+30\) mV in 10-mV increments. B: current voltage relationship of the peak current. C: current-voltage (I-V) relationship of the steady-state current measured 50 ms after the start of the pulse. D: voltage dependency of the time constant of inactivation. E: normalized I-V relationship of the peak current (n = 12). F: normalized I-V relationship of the steady-state current (n = 12).

**Fig. 6.** Evoking the ERG K\(^+\) currents depends on holding potential. Left: at control conditions, the cell was held at 0 mV and voltage steps were applied from \(-120\) mV up to \(-30\) mV in 10 mV increments. Marked inward currents are recorded as in Fig. 5. Right: when the holding potential was set at \(-70\) mV and voltage steps were applied from \(-120\) up to \(-50\) mV, no significant inward current was recorded.

**Fig. 7.** Current profiles at different extracellular K\(^+\) concentrations. Whole cell currents were obtained from a holding potential of 0 mV. Typical peak I-V plots of the inwardly rectifying currents showed a shift in reversal potential with changes in external K\(^+\) concentration ([K\(^+\)]\(_o\)). The reversal potential for [K\(^+\)]\(_o\) = 5 mM was \(-65.0 \pm 12.4\) mV (n = 7, ■); for [K\(^+\)]\(_o\) = 50 mM, it was \(-23.0 \pm 8.3\) mV (n = 9, ●); for [K\(^+\)]\(_o\) = 140 mM, it was \(0.7 \pm 2.2\) mV (n = 7, ▲).
current by only 15 ± 3%, and a concentration of 10 mM caused 41 ± 7% inhibition (n = 11) (Fig. 10). Omission of Mg$^{2+}$ from the bathing solution had no effect on the currents (n = 7). The ERG K$^+$ current was abolished by 100 μM of La$^{3+}$ (n = 10) and also by 100 μM Gd$^{3+}$ (n = 8) (not shown).

**Effect of E-4031 on outward current.** Depolarizing pulses from −80 to +180 mV from a holding potential of −70 mV evoked large outward currents with little time-dependent inactivation. This contrasts with smooth muscle cells from the same culture dish, which usually show large transient outward currents (14, 15). A significant portion of the outward currents (63 ± 3%) was blocked by TEA (n = 10), and in the presence of TEA, a significant part of the remaining outward currents (44 ± 7%; calculated at +100 mV) was blocked by E-4031 (n = 3) (Fig. 11). The E-4031-sensitive steady-state portion of the outward currents was very small in the physiological range of voltages.

**Blockers of the ERG K channel affect spontaneous activity in single ICC.** To assess whether the rhythmicity of spontaneous inward currents was affected by blockers of the ERG K$^+$ current, the effects of E-4031 and cisapride were studied. In the presence of high extracellular K$^+$ solutions (140 mM), rhythmic inward currents were observed as shown in Figs. 1F and 12. Under these conditions, the single ICC had an average resting potential of −7.2 mV (n = 5), a capacitance of 20 ± 5 pF (n = 5), and an input resistance of 330 ± 40 MΩ (n = 5). Cisapride (1 μM) caused a reduction in the frequency of the rhythmic inward currents from 22 ± 5 to 10 ± 2 per min (Fig. 12, n = 5). Duration of the rhythmic inward current was increased. In current clamp experiments, recording periodic membrane potential oscillations in a single ICC, E-4031 (1 μM) markedly increased the slow-wave duration, resulting in a decrease in frequency (Fig. 12).

**Slow-wave activity in tissue.** We (21) previously showed that interstitial cells of Cajal are essential for the generation of slow-wave activity in the intestine. The spontaneous inward currents observed in ICC are likely to initiate the generation of slow waves. Because slow waves do not occur without the presence of ICC, the slow waves can be seen as a functional expression of ICC activity, although muscle cell activity and neuronal activity may help shape the slow waves once propagated into the smooth muscle layers. E-4031 (1 μM) and cisapride (1 μM) caused a decrease in slow-wave frequency (Figs. 13 and 14) consistent with the effect of cisapride and E-4031 on the rhythmic inward currents in ICC. Both drugs as well as lanthanum also caused a marked increase in the slow-wave duration and an increase in the extent of action potential generation superimposed on slow waves (Fig. 14). This is caused by cell depolarization. Interestingly, the K channel blocker TEA did not affect the slow-wave frequency (Fig. 14). Neither TEA nor 4-AP affected the slow-wave duration. Both TEA and 4-AP were less effective in increasing tissue excitability compared with E-4031 (Fig. 14).
DISCUSSION

The present study revealed that interstitial cells of Cajal in the mouse small intestine harbor the ERG K channel on the basis of the following evidence: 1) RT-PCR on the mouse intestinal musculature revealed expression of a 198-bp ERG mRNA PCR product similar to that observed in other organs (27); 2) HERG-like immunoreactivity was present in ICC associated with Auerbach’s plexus (ICC-AP); 3) in the presence of TEA and 4-AP, an inwardly rectifying K current was identified that was sensitive to E-4031 and cisapride; 4) its transient component showed fast activation and fast, voltage-sensitive inactivation; and 5) the current was relatively insensitive to Ba$^{2+}$ and was abolished by La$^{3+}$. These are properties of ERG K$^+$ currents (1, 18, 28). Whereas Gd$^{3+}$ at 1 μM inhibits nonselective cation channels in ICC (7), at 100 μM, it also abolished the ERG K$^+$ currents. Interestingly, the immunohistochemistry indicated that a HERG-like protein was abundantly present in many ICC, whereas the density in smooth muscle cells from the mouse small intestine was much less. The ERG K channel is present in smooth muscle cells of the esophagus (1) and gall bladder (17). In the heart, heterogeneous distribution

![](Fig. 9. Inhibition of the inwardly rectifying K$^+$ current by cisapride (1 μM). Whole cell currents were measured in 140 mM K$^+$ solutions. The bath solution contained 5 mM TEA, 3 mM 4-AP, and low Ca$^{2+}$ (0.1 mM). The cell was held at 0 mV, and voltage steps were applied from −120 up to +30 mV. A: recordings were obtained after a voltage step from 0 to −110 mV in the absence and the presence of cisapride. The recording in the presence of cisapride was shifted on the time axis for easy recognition. B: subtracting the 2 current profiles in A shows the cisapride-sensitive current. C: I-V relationships of the peak current: ●, control; ○, with cisapride. D: I-V relationships of the steady-state current: ●, control; ○, with cisapride. E: I-V relationships of the cisapride-sensitive currents: ●, peak currents; ○, steady-state currents. The steady-state currents were obtained 50 ms after the start of the pulse.

![Fig. 10. Effect of barium. Whole cell currents were measured in 140 mM K$^+$ solutions. The bath solution contained 5 mM TEA, 3 mM 4-AP, and low Ca$^{2+}$ (0.1 mM). The cell was held at 0 mV, and voltage steps were applied from −120 up to +30 mV. Ba$^{2+}$ (100 μM) had little effect on the inward currents. Even in the presence of 10 mM Ba$^{2+}$, the inwardly rectifying currents were still present.](AJP-Gastrointest Liver Physiol • VOL 285 • DECEMBER 2003 • www.ajpgi.org)
of the ERG currents is also observed (25). The abundant presence of ERG K\textsuperscript{+}/H\textsubscript{11001} currents in ICC-AP likely reflects the importance of the ERG K channel in the regulation of pacemaker activity. However, double staining of c-kit and ERG proteins revealed that not all ICC-AP were ERG positive, consistent with the fact that we did not observe the ERG K\textsuperscript{+} currents in all ICC-AP.

The inward rectifier K channels preferentially conduct inward current at voltages negative to the K\textsuperscript{+}/H\textsubscript{11001} equilibrium potential (24). In the heart, outward currents inactivate quickly but recover from inactivation on hyperpolarization. These features give the ERG channel a role in the regulation of the resting membrane potential and a contribution to the terminal phase of the repolarization of the cardiac action potential (18). Mutations in the HERG gene can cause sudden death because of a prolonged QT interval on the electrocardiogram, indicating their importance in cardiac pacemaker frequency regulation (8). In neurons, ERG activity produces afterhyperpolarizations in response to neuronal action potentials and hence tends to reduce the action potential frequency (2). The present study shows that ERG channels are involved in the regulation of the pacemaker frequency in the ICC and hence the slow-wave frequency in the gut musculature. ERG channel blockade decreased the frequency of

![Fig. 11. E-4031 reduced outward K\textsuperscript{+} current. K\textsuperscript{+} currents from an ICC were obtained by depolarizing voltage steps from -40 to +100 mV in 10 mV increments in high K\textsuperscript{+} solutions. The holding potential was -40 mV. A prepulse was given for 10 ms to -90 mV followed by 45-ms pulses up to +180 mV. The extracellular solution was normal Tyrode solution, and the pipette contained 140 mM K\textsuperscript{+}. I-V relationships of the peak currents are shown. Peak and steady-state outward currents at physiological potentials are small.](image1)

![Fig. 12. Spontaneous activity of ICC affected by blockers of the ERG K channels. At a holding potential of -60 mV, spontaneous rhythmic inward currents were observed. Cisapride 1 \mu M decreased the frequency of the inward currents. In the control trace, the frequency is 22 per min; after superfusing with cisapride 1 \mu M, the frequency dropped to 10 per min. In current clamp, spontaneous voltage oscillations were observed. The resting membrane potential was -50 mV. E-4031 decreased the slow-wave frequency by marked prolongation of the slow-wave plateau.](image2)

![Fig. 13. E-4031 markedly increases tissue excitability. The first trace shows normal slow-wave activity with superimposed action potentials. In the presence of E-4031 (1 \mu M, 30 min), slow-wave duration increased, slow-wave frequency decreased, and number and amplitude of action potentials increased. Increasing the concentration of E-4031 caused periodic occurrence of slow waves with very long duration (15–30 s).](image3)

![Fig. 14. Effect of K channel blockers on tissue slow-wave parameters: TEA (5 mM), 4-AP (4 mM), lanthanum (100 \mu M), cisapride (1 \mu M), and E-4031 (1 \mu M). *Significantly different from control (P < 0.05–0.01).](image4)
spontaneous rhythmic inward currents and associated membrane potential oscillations (slow waves) in ICC and markedly reduced the slow-wave frequency in tissue. The channel is likely maximally activated on cell hyperpolarization. ICC in the mouse small intestine go from approximately $-40$ mV to approximately $-80$ mV 50 times a minute without action potentials, and in the presence of action potentials, the voltage drop is from $-0$ mV to $-80$ mV.

When extracellular electrodes are gently sucked onto the mouse small intestine musculature, regular slow-wave activity is consistently recorded. Without any stimulation, few action potentials are seen on the slow wave. When E-4031 is added, the slow wave increases in amplitude, its plateau phase is depolarized, and abundant action potentials appear on the plateau. The plateau phase of the slow wave is highly voltage sensitive. A small hyperpolarization by field stimulation (10) or an increase in K conductance (4) abolishes the sensitive. A small hyperpolarization by field stimulation (10) or an increase in K conductance (4) abolishes the plateau phase of the slow wave is highly voltage sensitive. A small hyperpolarization by field stimulation (10) or an increase in K conductance (4) abolishes the plateau completely. Hence under normal conditions, ERG K channel activity significantly decreases cell excitation. After comparison of the effect of E-4031 to TEA or 4-AP (Fig. 14), it appears that ERG channel activity is much more involved in regulation of excitability than K channels blocked by TEA or 4-AP. In a very similar way, neurons that have ERG channel activity are much less likely to respond to continuing depolarization with bursts of action potentials (2). In fact, severe neuronal hyperactivity may be due to loss of ERG activity. The change in slow-wave duration is likely due to inhibition of ERG channel activity in ICC. Interestingly, in the gall bladder, E-4031 caused marked increase in action potential generation and also periodic occurrence of prolonged depolarization with superimposed action potentials (17). The marked increase in tissue excitability on E-4031 revealed by the increase in action potential activity likely is also a reflection of the inhibition of ERG K channels in smooth muscle cells.

Use of explant cultures to obtain isolated ICC did not require any digestive enzymes, thus avoiding any potential enzymatic alteration to the surface c-kit protein and ion channels. ICC were identified as c-kit positive, triangled shaped with multiple branches and with spontaneous rhythmic contractile activity. Use of c-kit antibodies (1:500 dilution) coupled to Alexa 488 did not affect the unique characteristic intrinsic pacemaker activity. At an antibody dilution of 1:100 or incubation longer than 2 h at 37°C, a marked reduction in contractility was observed, consistent with other studies (23). We observed that neonatal ICC grown from explants were more resistant to antibody staining than chemically isolated ICC from adult mice (12, 15), possibly related to the presence of more multipotent growth factors (26). There are advantages and disadvantages to the use of single cells obtained through cell culture or chemical dissociation. Both procedures can lead to a change in cell properties, the first through changes over time in culture medium and the second by damage caused by enzymatic digestion. The key is to identify the phenomenon under study in the cells chosen. In our hands, cells derived from short-term culture (2–5 days) possess the rhythmic inward current, which is what is needed to identify currents related to this pacemaker activity.

With the elucidation of the ERG K channel in the present study, there are now two inwardly rectifying K channels identified in ICC. Recently, a barium-sensitive “classical” inward rectifier was identified in canine colonic ICC. Flynn et al. (6) reported the presence of a Ba$^{2+}$-sensitive conductance encoded by Kir2.1, which contributed to the generation and maintenance of negative membrane potentials between slow waves. A similar current was also suggested to be in ICC isolated from the mouse small intestine (12). Differences between the Kir2.1 and the ERG K$^+$ current in our present study are the sensitivity to Ba$^{2+}$ ions, the kinetics of opening and closing, and the sensitivity to E-4031 and cispamide. The high concentration of Ba$^{2+}$ needed to inhibit the ERG current was similar to that observed in esophageal smooth muscle (1). HERG-like currents are sensitive to lanthanum, as were the ERG K$^+$ currents observed in the present study. We also discovered that the ERG K$^+$ currents in ICC are sensitive to 100 μM gadolinium, but not to 10 μM, which blocks nonselective cation channels in these cells (7). Compared with the ERG currents in the heart (25), esophageal smooth muscle cells (1), and glial cells (28), the activation kinetics (on hyperpolarization) and the subsequent inactivation kinetics of the ERG currents in ICC are very fast. The present study indicates that ERG K inward rectifiers are a dominant regulator of pacemaker activity and smooth muscle cell excitability in the intestinal musculature.

H. I. Akbarali contributed to this work as a visiting professor. Present address for H. I. Akbarali: Dept. of Physiology, Univ. of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.

DISCLOSURES

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