Ether-a-go-go-related gene 3 is the main candidate for the E-4031-sensitive potassium current in the pacemaker interstitial cells of Cajal

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Submitted 22 May 2008; accepted in final form 24 July 2008

White EJ, Park SJ, Foster JA, Huizinga JD. Ether-a-go-go-related gene 3 is the main candidate for the E-4031-sensitive potassium current in the pacemaker interstitial cells of Cajal (ICC). Am J Physiol Gastrointest Liver Physiol 295: G691–G699, 2008. First published July 31, 2008; doi:10.1152/ajpgi.90348.2008.—The interstitial cells of Cajal (ICC), as pacemaker cells of the gut, contribute to rhythmic peristalsis and muscle excitability through initiation of slow-wave activity, which subsequently actively propagates into the musculature. An E-4031-sensitive K⁺ current makes a critical contribution to membrane potential in ICC. This study provides novel features of this current in ICC in physiological solutions and seeks to identify the channel isoform. In situ hybridization and Kit immunohistochemistry were combined to assess ether-a-go-go-related gene (ERG) mRNA expression in ICC in mouse jejunum, while the translated message was assessed by immunofluorescence colocalization of ERG and Kit proteins. E-4031-sensitive currents in cultured ICC were studied by the whole cell patch-clamp method, with physiological K⁺ concentration in the bath and the pipette. In situ hybridization combined with Kit immunohistochemistry detected m-erg1 and m-erg3, but not m-erg2, mRNA in ICC. ERG3 protein was colocalized with Kit-immunoreactive ICC in jejunal sections, but ERG1 protein was visualized only in the smooth muscle cells. At physiological K⁺ concentration, the E-4031-sensitive outward current in ICC was different from its counterpart in cardiac and gut smooth muscle cells. In particular, inactivation upon depolarization and recovery from inactivation by hyperpolarization were modest in ICC, and the current kinetics were far different from the basal interstitial cells of Cajal (ICC), as pacemaker cells of the gut, contribute to rhythmic peristalsis and muscle excitability through initiation of slow-wave activity, which subsequently actively propagates into the musculature. An E-4031-sensitive K⁺ current makes a critical contribution to membrane potential in ICC. This study provides novel features of this current in ICC in physiological solutions and seeks to identify the channel isoform. In situ hybridization and Kit immunohistochemistry were combined to assess ether-a-go-go-related gene (ERG) mRNA expression in ICC in mouse jejunum, while the translated message was assessed by immunofluorescence colocalization of ERG and Kit proteins. E-4031-sensitive currents in cultured ICC were studied by the whole cell patch-clamp method, with physiological K⁺ concentration in the bath and the pipette. In situ hybridization combined with Kit immunohistochemistry detected m-erg1 and m-erg3, but not m-erg2, mRNA in ICC. ERG3 protein was colocalized with Kit-immunoreactive ICC in jejunal sections, but ERG1 protein was visualized only in the smooth muscle cells. At physiological K⁺ concentration, the E-4031-sensitive outward current in ICC was different from its counterpart in cardiac and gut smooth muscle cells. In particular, inactivation upon depolarization and recovery from inactivation by hyperpolarization were modest in ICC. In summary, the E-4031-sensitive currents in cultured ICC were studied by the whole cell patch-clamp method, with physiological K⁺ concentration in the bath and the pipette. In situ hybridization combined with Kit immunohistochemistry detected m-erg1 and m-erg3, but not m-erg2, mRNA in ICC. ERG3 protein was colocalized with Kit-immunoreactive ICC in jejunal sections, but ERG1 protein was visualized only in the smooth muscle cells. At physiological K⁺ concentration, the E-4031-sensitive outward current in ICC was different from its counterpart in cardiac and gut smooth muscle cells. In particular, inactivation upon depolarization and recovery from inactivation by hyperpolarization were modest in ICC. In summary, the E-4031-sensitive currents in cultured ICC were studied by the whole cell patch-clamp method, with physiological K⁺ concentration in the bath and the pipette. In situ hybridization combined with Kit immunohistochemistry detected m-erg1 and m-erg3, but not m-erg2, mRNA in ICC. ERG3 protein was colocalized with Kit-immunoreactive ICC in jejunal sections, but ERG1 protein was visualized only in the smooth muscle cells. At physiological K⁺ concentration, the E-4031-sensitive outward current in ICC was different from its counterpart in cardiac and gut smooth muscle cells. In particular, inactivation upon depolarization and recovery from inactivation by hyperpolarization were modest in ICC. Therefore, we also assessed ERG2 and ERG3 isoforms as described in the central nervous system (7) and pancreas (14), since electrophysiological characteristics of these channels are largely unknown outside expression systems.

The aim of this study was to search for potentially unique features of the E-4031-sensitive currents using physiological ionic conditions and voltage-step protocols focusing on membrane potentials in the range of slow wave activity generated in ICC and to investigate a potentially unique expression of ERG K⁺ channel α-subunits, ERG1, ERG2, and ERG3, and β-subunits, MiRPI and MinK, in mouse jejunum tissue sections.

METHODS

Animals. All procedures used to obtain mouse tissue were approved by the McMaster University Animal Research Ethics Board. Neonatal (3- to 4-day-old) and adult (6- to 8-wk-old) CD1 mice were killed by cervical dislocation. For primary cultures, the neonatal jejunum muscularis was isolated and cut into small (~1-mm²) pieces in M199 supplemented with 10% FBS, 1% glutamine, and 1% antibiotic-antimycotic (GIBCO, Invitrogen, Burlington, ON, Canada). Tissue explants were cultured for 5–6 days on 20-mm-diameter coverslips at 37°C in 95% O₂-5% CO₂ before use. Generation of 35S-labeled m-erg cRNA probes. Total RNA was extracted from jejunum tissue using a combination of TRIzol and the
RNase digestion. The digested sample was then added to 7 M urea for 15 min. The resultant single-strand DNA was used in PCR to generate probe sequences for m-erg1, m-erg2, and m-erg3. All primers were designed to use an annealing temperature of 56°C. Specific primers were designed for m-erg1 from GenBank sequence NM_013569 with forward (5'-GTACCAAGGTTGCTCTGAT-3') and reverse (5'-GTACCAAGTGTAGCTAGCAGG-3') primers to generate a 164-bp product, for m-erg2 from GenBank sequence NT_039521 with forward (5'-CAATGACAGCCATACAGGTT-3') and reverse (5'-CACCAATCCGCTTCTC-3') primers to generate a 124-bp product, and for m-erg3 from GenBank sequence AF291608 with forward (5'-CAACAGACTCCATGGTGAA-3') and reverse (5'-GCCAAGCTCTCTGAAGTCCTG-3') primers to generate a 164-bp product. The PCR included 1× PCR buffer, 1 mM MgCl2, 0.2 mM dNTPs, forward and reverse primers of the outside reaction at 1 μM each, 1 U of platinum Taq DNA polymerase, and up to 25 μl of nuclease-free water. The thermocycler program included initiation at 94°C for 2 min followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final polish step at 72°C for 2 min. Sense and antisense 35S-labeled cRNA probes were produced by SP6 or T7 RNA polymerase-mediated in vitro transcription reactions using purified linearized pGEM-T-Easy vector (Promega, Fisher Scientific, Nepean, ON, Canada) containing the m-erg1, m-erg2, or m-erg3 probe sequence generated from PCR products. The in vitro transcription-labelling reaction contained 1× transcription buffer, 10 mM DTT, 12 U of RNAsin, 2.5 mM NTP mix, 0.5 μg of linearized plasmid, 7.5 μl of 35S-labeled UTP (187.5 μCi), and 7.5–10 U of SP6 or T7 RNA polymerase. After 30 min of incubation at 37°C, RNA polymerase (7.5–10 U) was added to this mixture, and incubation continued at 37°C for 30 min. Plasmid DNA template was removed in a reaction containing 20 U of RNasin and 0.5 U of RQI DNase (Promega, Fisher Scientific) which was incubated at 37°C for 10 min. Unincorporated NTPs were removed using Probe-Quant G-50 Micro-Columns (GE Health, Baie d’Urfé, PQ, Canada). A scintillation counter was used to measure 35S labeling. Combined Kit immunostain and in situ hybridization for ERG mRNA. Cryosections (10 μm) were fixed with 4% formaldehyde, rinsed in PBS, and incubated in 5 μg/ml ACK4 with 80 U of RNaseOUT (Invitrogen) diluted in PBS for 30 min. Slides were washed in PBS, incubated in goat anti-rabbit IgG-biotin for 30 min, washed in PBS, incubated in avidin-biotin complex for 20 min with secondary antibodies, and blocked with 5% normal goat serum in PBS for 1 h. Each tissue section was examined for Kit and ERG or β-subunit (MiRP1 and MinK) immunofluorescence using antibodies and concentrations described in Table 1. Briefly, tissues were incubated overnight in primary antibody at 22°C, washed in PBS, incubated in secondary antibody for 1 h at 22°C, washed in PBS, incubated with 10 μM DRAQ5 (Biostatus, Leicestershire, UK) for 10 min, and rinsed in PBS; then coverslips were applied with antifade mounting medium (Biomedia, Foster City, CA). Images were captured using an LSM 510 confocal microscope multitracking program with laser excitation wavelengths of 488 nm (Ar), 543 nm (HeNe1), and 633 nm (HeNe2) and ×20, ×63, and ×100 objectives. An image resolution of 512 × 512 or 1,024 × 1,024 pixels was used, and identical confocal microscope acquisition settings were used to capture images for all three ERG isoforms. Images were captured and illustrated as z stacks, but all colocalization analysis was carried out on a minimum of three z slices (each optical slice = 1 μm) within each z stack using ImageJ (National Institutes of Health, Bethesda, MD). Colocalization data were quantified using Mander’s colocalization coefficient showing the intensities of red pixels (Kit+ regions in the image) with a green intensity above 0 (ERG+ signal) divided by the sum of red pixels with intensity above 0. This value is expressed as a percentage of colocalized pixels relative to the total Kit-immunoreactive red pixels in the selected region. After analysis, images were adjusted for brightness and contrast using Corel Draw 11 (Corel, Ottawa, ON, Canada) for illustration purposes. Electrophysiology. Whole cell currents were measured by patch clamp from single cultured ICC situated close to neonatal explants. Patch pipettes were pulled on a micropipette puller (model P-87, Sutter) from thin-walled glass capillary tubing with filament; pipette resistance was 3–5 MΩ. Data were acquired using an EPC-9 amplifier (HEKA Elektronik) controlled by a personal computer running EPC Instruments software (Pulse/PulseFit) at 1 kHz (ramp pulse) or 10 kHz (step pulse) and filtered at 2.9 kHz with an analog four-pole filter. Capacitance and series resistance compensation was performed with the EPC-9 amplifier. Series resistance was measured with 0.3 M ammonium acetate and 100% ethanol and air-dried. For determination of the cellular localization of hybridized probes, slides were dipped in Amersham Hypercoat LM-1 emulsion (GE Health), stored in darkness for 4 wk at 4°C, and then developed in D19 (Kodak, Rochester, NY), fixed in nonhardening fixer (Kodak), counterstained in toluidine blue, and mounted in Permount. Slides were examined using a Zeiss Axiosvert microscope.
compensated up to 80%. Cells were visualized with an inverted microscope (Axiovert 25, Zeiss). The recording chamber was constantly perfused with the extracellular solution (ECS) at a rate of 1 ml/min. A perfusion device (Valvelink 8) was used to apply test solutions directly to cells. The recording chamber was grounded via an agar-salt bridge (3% agar and 1 M KCl), minimizing liquid junction potentials produced by test solutions. The cells were continuously bathed with ECS at room temperature (23–25°C). ECS contained (in mM) 135 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, and 10 dextrose, with pH adjusted to 7.35–7.40 with NaOH. Intracellular solution contained (in mM) 70 KCl, 70 potassium aspartate, 4.5 magnesium ATP, 10 EGTA, and 5 HEPES, with pH adjusted to 7.25–7.30 with KOH. The test drug, E-4031, was obtained from Sigma-Aldrich. Data were analyzed using Origin software (version 6.0, Microcal Software, Northhampton, MA).

RESULTS

**ERG mRNA expression in the small intestine muscle layer.** In situ hybridization combined with bright-field immunohistochemistry spatially localized m-erg1, m-erg2, and m-erg3 mRNA in mouse jejunum tissue in relation to Kit+ ICC. For positive controls, m-erg1 mRNA signal was confirmed in heart tissue, and m-erg2 and m-erg3 mRNA signals were confirmed in brain (data not shown). In mouse jejunum tissue, m-erg1 and m-erg3 mRNA signals were present throughout the smooth muscle cell layers and within Kit+ cells in the myenteric plexus (Fig. 1, A and C). The negative control sense probes for m-erg1 and m-erg3 showed minimal signal (Fig. 1, D and F). The m-erg2 signal was not present (Fig. 1B), and nonspecific

Fig. 1. Colocalization of m-erg mRNA and Kit protein in adult mouse jejunum tissue sections. Note the presence of m-erg1 and m-erg3 mRNA signals, visualized as black grains and highlighted by black arrowheads, in smooth muscle cells and overlapped with Kit-immunoreactive cells (visualized by diaminobenzidine brown precipitate) in the myenteric plexus and the absence of m-erg2 signal. Antisense in situ hybridization signals are shown in A–C and sense probe signals are shown in D–F for m-erg1, m-erg2, and m-erg3, each combined with Kit immunohistochemistry. Scale bars, 25 μm. LM, longitudinal muscle; AP, Auerbach’s (or myenteric) plexus; CM, circular muscle; MUC, mucosa.
signal was observed with the negative control sense probe (Fig. 1E). mRNA signals from mouse jejunum tissue sections subjected to only in situ hybridization produced signal patterns similar to those of the combined immunohistochemistry-in situ hybridization protocol for m-erg1, m-erg2, and m-erg3 anti-sense and sense probes (data not shown).

**ERG protein and β-subunit expression.** Immunofluorescence was used to investigate the presence of immunoreactivity for ERG1, ERG2, and ERG3 proteins and the β-subunits MiRP1 and MinK in relation to Kit-immunoreactive ICC in adult mouse jejunum tissue sections. The β-subunits MiRP1 and MinK were not detectable within the muscularis by immunofluorescence (data not shown). ERG1 was immunoreactive within smooth muscle cells, whereas 27 ± 14.2% (SD) of Kit-immunoreactive red pixels colocalized with ERG1-immunoreactive green pixels when the complete muscularis was selected (Fig. 2A, regions i and ii). In case colocalization artifact was created by selection of Kit-immunoreactive regions that overlapped with the ERG1-immunoreactive regions in smooth muscle cells, this colocalization was compared with selection of only the ICC cell bodies. Selection of a specific ICC as the region of interest produced 6 ± 5.2% (SD) colocalization of Kit and ERG1 (Fig. 2A, region ii). In the peptide-preincubated ERG1 antibody control, the proportion of ERG1 pixels colocalized with Kit in the complete muscularis was 4.5 ± 3.4% (SD) (Fig. 2B), and the immunoreactivity in the smooth muscle cells was eliminated. ERG2 was detected within the smooth muscle cells and colocalized with 11.5 ± 5.7% (SD) of Kit-immunoreactive red pixels in the muscularis layer (Fig. 2C), but colocalization was similar (11.4 ± 6.4% (SD)) in the peptide-preincubated ERG2 antibody control (Fig. 2D). This suggested that the low signal in ICC was nonspecific, although the ERG2 signal in the smooth muscle cells was greatly reduced in the peptide-preincubated ERG2 antibody control. ERG3 was located in smooth muscle cells and ICC in the myenteric plexus, with 47.9 ± 12.2% (SD) of Kit-immunoreactive red pixels colocalizing with ERG3 signal in the complete muscularis layer (Fig. 2E). Selection of specific ICC as a region of interest (similar to region ii in Fig. 2A) produced a colocalization value of 95.4 ± 6.7% (SD). With preincubation of the anti-ERG3 antibody with peptide antigen, colocalization of Kit-immunoreactive red pixels was reduced to 7.9 ± 4.5% (SD) (Fig. 2F), and signal was reduced in the smooth muscle cells.

**Effect of E-4031 on electrical properties of the small intestine musculature.** With use of a standard microelectrode to penetrate a smooth muscle cell, the ICC-initiated slow-wave activity was readily observed in a tissue preparation of the

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**Fig. 2.** Ether-a-go-go-related gene (ERG) protein expression in adult mouse jejunum costained with Kit and DRAQ5 nuclear stain. ERG1 and ERG2 were predominant in smooth muscle cells; ERG3 was distributed throughout the muscularis in smooth muscle cells and nerve plexus, including interstitial cells of Cajal (ICC). A, C, and E: jejunum tissue sections immunostained with ERG1, ERG2, and ERG3, respectively, with Kit immunofluorescence and nuclear stain shown in separate and merged images. B, D, and F: sections in which each ERG primary antibody was preincubated with peptide (pep) antigen, with Kit immunofluorescence and DRAQ5. DRAQ5 far-red nuclear stain was pseudocolored blue. Regions i and ii, areas analyzed for colocalization of Kit and ERG1 in complete muscularis or ICC cell body only, respectively. Similar analysis was applied for ERG3, but only region i was analyzed for ERG2. Scale bars, 10 μm.
mouse small intestine musculature (Fig. 3A). The methanesulfonanilide compound E-4031 caused a significant depolarization of the musculature at 1 μM (7 ± 2 mV, n = 6), probably due to block of ERG channels in smooth muscle cells as well as ICC; further depolarization was seen with 2 μM E-4031. In concert with the depolarization, changes in the shape of the slow wave were noted, likely due to effects of E-4031 on ICC; further depolarization was seen with 2 μM E-4031, prolongation of the slow wave became significant and action potentials appeared superimposed on the slow wave. Hence, E-4031-sensitive currents have a marked effect on tissue excitability.

**Electrophysiological evidence of E-4031-sensitive K+ currents.** The whole cell patch-clamp technique was utilized previously on isolated ICC to detect E-4031-sensitive currents in cultured ICC using commonly employed, but nonphysiological, K+ concentrations in the experimental solutions (12, 31). To understand the characteristics of the current under physiological ionic conditions and to compare the currents with other cell types under similar conditions, 5 mM extracellular K+ and 140 mM intracellular (pipette) K+ were used. Under these conditions, depolarization from −60 mV evoked outward current starting at −30 mV that was partially inhibited by E-4031 (Fig. 3B).

The E-4031-sensitive currents at each test potential were obtained by digital subtraction of the current traces in the presence of E-4031 from those in the absence of E-4031 (Fig. 3C). The threshold for activation of outward current was between −30 and −20 mV. Interestingly, the E-4031-sensitive currents (Fig. 3C) did not show dramatic inactivation as seen in some expression systems (19, 21). On repolarization (at the end of the voltage steps), small tail currents, which tend to be very large in expression systems because of recovery of inactivation, were seen in ICC (Fig. 3C). The E-4031-sensitive current between −30 and −50 mV was inward in direction, resulting in a negative slope of the current-voltage (I-V) plot (Fig. 3C).

**K+ selectivity of the ERG-like current.** To assess the K+ selectivity of the E-4031-sensitive currents, current profiles in the presence of 0 and 140 mM extracellular K+ were compared (Fig. 4). The reversal potential was −1.9 ± 3.2 mV (n = 5) with 140 mM K+, in which the expected equilibrium potential for K+ is 0 mV (Fig. 4B). Removal of extracellular K+ moved the reversal potential to less than −80 mV, and the distinct negative slope of the...
E-4031-sensitive I-V plot was no longer present (Fig. 4A). Thus the channel was K⁺ selective, and the negative slope of the E-4031-sensitive I-V plot originated from K⁺ current.

Activation kinetics. The activation kinetics of the E-4031-sensitive current were measured at the start of the depolarizing pulses (Fig. 5). The activation time course between −20 and 40 mV was analyzed by two methods. The first method fitted the rising phase of a current trace as a single-exponential function. The mean current traces (n = 5; Fig. 6A) were fitted to obtain a time constant, which was plotted as a function of membrane potential shown in Fig. 5B. The activation time constants of the E-4031-sensitive currents were voltage dependent, and activation was rapid at more positive voltages. The mean time constants were 13.4 ± 0.6 ms at −20 mV and <10 ms at −10 to 40 mV (n = 7). The second method used the time to peak from the original current trace. The times to peak were also plotted as a function of membrane potential shown in Fig. 5C. The time to peak of the E-4031-sensitive currents varied strongly with membrane potential, in a manner similar to the time constant. Time to peak was 74.4 ± 15.8 ms at −20 mV and decreased to <25 ms at −10 to +40 mV.

The voltage dependence of activation was studied by activation of the E-4031-sensitive currents through 700-ms depolarizing steps to potentials between −30 and +50 mV (Fig. 5D). The voltage dependence was evaluated by measurement of the amplitude of the tail current elicited on return to −50 mV (n = 5), which reflects the degree of activation at the preceding depolarizing test potential. The activation variable was then obtained by normalization of the amplitude of the tail current at each test potential with reference to its peak amplitude at +50 mV and plotted as a function of membrane potential to provide the steady-state activation curve (Fig. 5E).

Inactivation kinetics. To study the voltage dependence of steady-state inactivation of E-4031-sensitive currents, currents were evoked from −80 to 30 mV. Inactivation was allowed for a period of 700 ms; then the voltage was stepped up to 30 mV (Fig. 6A). When the voltage step to 30 mV was launched from −60 mV, significant current was generated; when it was launched from −20 mV, the current evoked was much smaller, indicating significant inactivation. To construct a typical inactivation curve, the peak currents at 30 mV were normalized and plotted vs. the prepulse potentials (the potentials from which the depolarization to 30 mV was launched) to provide the steady-state inactivation curve (Fig. 6B). The inactivation curve was well fitted by a single-exponential Boltzmann equation, with fit parameters as follows: \( V_{1/2} = −40.3 ± 0.5 \text{ mV} \) and \( k = 5.7 ± 0.4 \) (n = 5), where \( V_{1/2} \) is the voltage at which activation is half-maximal and \( k \) is the slope factor.

Deactivation kinetics. Deactivation kinetics were examined by measurement of the voltage dependence of the time course of the E-4031-sensitive current decay. The cells were voltage stepped to 50 mV for 700 ms to fully activate the channel and then stepped to different membrane potentials from −70 to −40 mV, which is the membrane potential range of the slow decay.
wave (Fig. 6C). Time constants of deactivation were determined by fitting a single-exponential equation to the initial current decay at each potential. The initial phase of deactivation is well fitted by a single-exponential equation. Time constants were plotted as a function of the test potential in Fig. 6D. The rate of deactivation was dependent on membrane potential, with the currents decaying more rapidly as the membrane potential became more negative. The time constant was 60 ms at the membrane potentials examined.

DISCUSSION

In the elucidation of ionic components that contribute to the pacemaking electrical slow wave of ICC in the myenteric plexus, it is essential to characterize the electrophysiological and molecular properties of the channels involved. Despite the culture environment, ICC in the neonatal mouse jejunum explant culture produce rhythmic inward currents and express Kit protein, both of which are essential characteristics of ICC in the adult mouse. Thus ICC in newborn mice have the components necessary for pacemaker function that are typical of the adult mouse. The examination of ERG channel expression in adult tissue confirmed that the channels identified functionally at a developmentally earlier stage are present at the molecular level in the mature animal. In the present study, we show that the E-4031-sensitive current in ICC in physiological ionic solutions shares features with the ERG3 isoform of the ERG K⁺ channel family. This finding is consistent with the molecular identification of ERG3 protein by immunoreactivity and erg3 mRNA by in situ hybridization. The identification of erg1 mRNA in ICC suggests that this isoform could contribute to the E-4031-sensitive current, but we could not detect ERG1 protein in ICC, and the electrophysiological characteristics of channel activation, inactivation, and deactivation were different from typical ERG1 features. In ICC, the electrophysiological evidence obtained in physiological solutions suggests that the E-4031-sensitive currents restrict ICC excitability by limiting membrane depolarization and by limiting the duration of the slow-wave potential, thus contributing to the control of muscle excitability.

Under physiological ionic conditions, ICC displayed a marked depolarization-activated E-4031-sensitive current that shared many features with ERG3 currents but differed from typical ERG1 currents. In most cells natively or artificially expressing the ERG1 channel, the currents inactivate immediately on activation, and they recover from inactivation by repolarization. In ICC, the activation is relatively fast, with a time constant of activation of 13 ms at −20 mV; similarly, ERG3 channels expressed in oocytes display a time constant of activation of 33 ms (20), whereas the corresponding values were −200 ms for ERG1 in mouse cardiac cells and 770 ms for ERG1 expressed in HEK 293 cells (30). Time constants for ERG1 and ERG2 currents expressed in oocytes were >300 ms (20). The I-V relationship of many E-4031-sensitive outward currents displays a bell shape, with a maximum at −0 mV, which is thought to be due to strong inactivation of the current at more depolarized potentials (8, 18). The absence of this inactivation phenomenon in ICC is similar to observations in murine portal vein myocytes (16) and subendocardial Purkinje
myocytes (25). Consequently, during the recovery from inactivation, the outward “resurgent” current evoked on hyperpolarization was not as pronounced in ICC as in the ERG1-expressing mouse sinoatrial node cells (25), whereas in *Xenopus* oocytes under physiological ionic conditions the ERG3 channel showed a minor resurgent current compared with ERG1 and ERG2 (20). Another factor contributing to the relatively minor resurgent current was the rapid deactivation (inactivation of the resurgent current) observed in ICC with a time constant of 56 ms at −40 mV, while the comparable deactivation time constants at −40 mV were 119 and 200 ms in guinea pig atrium and HERG-expressing HEK 293 cells, respectively (17, 30). The deactivation time constant of ERG3 currents expressed in Chinese hamster ovary cells was measured at 18.7 ms at −120 mV (28), whereas we measured the time constant at 19.1 ms at −70 mV for the E-4031-sensitive current in ICC (12). The comparable time constants for ERG1 and ERG2 at −120 mV were 74 and 89 ms, respectively (28). Marked E-4031-sensitive currents evoked on hyper- or repolarization are thought to promote action potential repolarization in sinoatrial node cells and, subsequently, increase the frequency of action potentials. This is likely less of a factor in ICC. At the molecular level, there is evidence for ERG1 mRNA in ICC, but no ERG1 protein is detected. These data are consistent with immunofluorescence studies in human jejunum, which also demonstrated strong ERG1 immunoactivity in smooth muscle cells (6). The expression of ERG1 immunoactivity in ICC previously reported using a bright-field immunohistochemical technique (31) could not be confirmed by the present study, likely because of accuracy limitations in serial-section staining compared with the present colocalization immunofluorescence technique. Nevertheless, the presence of ERG1 protein cannot be completely excluded because of the detection of m-erg1 mRNA and a related observation of m-erg1 mRNA expression enrichment in the ICC associated with Auerbach’s plexus (3). Perhaps the translation of the ERG1 transcript could be negatively regulated by a microRNA mechanism to prevent the expression of ERG1 protein in ICC. Alternatively, there is potential for an ERG1 protein in ICC with a unique carboxyl terminus that remains undetectable with commercially available antibodies.

Since smooth muscle cells depolarized from a resting membrane potential of −57 mV in response to E-4031 (Fig. 3A), the activation threshold of the E-4031-sensitive currents in smooth muscle cells is likely lower than that of ICC. Indeed, marked E-4031-sensitive currents were evoked at the resting membrane potentials of smooth muscle cells in esophageal smooth muscle (2). The activation threshold measured in ICC (Fig. 3B), however, was measured at room temperature, and the ICC were isolated from tissue; hence, the value in vivo may be different.

Differences between kinetics of the currents studied here and kinetics studied in other systems may also be due to variation in intracellular factors or other channel subunits. We did not detect the ERG channel β-subunits MiRP1 and MinK, which can alter ERG channel activity (1, 11). Other possible explanations for the differences are recording conditions (temperature, extracellular pH, extracellular cation concentrations, and voltage protocol), which can have important effects on the ERG channel gating (26, 29, 30). In addition, differences in current characteristics may be due to cell-specific regulation of the channel protein through the cyclic nucleotide-binding domain, phosphorylation sites, or the Per-Arnt-Sim domain, which modifies gating characteristics (13, 27).

An interesting feature of the E-4031-sensitive currents observed in ICC was the inwardly directed K+ current between −30 and −50 mV, apparently occurring against the electrochemical gradient and in a time-dependent manner, resulting in a negative slope of the I-V plot (Fig. 3C). Other K+ channel blockers (4-aminopyridine and tetraethylammonium) did not result in a negative slope of the I-V curve of blocker-sensitive current (S. J. Park, unpublished observations). This current could be generated by the Na⁺-K⁺ pump, which can use active transport to produce K⁺ influx against the electrochemical gradient. In general, Na⁺-K⁺ pump currents are outward in direction (23). U87-MG cells exhibited K⁺ influx against the electrochemical gradient (5), which the authors suggested could be due to the absence of Na⁺ in the intracellular solution, with the Na⁺-K⁺ pump functioning as an extracellular K⁺/intracellular K⁺ exchanger (15). K⁺ influx can also occur if the channel is strongly rectifying in this region. In murine portal vein myocytes, the I-V curve of the E-4031-sensitive currents also exhibited this unique negative slope (16). The inward current in ICC occurs at −50 to −30 mV and may contribute to the plateau potential of the slow wave.

This study provides evidence for a unique ERG K⁺ current in ICC that contributes to the characteristics of gut pacemaker activity. Differential ERG channel expression in ICC compared with intestinal smooth muscle cells and cardiac muscle cells may make ERG3 a unique target to regulate ICC excitability without affecting the cardiac action potential. Since it is the pacemaker activity from ICC that propagates into the musculature and, in part, defines muscle excitability, drugs affecting specifically the ERG3 channel in ICC may have potential to optimize prokinetic drug function for patients.

ACKNOWLEDGMENTS
Jing Ye and Yaohui Zhu provided the data presented in Fig. 3A.

GRANTS
The research was supported by Canadian Institutes of Health Research (CIHR) Operating Grant MOP-12874 (to J. D. Huizinga). E. J. White was supported by a CIHR-Canadian Digestive Health Foundation-partnered doctoral research award. S. J. Park was supported by a CIHR-Canadian Association of Gastroenterology postdoctoral fellowship.

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