Clotrimazole-sensitive K$^+$ currents regulate pacemaker activity in interstitial cells of Cajal

Yaohui Zhu, Jing Ye, and Jan D. Huizinga

Intestinal Disease Research Programme, Department of Medicine, McMaster University, Hamilton, Ontario, Canada

Submitted 9 November 2006; accepted in final form 6 February 2007

Zhu Y, Ye J, Huizinga JD. Clotrimazole-sensitive K$^+$ currents regulate pacemaker activity in interstitial cells of Cajal. Am J Physiol Gastrointest Liver Physiol 292: G1715–G1725, 2007. First published March 6, 2007; doi:10.1152/ajpgi.00524.2006.—Interstitial cells of Cajal (ICC) are pacemaker cells for gut peristaltic motor activity. Compared with cardiac pacemaker cells, little is known about mechanisms that regulate ICC excitability. The objective of the present study was to investigate a potential role for clotrimazole (CTL)-sensitive K currents (I_{CTL}) in the regulation of ICC excitability and pacemaker activity. ICC were studied in situ and in short-term culture by using the whole cell patch-clamp configuration. In situ, ICC exhibited spontaneous transient inward currents followed by transient outward currents. CTL blocked outward currents, thereby increasing the net inward currents, and depolarized ICC, thereby establishing CTL-sensitive channels as regulators of ICC pacemaker activity. In short-term culture, a I_{CTL} was identified that showed increased conductance when depolarized from the resting membrane potential to 0 mV and subsequent inward rectification at further depolarized potentials. The I_{CTL} markedly increased with increasing intracellular calcium and was insensitive to the ether-à-go-go-related K channel blocker E-4031 and the large-conductance calcium-activated K channel blocker ibetoxin. I_{CTL} contributed 3–9 nS to the whole cell conductance at 0 mV membrane potential under physiological conditions; it was fast activating (τ = 88 ms), showed little time-dependent inactivation, and exhibited a deactivation time constant of 38 ms. The nitric oxide donor sodium nitroprusside (SNP) increased I_{CTL}. Single-channel activity, activated by calcium and SNP, was inhibited by CTL, with a single-channel conductance of ~38 pS. In summary, ICC generate a I_{CTL} on depolarization through an intermediate-conductance calcium-activated K channel that regulates pacemaker activity and ICC excitability.

MATERIALS AND METHODS

Cell culture and in-situ ICC-Auerbach’s plexus preparation. Explant preparations from the jejunum of CD1 neonatal mice were isolated by sharp dissection without enzymatic digestion as described elsewhere (12, 35). Recordings were obtained from single, mechanically active ICC identified by vital staining with c-kit antibody coupled to Alexa 488 (12) or by morphological criteria before patching and methylene blue staining afterward (12). The superfusion with methylene blue (10–100 mM, 15–40 min) was followed through the microscope and was switched back to physiological solution once the cell stained pink. Washout of methylene blue left the ICC associated with Auerbach’s plexus (ICC-AP) dark due to irreversible accumulation in the sarcoplasmic reticulum. In live tissue, methylene blue selectively accumulates in the sarcoplasmic reticulum of ICC-AP but not ICC associated with the deep muscular plexus (16, 36). Some experiments were conducted in a longitudinal muscle-myenteric plexus preparation, developed by W. A. Kunze and colleagues (20), to record from ICC-AP in intact tissue of the mouse jejunum.

Electrophysiology. Before patch-clamping a cell, offset potentials were eliminated. The transients of the seal formation were minimized by adjustment of the access resistance controls of the amplifier (Axopatch 1D and Axopatch 200B; Axon Instruments, Sunnyvale, CA). The standard whole cell patch-clamp configuration was used on cultured cells that were continuously superfused with modified Tyrode solution (subsequently referred to as the standard bath solution) containing (in mM) 135 NaCl, 5.4 KCl, 2.0 CaCl$_2$, 0.8 MgCl$_2$, 3.0 NaH$_2$PO$_4$, 5.0 HEPES, and 5.5 glucose (pH 7.35 with NaOH). The standard pipette solution contained (in mM) 115 K-gluconate, 25 KCl, 5 NaCl, 1 CaCl$_2$, 10 HEPES, and 2.5 glucose (pH 7.35 with NaOH). The standard pipette solution contained (in mM) 115 K-gluconate, 25 KCl, 5 NaCl, 1 CaCl$_2$, 10 HEPES, 2.5 ATP-Mg, and 1.12 EGTA. In some experiments a 0 mM Mg$^{2+}$ solution was used, containing (in mM) 100 K-aspartate, 30 KCl, 5 NaCl, 1 CaCl$_2$, 10 HEPES, 2.5 ATP-Mg, and 1.12 EGTA. In some experiments a 0 mM Mg$^{2+}$ solution was used, containing (in mM) 100 K-aspartate, 30 KCl, 5 NaCl, 1 CaCl$_2$, 10 HEPES, 2.5 ATP-Mg, and 1.12 EGTA. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: J. D. Huizinga, McMaster Univ., HSC-3N5C, 1200 Main St. West, Hamilton, ON L8N 3Z5, Canada (e-mail: huizinga@mcmaster.ca).
were expressed as means ± SE, where \( n \) is number of cells. The statistical significance of differences in the means was determined by the Student’s \( t \)-test or the ANOVA test for repeated measures wherever appropriate. Differences were considered significant at \( P < 0.05 \). CTL (Sigma) was dissolved in DMSO with bovine serum albumin or dissolved in Tyrode solution by using ultra sonic vibration.

**RESULTS**

**K currents in positively identified ICC.** Isolated, single ICC were obtained from neonatal explants in short-term culture, in a nonenzymatic manner (Fig. 1, A and B). The ICC were identified by their shape as small triangular cells with long

---

**Fig. 1.** Clotrimazole (CTL)-sensitive outward currents (\( I_{\text{CTL}} \)) recorded from interstitial cells of Cajal (ICC) associated with Auerbach’s plexus (AP). A: c-kit-positive ICC in short-term culture (4 days). Recordings from the asterisk-marked cell are shown in D–F. B: other ICC were identified by morphology, recorded from, and identified by methylene blue uptake after recordings were obtained. ICC attached to deep muscular plexus do not accumulate methylene blue (34). C: voltage protocol for current activation. Depolarizing pulses of 400-ms duration were applied from a holding potential of \(-80 \text{ mV} \), stepping from \(-80 \) to \(+100 \) in 20-mV increments. D–F: recordings using standard bath and pipette solutions as indicated in MATERIALS AND METHODS. Note inward rectification at depolarized potentials. These traces are representative of 96% of CTL-sensitive currents obtained. D: in standard bath solution. E: in the presence of 1 \( \mu \text{M} \) CTL. F: \( I_{\text{CTL}} \) (\( E \) subtracted from \( D \)). G: steady-state current-voltage (\( I-V \)) curves of \( D \) (■), \( E \) (●), and \( F \) (▲). H: average values of \( I_{\text{CTL}} \) from 6 experiments. I: conductance-voltage (\( G-V \)) curve, calculated as \( G = I/(V - E_K) \) (\( n = 6 \)).
Voltage steps up to 1100 mV caused a maximum amplitude of 436800 pA when physiological solutions were used (Fig. 1). Evoked outward currents included K currents as described in the present study. The relative contribution of the different channels to the total outward current in ICC was variable, similar to recent observations in neurons (31).

**CTL-sensitive currents in ICC.** Outward currents were evoked on depolarization from a holding potential of −80 mV. Voltage steps up to +100 mV were applied in either 20- or 10-mV increments (Fig. 1C). In 70 out of 185 ICC tested, a I CTL contributed to ±50% of the total outward current with a maximum amplitude of 436 ± 122 pA, ranging from 300 to 800 pA when physiological solutions were used (Fig. 1, D–G). At 0 mV membrane potential, this contributed ~3-9 nS to the whole cell conductance. Of the 70 cells studied, 67 showed a current profile, as shown in Fig. 1D. Addition of CTL inhibited a significant portion of the outward currents (Fig. 1E); a typical profile of I CTL in ICC is shown in Fig. 1F. When current was evoked from −80 to 0 mV, 10 nM CTL inhibited the maximum current by 22 ± 3% (n = 3; P < 0.05), 100 nM CTL inhibited the current by 35 ± 16% (n = 9; P < 0.01), and 1 μM CTL inhibited the current by 78 ± 19% (n = 23; P < 0.01). The I CTL in these cells exhibited a steep current-voltage relationship; that is, the voltage range that saw current develop from small to maximal was narrow, from approximately −40 to approximately 0 mV (Fig. 1, G and H). The whole cell conductance increased on depolarization from −70 to 0 mV and dropped to ~50% of maximal value when further depolarized to 80 mV (Fig. 1F). Hence the I CTL in ICC showed marked inward rectification at depolarized potentials positive to 0 mV. The shape of the curve was not dependent on the anion present (41) nor on Mg2+, through 4-aminopyridine-sensitive currents (Park S, Zhu Y, and Huizing JD, unpublished observations), and through CTL-sensitive currents as described in the present study. The relative contribution of the different channels to the total outward current in ICC was variable, similar to recent observations in neurons (31).

**Fig. 2.** Effect of CTL on spontaneous activity of ICC-A-P. A–C: current-clamp recordings revealed spontaneous rhythmic oscillations in membrane potential. The dotted line indicates −70 mV membrane potential. A: under control conditions, regular slow-wave activity was recorded. B: on addition of 100 nM CTL, depolarization of the cell was observed and the frequency of the slow waves decreased. C: in the presence of 1 μM CTL, the cell depolarized further and prolonged the plateau and duration. The slow waves became reduced in amplitude. D–E: voltage-clamp recordings revealed spontaneous rhythmic inward currents identifying the pacemaker activity of ICC as shown previously (33). The dotted line indicates the baseline of holding current. D: spontaneous rhythmic inward currents followed by transient outward currents when ICC was held at −50 mV with the use of standard solutions. E: 2 min after uninterrupted recording, CTL (1 μM) was added. Note block of outward component and the increase in amplitude of inward current.
Fig. 3. K dependence of mixed whole cell outward currents from ICC exhibiting inward rectification at depolarized potentials. A–C: typical outward current profile of an ICC exhibiting inward rectification at depolarized potentials. Tail currents appeared when E<sub>K</sub> was made more positive by elevating K<sup+</sup> concentration in external solution ([K<sup+</sup>]<sub>e</sub>) to 40 mM (B) and 100 mM (C) by replacing Na<sup+</sup> with K<sup+</sup>. D: I-V relationships obtained by plotting the peak current vs. test potential ([K<sup+</sup>]<sub>e</sub>) (n = 5; ■, 5.5 mM K<sup+</sup>; ●, 40 mM K<sup+</sup>; ▲, 100 mM K<sup+</sup>; n = 4). The current amplitude was taken at 160 ms (at 160 ms). E: plot of log [K<sup+</sup>]<sub>e</sub> vs. reversal potential (E<sub>rev</sub>) (n = 12). Experimental values of E<sub>rev</sub> were 5.5 mM K<sup+</sup>, −82.6 ± 1.5 mV; 10 mM K<sup+</sup>, −61.6 ± 2.3 mV; 40 mM K<sup+</sup>, −29.6 ± 3.3 mV; 100 mM K<sup+</sup>, −13.5 ± 2.3 mV; 140 mM K<sup+</sup>, 2.9 ± 2.4 mV. The solid line represents fitting using the Nernst equation and has a slope of 46.0 ± 2.4 mV per decade. Values are means ± SE. F and G: to obtain reversal potentials using tail currents to avoid potential channel inactivation during depolarizing pulses, a short depolarizing pulse was given (100 ms, +20 mV) followed by repolarizing pulses (200 ms) from −20 to −80 in 20-mV increments. These protocols were executed with [K<sup+</sup>]<sub>e</sub> = 5.5 mM (F) and [K<sup+</sup>]<sub>e</sub> = 100 mM (G). The tail-current amplitude was taken at t = 0 obtained by extrapolation (arrows). H: I-V plot of the tail currents averaged from paired experiments of 7 cells (■, [K<sup+</sup>]<sub>e</sub> = 5.5 mM; ●, [K<sup+</sup>]<sub>e</sub> = 100 mM). The calculated E<sub>K</sub> values were −81.5 and −8.5 mV, respectively, corrected for the junction potential.
with extracellular potassium concentration ([K\text{\textsuperscript{+}}], 5.5 mM (n = 70), close to K\text{\textsuperscript{+}} equilibrium potential (E\text{\textsubscript{K}}) calculated by the Nernst equation. The conductance, as calculated by \( G = I/(V - E\text{\textsubscript{K}}) \) where G is conductance and V is voltage, was 3.9 ± 1.2 nS at 0 mV.

Of the 70 cells, 3 exhibited a current profile that did not show inward rectification at depolarized potentials. CTL (1 \( \mu \text{M} \)) inhibited the current by 56.8 ± 9.4%; the current-voltage relationship of this CTL-sensitive current was linear positive to −40 mV. Experiments shown in the remainder of this study do not pertain to this current profile.

ICC that showed inward rectification at depolarized potentials did not significantly respond to a combination of IbTX (1 \( \mu \text{M} \); blocking BK\text{\textsubscript{Ca}}) and E-4031 (1 \( \mu \text{M} \); blocking ERG channels) but showed inhibition by 1 \( \mu \text{M} \) CTL of 48 – 87% (n = 7), indicating that the I\text{\textsubscript{CTL}} was not sensitive to IbTX and E-4031. Ba\text{\textsuperscript{2+}} reduced I\text{\textsubscript{CTL}}, and quantification at a pulse potential of +20 mV showed inhibition by 44 ± 18 and 90 ± 3% at 30 and 100 \( \mu \text{M} \), respectively (n = 5).

The physiological significance of I\text{\textsubscript{CTL}}. The significance of the I\text{\textsubscript{CTL}} for the electrophysiology of ICC was revealed by the effects of CTL on spontaneous rhythmic currents (voltage clamp) or associated rhythmic depolarizations (current clamp) in ICC from the longitudinal muscle-myenteric plexus preparation. ICC-AP were identified at the edges of the myenteric plexus ganglia, aided by staining with the use of c-kit antibody (ACK2) or methylene blue.

Rhythmic inward currents were recorded for 4–90 min, and thereafter the activity diminished, presumably because of washout of essential intracellular components. In the experiment shown in Fig. 2, D and E, rhythmic inward currents were maintained for 60 min at −50 mV. The inward currents were followed by “rebound” outward currents. Addition of CTL (1 \( \mu \text{M} \)) abolished the outward currents and increased the ampli-

![Fig. 4. Ca\text{\textsuperscript{2+}} dependence of mixed whole cell outward currents from ICC exhibiting inward rectification at depolarized potentials. A: chelating intracellular calcium by using BAPTA-AM (5 mM). a: A ramp protocol was employed as indicated. The holding potential of −60 mV was held for 450 ms, then the voltage ramp started at −120 and developed to +120 mV over 1 s. b: After exposing the cell to BAPTA-AM, within 3 min the magnitude of outward current decreased. Recording shown was obtained at 5 min. c: a and b superimposed. B: changing extracellular calcium. a: Current profile obtained by using standard solutions, but with extracellular Ca\text{\textsuperscript{2+}} concentration ([Ca\text{\textsuperscript{2+}}], 2 mM. b: [Ca\text{\textsuperscript{2+}}] changed to 10 nM, which caused marked reduction in current amplitude. c: [V\text{\textsubscript{Ca}}] curves from 4 cells (\( \bullet \), [Ca\text{\textsuperscript{2+}}] = 2 mM; \( \bullet \bullet \), [Ca\text{\textsuperscript{2+}}] = 10 nM). d: Control recordings from a different series of experiments with [Ca\text{\textsuperscript{2+}}] = 2 mM. e: Increase in [Ca\text{\textsuperscript{2+}}] to 10 mM did not further increase current amplitude but resulted in time-dependent inactivation. f: Comparison of current profiles using [Ca\text{\textsuperscript{2+}}] = 2 and 10 mM evoked by depolarization from 0 and +80 mV. Time constants of current decay were determined by using single exponential fitting (n = 7).]
tude of the inward currents by 10–30% (Fig. 2E; n = 4; P < 0.05). Hence CTL-sensitive K+ currents influenced the amplitude of the pacemaker currents. When ICC were recorded in current clamp, typical slow-wave activity was recorded from a membrane potential of −70 ± 6.2 mV, the frequency of the slow wave was 14 ± 6 cycles/min, the amplitude was 27 ± 11 mV, and the duration was 2.2 ± 1.2 s. CTL (1 μM) depolarized the cells 10 ± 2 mV, the plateau duration of slow waves increased to 4 ± 2 s, and the frequency decreased to 6 ± 3 cycles/min (Fig. 2, A–C; n = 5).

K and Ca dependence of total mixed whole cell outward currents in ICC with a marked inward rectification at depolarized potentials. In 12 ICC that showed marked inward rectification at depolarized potentials, each isolated from different cultures, the K+ and Ca2+ dependency of the outward currents was investigated (Fig. 3A). In the absence of K-channel blockers, the total current reversal potential (Erev) shifted in the positive direction when the [K+]o was increased from 5.5 mM (−81.5 ± 6.2; Fig. 3A) to 40 mM (−31.4 ± 4.0; Fig. 3B) and 100 mM (−8.5 ± 1.8 mV; Fig. 3C), all close to the expected EK values; hence the outward currents were primarily carried by K+ ions. A plot of Erev vs. [K+]o was linear; a 10-fold change in [K+]o induced an Erev shift of 46 ± 4.2 mV (n = 12; Fig. 3E). To minimize inaccuracies from potential-channel inactivation at depolarized potentials, reversal potentials were also calculated by using the tail currents of the current profiles at [K+]o = 5.5 mM (Fig. 3F), 40 mM (not shown), and 100 mM (Fig. 3G). The calculated reversal potentials were −81.0 ± 2.1 (5.5 mM [K+]o), −27.0 ± 1.7 (40 mM [K+]o), and −10.7 ± 2.4 mV (100 mM [K+]o) (n = 15; Fig. 3H). EK values obtained by using the Nernst equation were −81.5, −31.5, and −8.4 mV, respectively.

Ramp protocols were employed to study the effect of reducing intracellular Ca2+ by BAPTA-AM (5 mM, Fig. 4Aa). The holding potential was kept at −60 mV for 450 ms, followed by the voltage ramp starting at −120 up to 120 mV over 1 s. After exposing the cell to BAPTA-AM for 3 min, the magnitude of outward currents decreased 33 ± 10% at 0 mV (n = 6; Fig. 4A, b and c). Reducing extracellular Ca2+ also had a marked effect on current amplitude. Compared with the response to depolarizing voltage steps at a physiological extracellular Ca2+ concentration ([Ca2+]o) of 2 mM (Fig. 4Ba), a [Ca2+]o of 10 nM significantly reduced the current amplitude (n = 5; Fig. 4B, b and c). Increasing [Ca2+]o from 2 mM (Fig. 4Bd) to 10 mM did not increase this current (Fig. 4Be), although the time constant of inactivation decreased markedly (n = 4; Fig. 4Bf).

Isolation of I CTL. Within the mixed outward currents, the I CTL was almost exclusively a K current, investigated by studying reversal potential changes of I CTL in response to changes in extracellular K+, using tail-current protocols (Fig. 5, A and B). The tail currents revealed a reversal potential of −72 ± 2.1 mV with [K+]o = 5.5 mM, −14 ± 1.7 mV with

Fig. 5. K dependence of I CTL. Reversal potentials at different extracellular K+ concentrations were obtained by using tail-current measurements evoked by applying a short, depolarizing pulse (100 ms) to +20 mV followed by repolarizing pulses (200 ms) from −20 to −80 in 20-mV increments. CTL-sensitive currents were obtained by subtraction. A: I CTL at [K+]o = 5.5 mM. B: I CTL at [K+]o = 100 mM. C: I-V plot of tail currents measured by extrapolation of the tail currents to t = 0 of repolarization (n = 4). A, [K+]o = 5.5 mM; ◆, [K+]o = 100 mM. The calculated EK values were −86.3 mV and −12.5 mV, respectively, corrected for the junction potential. D: deactivation time constants (τ) obtained from CTL-sensitive tail currents. □, [K+]o = 5.5 mM; ◆, [K+]o = 100 mM.
[K⁺]₀ = 40 mM, and −10.9 ± 2.4 mV with [K⁺]₀ = 100 mM [K⁺]₀ (Fig. 5C). The time constant of activation from −60 mV to +20 mV was 87.5 ± 8.0 ms, ranging from 79 to 96 ms at 40 mV (n = 4). Repolarization to different potentials revealed tail currents (extrapolated to t = 0 of repolarization) that showed a deactivation time constant of 36.7 ± 6.5 ms when the potential was switched from +20 to −80 mV (Fig. 5D). The time constant was voltage sensitive (Fig. 5D); it was 120 ± 5 ms when the tail current was assessed with a voltage drop from +20 to −20 mV. The time constant of deactivation of ERG currents, studied during this same period, was 7.4 ± 4.8 ms (n = 7).

Pacemaker ICC undergo rhythmic changes in intracellular calcium (39, 46). To elucidate the role of intracellular Ca²⁺ in channel activation, ICC cells were patched twice by using different pipettes with a different pipette (intracellular) solution. Attempts to do this succeeded on 12 cells. The extracellular solution contained 2 mM Ca²⁺. The first pipette contained an intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) of ~10 nM obtained by using EGTA (11.2 mM; Fig. 6Aa) or BAPTA (2 mM). The second pipette contained [Ca²⁺]ᵢ = 1 µM (Fig. 6Ab). Outward currents were markedly increased with increase of intracellular calcium (Fig. 6Ac). In six cells, the seal was maintained long enough to assess the effect of CTL. CTL inhibited the calcium-induced current by 40.0 ± 12.6% (P < 0.01). Consistent with these results, the calcium ionophore A-23187 (10 µM) caused a time-dependent increase in the outward currents (Fig. 6B, a–c). The A-23187-induced currents were sensitive to CTL (Fig. 6B, d and e).

Effect of nitric oxide on IᵣCTL. Nitric oxide is an important second messenger for ICC that may increase intracellular calcium, thereby influencing K conductance (25). ICC also have synapse-like contact with nitricergic nerves, indicating a potential role for nitricergic nerves in affecting K⁺ currents in ICC. The nitric oxide donor sodium nitroprusside (SNP; 100 µM) had a marked effect on the amplitude of depolarization-activated IᵣCTL (Fig. 7). Under physiological conditions (standard solutions), SNP (100 µM) increased the outward currents (Fig. 7B) by 162 ± 66% at +40 mV (n = 10; P < 0.001). IbTX and E-4031 reduced currents in the presence of SNP by <10%. Subsequent addition of CTL (1 µM) strongly inhibited the currents (Fig. 7, D and E; n = 7; P < 0.05) at 0 mV.

![Image](http://www.stanford.edu/)

**Fig. 6.** Sensitivity of IᵣCTL to intracellular calcium. A: sequential patching with intracellular Ca²⁺ concentrations ([Ca²⁺]ᵢ) of 10 nM and 1 µM. In 12 cells, with a current profile that showed inward rectification at depolarized potentials, it was possible to make 2 sets of whole cell recordings on the same cell with different pipettes sequentially, 1 with [Ca²⁺]ᵢ = ~10 nM and the other with [Ca²⁺]ᵢ = 1 µM. The free [Ca²⁺], was determined by using the Max-chelator program (http://www.stanford.edu/~cpatton/maxch.html). a: IᵣCTL using standard solutions except an intracellular pipette [Ca²⁺]ᵢ = ~10 nM. b: IᵣCTL using the second pipette with [Ca²⁺]ᵢ = 1 µM. c: I-V relationship with free [Ca²⁺]ᵢ = 10 nM (■) and [Ca²⁺]ᵢ = 1 µM (▲) (n = 12). B: increase in intracellular calcium using the calcium ionophore A-23187. a: Control recordings with pipette [Ca²⁺]ᵢ at ~10 nM with the use of 1.5 mM EGTA. b: 1 min after application of A-23187 (10 µM). c: 10 min after application of A-23187 (10 µM). d: Recordings obtained in c were followed by the application of CTL (1 µM). e: I-V-relationships with control (■), A-23187 (●), and A-23187 followed by CTL (▲) (n = 4).
CTL-sensitive single-channel activity. When cells were superfused with standard solution in which calcium was omitted, single-channel activity could be occasionally discerned in the whole cell recording (Fig. 8A). The single-channel conductance was $32 \pm 2 \text{pS}$ ($n = 6$). Addition of extracellular calcium evoked an increase in channel activity (Fig. 8B) without changing the single-channel conductance significantly ($38 \pm 5 \text{pS}; n = 6$; Fig. 8D). Thereafter, SNP was added and a marked increase in single-channel activity was observed (Fig. 8C; $n = 4$). The single-channel conductance in the presence of SNP was $39 \pm 2 \text{pS}$.

In a separate series of cell-attached experiments, single-channel activity was observed in the presence of high K (140 mM) in bath and pipette by using ramp protocols (Fig. 8, E and F) and pulse protocols (Fig. 8, G–J). SNP invariably increased single-channel activity, which showed a single-channel conductance of $40 \pm 2 \text{pS}$ that was inhibited by CTL (1 $\mu$M; $n = 5$). A typical experiment illustrating the effects of CTL in the cell-attached configuration, followed by omission of Ca$^{2+}$ in the inside-out configuration, is presented in Fig. 8, G–J. The single-channel currents were detected in the cell-attached configuration during an uninterrupted 2-min recording at different holding potentials and revealed a unitary conductance of 37.9 pS. CTL (1 $\mu$M) inhibited the single-channel activity (Fig. 8H). Channel activity recovered after switching to the inside-out configuration with washout of CTL; in subjecting the membrane patch to a solution without Ca$^{2+}$ but with EGTA (1 mM), the open probability reduced from 0.64 to 0.17 (Fig. 8J).

DISCUSSION

Isolated ICC in short-term culture associated with explants (48) and ICC in situ (present study), as well as chemically isolated ICC after short-term culture (15), show spontaneous rhythmic inward currents in voltage clamp and rhythmic voltage oscillations in current clamp. These rhythmic inward currents are often followed by a transient rebound outward current as shown in Fig. 2. This outward current was blocked in a dose-dependent manner by CTL, indicating that an $I_{\text{CTL}}$ is an important component of pacemaker activity in ICC. Indeed, CTL depolarized the ICC, prolonged the slow-wave duration, and increased the amplitude of the net inward currents, indicating that $I_{\text{CTL}}$ has a strong influence on the excitability of ICC. $I_{\text{CTL}}$ markedly increased when the intracellular calcium...
concentration was increased, and single-channel activity indicated the channel to be intermediate (~38 pS) in conductance; hence $I_{\text{CTL}}$ in ICC is generated by an intermediate-conductance K$_{\text{Ca}}$. The $I_{\text{CTL}}$ is small at membrane potentials of approximately ~50 mV, where the slow-wave plateau potential resides. However, with an estimated input resistance of ICC of 1 GΩ, small currents can have a physiologically significant effect: a 4-pA current can change the membrane potential 4 mV. The kinetics of the single-channel activity needs further study. The ion channels that are active in ICC are beginning to be identified, but little is still known about K channel activity (6, 29). It was noted that dialyzing calcium into ICC caused a persistent outward current, probably due to Ca-activated K channels expressed by ICC (29). The present study suggests that this may in part be mediated by a CTL-sensitive K$_{\text{Ca}}$ current. Immunohistochemical evidence suggests that ICC also harbor the BK$_{\text{Ca}}$ (5). In addition, there is a strong presence of the ERG K channel (21, 48). The present study concludes that in ~40% of ICC, the CTL-sensitive K$_{\text{Ca}}$ is dominant. The presence of a dominant K$_{\text{Ca}}$ can be predicted when the current-voltage curve shows inward rectification at depolarized potentials.

The electrophysiological basis for pacemaker activity in ICC is likely dominated by calcium-activated ion channels. ICC

Fig. 8. CTL-sensitive single-channel activity and effect of SNP. Recordings were obtained from ICC that generated whole cell outward currents with inward rectification at depolarized potentials. When total current amplitude was small, on occasion, single-channel activity was seen superimposed on the whole cell currents that increased on increase in [Ca$^{2+}$]$_{\text{o}}$ or SNP. Channel activity is shown in response to depolarizing pulses from ~80 to ~30, ~10, 30, and 50 mV. Standard bath solution except for [Ca$^{2+}$]$_{\text{o}}$. Pipette solution (in mM) was 115 K-gluconate, 25 KCl, 5 NaCl, 1 CaCl$_2$, 10 HEPES, 2.5 ATP-Mg, and 11.2 EGTA. A, top: control recordings with [Ca$^{2+}$]$_{\text{o}}$ = 1 μM. Middle: single-channel activity at ~20 mV. Bottom: amplitude histogram obtained with Axon Instruments software (Pclamp). The unitary conductance was calculated to be 36 pS as the slope of the relationship between single-channel amplitude and membrane potential. B, top: on increase in [Ca$^{2+}$]$_{\text{o}}$ to 100 μM, single-channel activity was seen with most voltage steps. Middle: single-channel activity at ~20 mV. Bottom: amplitude histogram; the unitary conductance was calculated to be 35.5 pS as the slope of the relationship between single-channel amplitude and membrane potential (D). C, top: this same cell responded to 100 μM SNP with increased channel activity, superimposed on increased whole cell currents. Middle: single-channel activity at ~20 mV. Bottom: amplitude histogram. Unitary conductance was calculated to be 36 pS. D: relationship between single-channel amplitude and membrane potential under conditions of given in B, E: ramp protocols in presence of 135 mM KCl in bath. Voltage-command ramps from ~90 to 90 mV were applied for 1.5 s, which elicited CTL-sensitive currents in the on-cell configuration. SNP caused marked increase in single-channel activity that was inhibited by CTL (1 μM). F: current traces of E shown separated. G–J: experiment conducted with high-K (140 mM) bath and pipette solutions. Traces shown occurred at a holding potential of ~80 mV in the cell-attached (G and H) and inside-out (I and J) configurations obtained consecutively from the same cell. G: control recording. H: CTL 1 μM. I: [Ca$^{2+}$], 10 μM. J: omission of [Ca$^{2+}$], + 1 mM EGTA. For all traces, bars represent 6 s and 8 pA.
harbor cytosolic Ca\(^{2+}\) oscillations that are synchronous with the spontaneous cyclic membrane depolarizations (39, 46). Membrane depolarizations are likely associated with calcium-activated chloride channels (12, 38, 50) as well as nonselective cation channels activated by a decrease in intracellular calcium (13), possibly mediated by ion channels from the transient receptor potential family (43). The effect of CTL on the pacemaker activity suggests that CTL-sensitive KCa channels influence the duration and frequency of the pacemaker activity. CTL was recently noted to affect TRPM2, a member of the transient receptor potential ion-channel family (10). However, the concentration of CTL needed to have this effect was much higher than needed for blockade of IKCa, and the effect was irreversible. The effect of CTL described in the present study was reversible.

It is of significant importance that nitric oxide can modulate the IKCa in ICC as reported in the present study. Not only does this indicate that nitric oxide as a neurotransmitter can reduce ICC excitability by increasing the activity of IKCa, but it also indicates that nitric oxide as an intracellular second messenger in ICC (5) can regulate ICC excitability. Nitric oxide may act directly or through increase in intracellular calcium (25). Inhibitory innervation of pacemaker ICC was shown in the canine colon (11) and was shown to be mediated by intramuscular ICC in the mouse small intestine (2); the present study helps clarify the mechanism of nitric oxide-mediated inhibition. It is not uncommon that K channels can be modified by nitric oxide (3, 28, 30). Stretch-dependent K channels (not activated by calcium) in murine colonic smooth muscle cells also were activated by SNP (14). Nitric oxide also suppressed activity of a calcium-stimulated chloride current in smooth muscle cells of the opossum esophagus (47). Hence it is likely that both nitric oxide and Ca\(^{2+}\) regulate pacemaker-associated ion channels in an interdependent manner. The overall effect of release of nitric oxide into the muscle layers of the small intestine will also be mediated by actions of nitric oxide on smooth muscle cells and ICC associated with the dorsal motor plexus.

The molecular nature of the KCa channel(s) in ICC is not yet known. It is likely that a variety of KCa channels are blocked by CTL. In many cell types, mostly nonexcitable cells, the KCa channel is voltage independent (32); in other cells, such as PC-12 cells (26) and breast cancer cells (MCF-7) (23), it is an outwardly rectifying channel. In mouse ileum smooth muscle cells, an intermediate-conductance K channel was shown to be Ca dependent with a unitary conductance of 40 pS (42). This study measured single-channel activity; since the open probability increased with depolarization, it appeared to be outwardly rectifying. Depolarization increased both open probability and mean open time of calcium-activated potassium channels in single smooth muscle cells of rabbit jejunum and guinea pig mesenteric artery (1). The importance of calcium-activated K channels in enteric nerves has long been recognized (4). A CTL-sensitive IKCa in myenteric sensory neurons in the mouse appeared to be poorly voltage sensitive (20). Its function is likely to mediate the slow afterhyperpolarization (20, 22). IKCa is also present in visceral sensory afferent neurons (9), where it is suggested to be voltage independent. Interestingly, in the present study, in three cells, an I\text{CTL} was present that had a linear current-voltage relationship positive to \(-40\) mV. However, in the vast majority of ICC, I\text{CTL} increased in amplitude on depolarization, starting at approximately \(-40\) mV, and showed inward rectification at depolarized potentials positive to 0 mV. Inward rectification at depolarized potentials is a property of several ion channels. Canine ERG channels studied in transfected HEK-293 cells (44) revealed depolarization-inducing inward rectification. The I\text{CTL} identified in the present study was not sensitive to E-4031, and ERG currents studied in ICC did not show inward rectification at depolarized potentials (Ref. 21 and Park S and Huizinga JD, unpublished observations).

There is little doubt that ICC are linked to the pathophysiology of gut motor dysfunction (18, 37). In tissue from numerous patients with a variety of motor disorders, ICC have been shown to be injured and/or decreased in number (27). Mechanistically, however, little is still known about the role that ICC might play in pathophysiology. Of interest is a recent publication that showed ICC of the human colon to be highly immunopositive for the small-conductance calcium-dependent K channel (SK3) (24). SK3 immunopositivity was markedly reduced in ICC in patients with Hirschsprung’s disease (24). Because of the importance of KCa channels in the regulation of excitability in ICC (present study), nerves (20), and smooth muscle (6), further studies may reveal a role for IKCa in the pathophysiology of intestinal motor disorders.

In summary, the present study demonstrates that an I\text{CTL} is present in ICC that is characterized as an intermediate-conductance KCa channel. A defining characteristic is a strong inward rectification at depolarized potentials. I\text{CTL} in ICC may be an important regulator of ICC excitability and pacemaker activity and is strongly affected by voltage, calcium, and nitric oxide.

ACKNOWLEDGMENTS

We have highly appreciated discussions with Dr. Wolfgang Kunze.

GRANTS

This research was supported by operating grants from the Canadian Institutes of Health Research.

REFERENCES


