Interstitial cells of Cajal generate a rhythmic pacemaker current

LARS THOMSEN, TIM L. ROBINSON, JONATHAN C.F. LEE, LAURA A. FARRAWAY, MARTIN J.G. HUGHES, DAVID W. ANDREWS & JAN D. HUIZINGA

Departments of Biomedical Sciences and Biochemistry, and the Intestinal Disease Research Program, McMaster University, Hamilton, Ontario L8N 3Z5, Canada
Correspondence should be addressed to J.D.H.; e-mail: huizinga@mcmaster.ca

Networks of interstitial cells of Cajal embedded in the musculature of the gastrointestinal tract are involved in the generation of electrical pacemaker activity for gastrointestinal motility[1,2]. This pacemaker activity manifests itself as rhythmic slow waves in membrane potential, and controls the frequency and propagation characteristics of gut contractile activity[3–6]. Mice that lack a functional Kit receptor fail to develop the network of interstitial cells of Cajal associated with Auerbach's plexus in the mouse small intestine[7,8] and do not generate slow wave activity[9,10]. These cells could provide an essential component of slow wave activity (for example, a biochemical trigger that would be transferred to smooth muscle cells), or provide an actual pacemaker current that could initiate slow waves. Here we provide direct evidence that a single cell, identified as an interstitial cell of Cajal by light microscopy, electron microscopy and expression of *Kit* mRNA, generates spontaneous contractions and a rhythmic inward current that is insensitive to L-type calcium channel blockers. Identification of the pacemaker of gut motility will aid in the elucidation of the pathophysiology of intestinal motor disorders, and provide a target cell for pharmacological treatment.

We isolated interstitial cells of Cajal (ICC) from the Auerbach's plexus area of the mouse small intestine (Fig. 1). ICC are mesenchymal cells[11,12] with myoid, smooth-muscle-like features[3]. They are single-layer networks in most neural plexuses associated with the gastrointestinal musculature, and three-dimensional networks within some smooth muscle layers. They are characterized in tissue by a small, often triangular or stellate-shaped cell body with several long processes branching out into secondary and tertiary extensions; these processes often contact smooth muscle cells. Isolated ICC, after three to four days in culture, resemble ICC *in situ*[3,5]. We confirmed the presence of ICC in culture by electron microscopy (Fig. 2), the standard for identification of ICC (refs. 3,5). The processes of the ICC showed a dominant presence of intermediate filaments, and contained numerous condensed type mitochondria and plasma membrane caveolae, which are established structural criteria for identification of ICC (ref. 2).

The ICC had distinct patterns of spontaneous rhythmic contractile activity, shown in video format (see *Nature Medicine* home page [http://medicine.nature.com]; Cajul rhythm). Contractile activity involved cell bodies and/or cell processes. Localized contractions in ICC may reflect local release of calcium from intracellular stores. Sarcoplasmic reticulum, associated with the plasma membrane, is very prominent in ICC and is thought to be involved in pacemaker activity[13].

To prove that the morphologically identified cells from which we recorded electrical activity were ICC, we used single-cell RT-PCR to demonstrate *Kit* mRNA expression in the identified cells, as ICC have the Kit tyrosine kinase membrane receptor[7,8,14,15]. *In situ* hybridization experiments in tissue localized *Kit* mRNA exclusively to the ICC within the musculature[7]. We did a series of single-cell RT-PCR experiments using mRNA harvested from morphologically identified ICC, intestinal smooth muscle cells, and from a P815 mast cell line used as a positive control[16] (Fig. 1). Agarose gel electrophoresis of the RT-PCR products of the mast cells and seventeen morphologically identified ICC revealed the 258-bp *Kit* mRNA band, indicating expression of *Kit* mRNA. Intestinal smooth muscle cells did not express *Kit* mRNA; this also showed that these experiments were not contaminated by the *Kit* product, and thus the bands found were not false positives.

We investigated this distinct cell type with the patch clamp technique, and found a spontaneous rhythmic inward current in 30 of 38 cells (Figs. 1*c* and 3*a*). The average current amplitude was 32 ± 8 pA. The frequency of the spontaneous inward current was always very regular, with an inter-cycle period of about 5 seconds, or a frequency of 12.1 ± 1.5 cycles per minute at 20 °C. Although slow wave activity in tissue of the mouse small intestine can be as high as 50 cycles per minute we found that the frequency slows down to about 10–15 cycles per minute at room temperature (J. Malysz and J.D.H, unpublished observations). Neither nifedipine (1 to 10 μM) nor verapamil (1 μM) affected the spontaneous inward current. Tissue experiments have provided indirect evidence that the upstroke of the slow wave is generated by inward currents that are insensitive to L-type calcium channel blockers[17,18].

During recording of the spontaneous inward current, the amplifier was switched to the current clamp mode, and spontaneous oscillations in the membrane potential were observed at the same frequency as the transient periodic inward current (Fig. 3). These data indicate that ICC can generate a spontaneous rhythmic current and slow waves' in the membrane potential.

The reversal potential of the spontaneous repetitive inward current was determined to be +10 mV (Fig. 3). The equilibrium potential for chloride was –75 mV, so it probably did not contribute to the inward current. The equilibrium potentials for the cations present in the intra- and extracellular solutions were: E_{Na} = +73 mV, E_{Cs} = –100 mV and E_{Ca} » +150 mV. This indicates that the inward current is carried by a mixture of cations. The I/V curve shows a linear relationship between membrane potential and current amplitude, indicating that the current amplitude is only related to the driving force of the ions (equilibrium potential minus membrane potential). Thus, the initiation or activa-

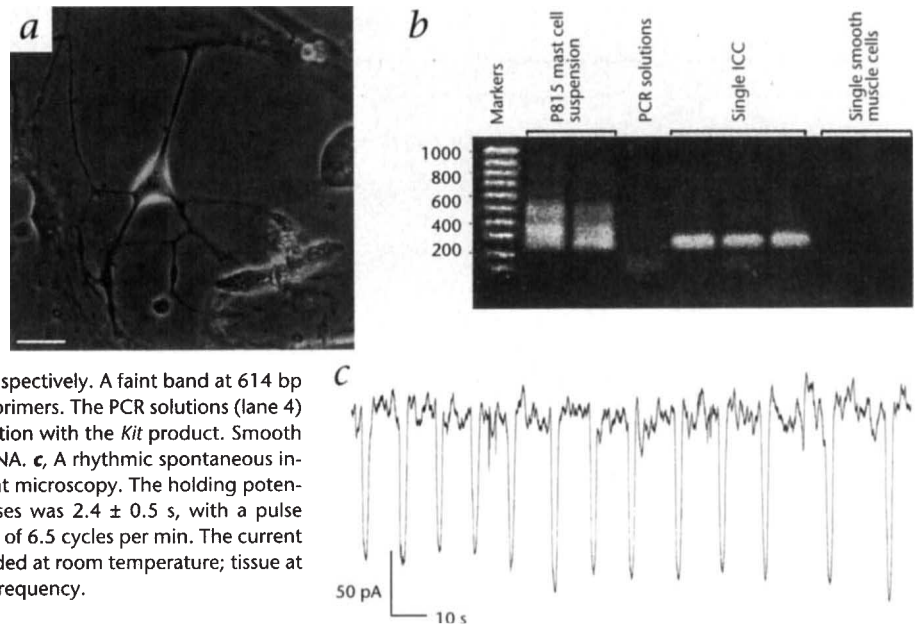

Fig. 1 Rhythmic pacemaker currents were recorded from isolated ICC identified by morphology and by the presence of *Kit* mRNA. ***a***, A morphologically identified interstitial cell of Cajal that was contracting spontaneously (see http://medicine.nature.com). Bar, 20 μm. ***b***, Morphologically identified ICC express *Kit* mRNA. Agarose gel electrophoresis of single-cell RT-PCR products (lanes 5, 6 and 7) revealed the 258 bp *Kit* mRNA band. A cell suspension from a p815 mast cell line (lanes 2 and 3) also produced the *Kit* mRNA band, as well as other bands, which may be alternatively spliced mRNA products at 525 bp and 348 bp, indicating excision of the 267 bp intron alone, and the 89 bp intron alone, respectively. A faint band at 614 bp may correspond to genomic DNA from the nested primers. The PCR solutions (lane 4) were amplified to demonstrate a lack of contamination with the *Kit* product. Smooth muscle cells (lanes 8 and 9) did not express *Kit* mRNA. ***c***, A rhythmic spontaneous inward current generated by an ICC identified by light microscopy. The holding potential was –80 mV. The duration of the current pulses was 2.4 ± 0.5 s, with a pulse interval of 6.9 ± 0.7 s, which resulted in a frequency of 6.5 cycles per min. The current amplitude was 100.7 ± 5.2 pA. This activity is recorded at room temperature; tissue at this temperature generates slow waves at a similar frequency.

tion of the inward current is voltage insensitive. This is the first time that such an inward current has been described in ICC. The study of the ionic basis of the tissue pacemaker activity had predicted the existence of such a current as the basis for the repetitive slow wave. Ion substitution and pharmacological experiments, both in the dog colon[17,18] and the mouse small intestine[19] indicated that the depolarizing phase of the slow wave was initiated by an inward current through a non-L-type calcium channel, or a non-specific cation channel. In addition, the slow waves occurred over a wide range of voltages, with little effect on frequency, indicating that the pacemaker current was not activated by a voltage change[20,21]. Thus, the spontaneous inward current generated by ICC observed here has the characteristics of a pacemaker current.

Other attempts to isolate ICC include a study of interstitial cells of the dog colon, in which an L-type calcium channel was identified which differed in kinetics from those of smooth muscle cells[22]. Although these experiments demonstrated some properties of these interstitial cells, they did not reveal a pacemaker current that must be insensitive to L-type calcium channel blockers[4,5]. *Kit*-positive cells were isolated from the mouse small intestine and were shown to have spontaneous chloride currents, but no voltage-activated Ca^{2+} currents[23]. Chloride currents are unlikely to represent pacemaker activity, as tissue data on slow waves have not demonstrated an essential role for chloride currents[19]. Furthermore, Tokutake *et al.*[23] tried to identify living isolated ICC by immuno-histochemical coupling to a Kit antibody FITC complex. However, this procedure leads to prominent staining of macrophages in the same culture because of uptake of the antibody-FITC complex (L.A.F. and J.D.H., unpublished data). Consequently, the essential step of positive identification of the cells studied was not achieved. We have demonstrated here that single isolated ICC, positively identified by morphology and presence of *Kit* mRNA, exhibit a spontaneous rhythmic inward current which has characteristics of the pacemaker current underlying tissue slow wave activity.

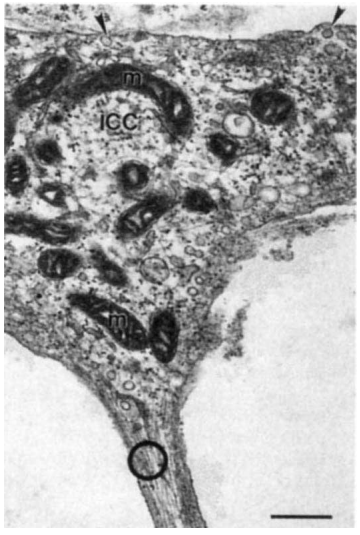

Fig. 2 Electron microscopic characterization of part of an isolated interstitial cell of Cajal from a four-day cell culture from the mouse small intestine, with focus on one process, showing a unique combination of ultrastructural characteristics. The cell membrane contains caveolae (arrowheads). The cytoplasm is characterized by the presence of numerous electron-dense mitochondria (m) and an aligned group of intermediate filaments (circle). Bar, 500 nm.

Methods

Cell isolation. Smooth muscle cells and ICC were isolated from the proximal small intestine of the mouse. The isolated external muscle layers were incubated in calcium-free HEPES-buffered saline solution with 1mg/ml trypsin (Sigma) for 45 min. Thereafter, incubation was in HEPES-buffered saline solution with 0.1 mM $CaCl_2$,1 mg/ml collagenase (Sigma) and 1mg/ml BSA (Sigma) for 20 min. The cells were then released by shaking, centrifuged and plated on coverglasses coated with rat-tail collagen, in M199 (10% fetal bovine serum (Canadian Life Technologies, Burlington Ont., Canada). The P815 mast cell line was purchased from American Type Culture Collection.

Electrophysiology. Patch clamp experiments were done in standard whole-cell configuration, with a pipette solution containing 130 mM Cs-gluconate, 10 mM EGTA, 5 mM HEPES, 10 mM NaCl, 4.5 mM ATP-Mg, and 0.1 mM GTP, adjusted to pH 7.35 with NaOH. The external solution contained 160 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 5 mM HEPES and 3 mM CsCl, adjusted to pH 7.35 with NaOH. The pipette resistance was 8–10 MΩ. Lowering the pipette resistance increased the rundown. With electrodes of 2–2.5 MΩ, no spontaneous activity was observed, and electrodes of 3–5 MΩ produced oscillations that seldom lasted longer than 30 seconds. Some ICC were connected to smooth muscle cells and the recorded activity could have been generated by the smooth muscle cells. However, the branches of the ICC often withdrew upon electrode attachment, and spontaneous activity was then

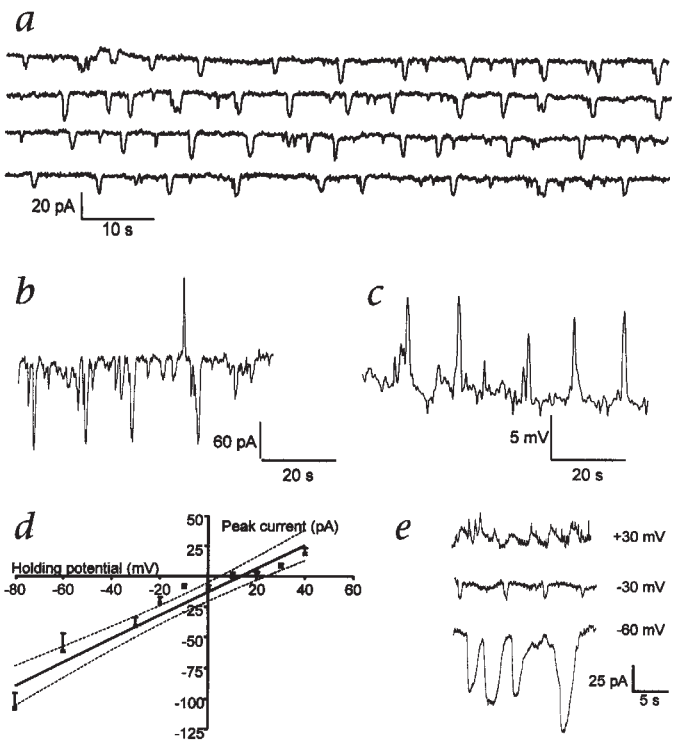

Fig. 3 Electrophysiology of isolated ICC. ***a***, Whole-cell currents recorded from a morphologically identified interstitial cell of Cajal, depicting spontaneous current oscillations every 5–10 seconds. The spontaneous rhythmic inward current continued for as long as 30 min. The holding potential was –30 mV. ***b***, Spontaneous voltage and current oscillations from a single ICC. In the same cell, successive voltage and current clamp recordings were made in the continued presence of 5 μM nifedipine. While the cell is voltage-clamped to a holding potential of –50 mV, a spontaneous rhythmic inward current can be seen to occur at about 5 cycles per min (large current oscillations). Occasionally a spontaneous outward current pulse was observed. ***c***, Switching to current clamp (I = 0), large spontaneous voltage depolarizations occurred also at about 5 cycles per min. ***d***, The linear I/V relationship of the spontaneous inward current showed that the ion channel carrying the current was not voltage-activated. The reversal potential of +10 mV indicates that the ion channel is conducting a non-specific cationic current. *n* = 6. Dotted lines indicate the average standard error. ***e***, A typical voltage clamp experiment, with currents recorded at +30, –30 and –60 mV holding potentials.

recorded from truly isolated ICC.

Single cell RT-PCR. We harvested mRNA from 1 ml of a P815 mast cell line suspension (American Type Culture Collection) using an mRNA extraction kit (RNeasy kit, Qiagen). We harvested mRNA from morphologically identified ICC and smooth muscle using patch pipettes with a resistance of 3–5 MΩ, filled with intracellular solution containing 140 mM CsCl; 5 mM HEPES; 10 mM NaCl, 3 mM ATP-Mg, 0.1 mM GTP, and 40 U RNAse inhibitor (RNAGuard; Pharmacia Biotech, Baie d'Urfé QC, Canada), pH 7.35. High-resistance electrodes made it difficult to harvest cell contents. The intracellular solution was drawn up into a syringe and stored on ice. mRNA was harvested by applying suction to the electrode with visual inspection, to monitor uptake of cell content into the pipet. The electrode containing the cell contents was removed and the tip was broken into a PCR tube[24] (Perkin-Elmer). The contents of the pipette (approximately 5 μl) were expelled by applying positive pressure.

For reverse transcription, the following were added to either the cell contents harvested using the patch pipette or to the mast cell mRNA suspension: 0.5 μg/μL oligo dT primers (18 dT; Mobix, McMaster University), 0.5 mM of each of the four deoxyribonucleoside triphosphates (dNTPs), 10 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0), 75 mM KCl, 3 mM $MgCl_2$. 100 U reverse transcriptase was added (Superscript RT; Life Technologies, Burlington ON, Canada), and the reaction was incubated for one hour at 36 °C.

For nested PCR, the first PCR reaction was undertaken by adding the following to the RT reaction mixture: 50 pmol of each outside primer (upstream base pair position 1642: 5'–TGTGATGGTGCTCACCTACA–3'; downstream base pair position 2088: 5'–GAGTCACGCTTCCTTCTCAA–3'), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8., 50 mM KCl, and 2.5 U Taq polymerase (MBI Fermentas, Flamborough ON, Canada); and running the reaction for 30 cycles (92 °C, 40 sec; 53 °C, 40 sec; 72 °C, 60 sec) followed by 15 min of final extension at 72 °C in a 2400 GeneAmp PCR system (Perkin-Elmer). The second PCR reaction used 2 μl of the first PCR product, to which we added 50 pmol of each inside primer (upstream base pair position 1695: 5'–GGAAGGTTGTCGAGGAGATA-3'; downstream base pair position 1952: 5'–CCTTCAGTTCCGACATTAGG–3'), 200 uM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8., 50 mM KCl, and 2.5 U Taq polymerase; and ran the reaction for 35 cycles (92 °C, 40 sec; 49 °C, 40 sec; 72 °C, 60 sec) followed by 15 min of final extension at 72 °C. The secondary PCR product (10 μl) was electrophoresed with size markers on 1.5% agarose gel stained with ethidium bromide. The *Kit* product from the first PCR reaction was 447 bp from cDNA, and approximately 12.9 kbp from genomic DNA. The *Kit* product from the second PCR reaction was 258 bp from cDNA, and 614 bp from genomic DNA. Two introns (a total of 356 bp) between the inside *Kit* primer sets produce the length difference between the cDNA and genomic DNA products in the second PCR reaction[25]. One intron is located between exons 11 and 12, at base pair position 1799, and is 267 bp in length. The other intron is located between exons 12 and 13, at base pair position 1905, and is 89 bp in length. The outside primers surround four introns: the two introns mentioned above, a 99 bp intron at base pair position 1677 between exons 10 and 11, and a 12 kbp intron at base pair position 2016 between exons 13 and 14. Thus, these four introns have a total of approximately 12.4 kbp, accounting for the length difference between cDNA and genomic DNA products in the first PCR reaction.

Electron microscopy. Mouse small intestine cell cultures were studied by electron microscopy using the 'inverted capsule embedding technique'. Cultured cells grown on glass cover slips were placed face-upwards in a small Falcon Petri dish and fixed *in situ* with 2% glutaraldehyde in 0.05M sodium cacodylate buffer, pH 7.4, containing 1.2 mM $CaCl_2$, for 40 min at room temperature. After fixation, tissue cultures were washed overnight in 0.1 M cacodylate buffer, containing 1.2 mM $CaCl_2$ in 0.05 M sodium cacodylate buffer (pH 7.4), postfixed with 1% OsO_4 in 0.05 M sodium cacodylate buffer (pH 7.4) for 40 min at room temperature, stained with saturated uranyl acetate for 30 min at room temperature, dehydrated in graded ethanol and embedded in Epon 812. Thin sections were cut on a Reighert-Jung Ultracut E microtome, stained with lead citrate, and examined in a JEOL-1200 EX Biosystem electron microscope at 80 kV.

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