Generation of slow waves in membrane potential is an intrinsic property of interstitial cells of Cajal

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Lee, Jonathan C. F., Lars Thuneberg, Irene Berezin, and Jan D. Huizinga. Generation of slow waves in membrane potential is an intrinsic property of interstitial cells of Cajal. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G409–G423, 1999.—To reveal the unique intrinsic properties of interstitial cells of Cajal (ICC), morphological and electrophysiological characteristics of isolated ICC from the adult mouse small intestine were investigated and compared with those of smooth muscle cells. All typical ultrastructural features of intact ICC were evident in isolated ICC throughout the isolation procedure and short-term culture. With the use of the nystatin perforated patch-clamp technique, ICC demonstrated spontaneous voltage oscillations that were not abolished by hyperpolarization nor by L-type calcium channel blockers. This rhythmic activity occurred at room temperature at a frequency of 13.9 ± 11.2 cycles/min, with an amplitude of 13.4 ± 11.2 mV at membrane potentials from −20 to −70 mV. Smooth muscle cells from the same culture only generated voltage-sensitive action potentials above the threshold potential of −35 mV. Hyperpolarization as well as the addition of L-type calcium channel blockers abolished the action potentials. In whole cell voltage-clamp recordings from ICC, a large nonactivating outward current was observed to be activated (5% threshold) at −49.6 mV with a halfactivation voltage of −18.7 mV and slope factor of 9.9 mV. In contrast, in smooth muscle cells, smaller outward currents with distinctive transient outward currents were present. In conclusion, the generation of L-type calcium channel blocker-insensitive slow waves in membrane potential is a unique intrinsic property of ICC.

RHYTHMIC ELECTRICAL activity underlies peristaltic and segmental contractions of the small intestine (1, 6, 8, 21, 38). This electrical activity consists of slow waves in membrane potential with superimposed action potentials. The slow wave gives the phasic contractile activity its characteristic frequency (38). The slow wave synchronizes circular muscle contractions along the longitudinal axis of the organ (7, 29) and directs the propagation of propulsive contractions in the aboral direction (8, 9, 35). Hence, the slow wave is referred to as the pacemaker.

Action potentials have been shown to occur in isolated smooth muscle cells (2, 3) and can be distinguished from slow wave activity. The action potentials have a fast rate of rise (−500 mV/s) and short duration (−1–50 ms). In tissue, they occur either superimposed on top of the slow wave plateau or they arise directly from depolarized resting membrane potentials such as in the longitudinal muscle of the colon (28). The smooth muscle action potentials occur when the membrane potential depolarizes past a voltage threshold (approximately −45 mV). Slow waves have a much slower rate of rise (−100 mV/s) and a relatively long duration (−1–2 s). Pharmacologically, slow waves can be distinguished from action potentials by their insensitivity to L-type Ca2+ channel blockers and their relative voltage insensitivity (19, 46). Slow wave activity is sensitive to agents that interfere with normal intracellular Ca2+ cycling, such as cyclopiazonic acid (CPA) (30). When stimulated, action potentials can increase in duration and can assume a slow wave-like appearance (34). However, this (17) remains susceptible to L-type Ca2+ channel blockers and disappears when the membrane potential is brought below the voltage threshold (33).

In the mouse small intestine, slow wave generation has been linked to the presence of a layer of interstitial cells of Cajal (ICC) within the Auerbach’s plexus region (13, 20). ICC are mesenchymal cells that have precursor cells in common with smooth muscle cells; through their development and as adult cells, they possess the Kit tyrosine kinase membrane receptor (44). The exact contribution of ICC to the electrical activity of the intestinal musculature has been difficult to unravel from tissue experiments for several reasons. First, ICC are part of an electrically coupled network of cells that includes smooth muscle cells. The electrical activity recorded from any cell within such a tissue is strongly influenced by activity from neighboring cells. Furthermore, ICC are densely innervated, which implies that activity observed in tissue may be influenced by neural activity. To reveal the exact role of ICC in the generation of the electrical activity, the intrinsic properties of ICC need to be studied. In a recent communication (39), chemically isolated ICC of the mouse small intestine were shown to exhibit a rhythmic inward current, providing direct evidence that ICC generate intrinsic electrical oscillatory activity. ICC were identified by showing that they contained kit mRNA. Several important questions must now be answered. Are special isolation procedures needed to obtain single ICC? Do ICC change during short-term culture? What are the characteristics of the “slow wave-like” activity associated with the observed current oscillations? Is the slow wave-like activity sensitive to L-type Ca2+ channel blockers and/or voltage? How does spontaneous activity of isolated ICC compare with that of smooth muscle cells from the same culture? Because it is not practical to characterize each ICC by the presence of kit mRNA, can ICC be identified by electrophysiological characteristics? It was the objective of this paper to answer these
questions. Some of the answers were published previously in abstract form (26).

METHODS

Dissociation of the Mouse Small Intestine

ICC are found at two locations, Auerbach’s plexus and the deep muscular plexus. The ICC associated with Auerbach’s plexus are involved in the generation of slow waves in the small intestine (20, 44) and cannot be directly dissected out in the mouse. Hence, it is important to realize that the external muscle layers must be completely dispersed to reveal the myenteric layer that is buried within the muscle layers. In addition, there is substantial connective tissue, in the form of reticular, elastic, and collagen fibers, between the main muscle layers (22).

Dissection of external musculature from small intestine. Adult female mice (25–30 g) were killed, and an ileal segment averaging 10 cm was removed proximal to the ileocecal junction. The mesentery was carefully removed, and the small intestinal segment was placed in preequilibrated M199 medium. Subsequently, the segment was opened flat by cutting along the mesenteric line. The segment was pinned flat with the mucosa facing the dissecting dish, and the muscularis externa was carefully removed by sharp dissection. Cross-sectional examination of the strip of muscularis externa by electron microscopy revealed that the muscularis externa was separated along the deep muscular plexus region. The deep muscular nerve plexus was retained on the submucosal layer and was absent from the stripped muscle tissue. The dissected muscle was carefully cut into small pieces (~1–2 mm) for enzymatic dissociation.

Enzymatic dissociation. The muscle pieces were washed briefly in solution A before incubation at 37°C in solution A plus 1 mg/ml trypsin for 15 min first and then for 30 min with fresh solution or enzyme. The supernatant was carefully removed, and the small intestinal segment was placed in fresh enzyme. Both problems were avoided by separating the enzyme combinations into two distinct solutions. The muscle pieces were washed briefly in solution A before incubation at 37°C in solution A plus 1 mg/ml trypsin (15 min) and then for 20 min with fresh enzyme. The cells were then mechanically dispersed by shaking. The cell suspension was layered on a 20% (wt/vol) Ficoll cushion and spun at 15

Enzyme combinations. The various enzymes that were tried either alone or as enzyme combinations included collagenase, papain, elastase, hyaluronidase, Pronase, trypsin, and DNase. Two enzymes emerged as essential in dissociating the intact tissue: a strong proteolytic enzyme and collagenase. Generally, the two proteolytic enzymes that showed good activity as well as minimal damage to cells were papain with dithiothreitol (DTT) and trypsin. Trypsin was later chosen because it does not require the addition of DTT. Single cells obtained from the trypsin treatment were released easily from the extracellular matrix and recovered from the enzymatic isolation faster compared with isolation procedures without trypsin. Collagenase was found to be necessary in releasing the cells. No other extracellular matrix-breaking enzyme (elastase, hyaluronidase, Pronase) was as effective as collagenase in releasing cells from mouse small intestinal tissue. It was noted that, although the enzyme digestion proceeded faster with both trypsin and collagenase together, the cells did not recover as well as cells derived from the current protocol. It was found that, for prolonged incubations, the addition of a stabilizing proteinaceous substance, such as BSA, was needed to promote viability in dissociated cells. Unfortunately, the addition of BSA blunted or stopped proteolytic activity. Also, the trypsin lowered the collagenase activity, possibly because trypsin was digesting the collagenase enzyme. Both problems were avoided by separating the essential trypsin and collagenase digestions into two distinct steps.

An important observation was that trypsin and collagenase worked optimally under slightly different conditions. Trypsin worked best in zero Ca2+ and neutral pH conditions, whereas collagenase worked better in the presence of Ca2+ and slightly more acidic to pH neutral conditions (5). Better trypsin activity was achieved by providing a divalent ion to stabilize the trypsin protein (see also Ref. 37). Because the obvious divalent ion, Ca2+, was excluded to maintain zero Ca2+ conditions, the next effective divalent ion, Mg2+, was chosen as the major divalent ion to replace Ca2+ for membrane and trypsin stabilization. The trypsin-containing solution (solution A) was deliberately adjusted to a physiologically neutral pH (~7.4). The collagenase enzyme was found to work best in higher Ca2+. To balance matrix dissociation (which requires Ca2+ restriction) with good collagenase activity (which requires Ca2+), the collagenase-containing solution (solution B) had a compromise in Ca2+ concentration (0.1 mM). BSA was added to the collagenase solution because it helped in stabilizing dissociated cells but did not affect collagenase activity.

Minimizing zero Ca2+ exposure. For good dissociation of tissue, it was found that subjecting the tissue to zero Ca2+ conditions was unavoidable. Allowing even nominal Ca2+ (~1–10 μM) did not result in optimal dispersion. Another strategy for successful cell harvest was developed after the finding that cellular Ca2+ levels overshoot once Ca2+-restricted tissue is reexposed to normal Ca2+ levels (11). To prevent Ca2+ toxicity, a Ca2+ channel blocker (2 mM Mn2+ in solution A) was used to slow Ca2+ release from the cell during Ca2+ restriction and to prevent Ca2+ overshoot after reintroduction of cells into normal Ca2+ solution. In addition,
dissociated cells were exposed to low Ca\(^{2+}\) levels (0.1 mM Ca\(^{2+}\) in solution B) before placement in 2.5 mM Ca\(^{2+}\) in medium M199.

**Light Microscopy**

Observation through a phase-contrast microscope was done using a Nikon inverted microscope (Diaphot-TMD) with either \(\times 200\) or \(\times 400\) power. The images were captured either with a conventional optical camera or with a Hitachi CAD solid-state color video camera.

**Electron Microscopy**

Electron microscopy study of mouse small intestine culture was performed on the cultured cells grown on glass coverslips in small Falcon petri dishes (3–5 days in culture). The cells were fixed in situ with 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) containing 1.2 mM CaCl\(_2\) for 40 min at room temperature. After fixation, the samples were washed overnight in 0.1 M cacodylate buffer (pH 7.4) containing 1.2 mM CaCl\(_2\), postfixed with 1% OsO\(_4\) in 0.05 M sodium cacodylate buffer (pH 7.4) for 40 min at room temperature, stained with uranyl acetate for 30 min at room temperature, dehydrated in graded ethanol and propylene oxide, and embedded in Spurr. The ultrastructure of ICC in culture and the ultrastructure of freshly dissociated ICC were compared with the ultrastructure of myenteric plexus ICC in situ (40) and the ultrastructure of ICC from dissected muscularis externa strips used for enzymatic dissociation. The tissue strips were fixed, processed, and embedded in Spurr in an identical manner. Thin sections were cut on a Reihert-Jung Ultracut E microtome, stained with lead citrate, and examined under a JEOL-1200 EX Biosystem transmission microscope at 80 kV.

**Electrophysiology**

Cells were not used beyond 5 days postisolation because it became difficult to form a tight seal with a glass pipette without enzyme pretreatment. The cells were continuously bathed with extracellular solution (ECS) at room temperature. Electrical activity in ICC was recorded using the nystatin perforated patch technique. Briefly, 2 µl of 3 mg/ml (wt/vol DMSO) nystatin stock were added to 1 ml of intracel-

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Fig. 1. Interstitial cells of Cajal (ICC) in primary culture. Single cells were dissociated from the adult mouse small intestines and grown in short-term culture (4–6 days). ICC typically attained their distinctive morphology after 2–3 days in culture. A: under the phase-contrast microscope, ICC could be distinguished from other cell types by being significantly more phase dark. The cell body was small and triangular. ICC generally extended 3–4 main branches from the cell body SMC; smooth muscle cell. B: ICC in short-term culture were seen with variations in cell body shape from strictly triangular to elongated triangles or a stellate shape. This ICC did not have well-developed processes. Processes were short, and not many contacts were made with neighboring cells. There was a distinctive triangular thickening at each bifurcation point of a process. The cell body was seen to contain a large nucleus with little surrounding cytoplasm. C: because the density of dissociated ICC in the cultures was deliberately low to obtain single ICC, it was rare to see ICC in contact with each other. When contacts between 2 ICC were established, contact was always made with processes and never between cell bodies. D: this ICC had a distinctive branching pattern, extending as a diffuse tree around the cell body and making several contacts with surrounding SMCs. All panels are shown at \(\times 400\), with magnification bar = 25 µm.
cular solution (ICS) to achieve a final concentration of 2 µg/ml. Pipettes were made using a Sutter micropipette puller to typical access resistances of 3–5 MΩ. After the pipette tip was filled with normal ICS by suction, the pipette was backfilled with nystatin containing ICS and inserted into the headstage of the patch amplifier (Axopatch 1B, Axon Instruments). The pipette was quickly lowered onto the cell surface, and suction was applied. When a tight seal was formed (~2–5 GΩ), the patch amplifier was switched over to current clamp. Holding potentials were chosen from observations of the membrane potential in current clamp. Results were acquired by Axotape (v1.2, Axon Instruments) at 50 Hz using the TM-1 DMA interface.

Evaluation of voltage clamp. Because of the branching nature of ICC and the smooth muscle cell contacts made by those branches, possible space clamp problems must be addressed. In voltage-clamp recordings from ICC, passive currents generated by ohmic response to the voltage protocols were recorded as well as active currents from the cell membrane. It was assumed that the patch-clamp amplifier delivered the voltage pulse to the pipette almost instantaneously (<50 µs, adequate for whole cell voltage clamp. The active currents evoked by the voltage protocols appeared normal for whole cell currents. Series resistance compensation circuitry was not used because it created instability in the voltage clamp.

Solutions. ECS contained (in mM) 125.0 NaCl, 5.0 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 11.0 glucose, 4.0 NaHCO3, 2.0 CaCl2, and 10.0 HEPES, pH 7.35. ICS contained (in mM) 129.0 potassium methanesulfonate, 5.0 NaCl, 2.0 magnesium acetate, 1.0 CaCl2, 11.0 EGTA, and 10.0 HEPES, pH 7.25. Solution A contained (in mM) 134.0 NaCl, 3.0 KCl, 5.0 taurine, 5.0 EDTA, 2.0 MnCl2, and 10.0 HEPES, pH 7.40. Solution B contained (in mM) 133.0 NaCl, 3.0 KCl, 5.0 taurine, 0.1 CaCl2, 2.0 MgCl2, 10.0 glucose, and 10.0 HEPES, pH 7.35. M199 medium contained 1× M199 medium, 26.0 mM NaHCO3, 2.0 mM glutamine, 0.25 mg/ml penicillin, and 10% FBS.

Drugs. Verapamil and nifedipine were dissolved in 70% EtOH as 10–2 M stock solutions. CPA was dissolved in DMSO as 5 × 10–2 M stock solution. Collagenase type I (no. C0130), trypsin type III (no. T8253), and other chemicals were purchased from Sigma-Aldrich Canada. Medium was obtained from GIBCO Life Sciences Canada.

Fig. 2. Electron micrographs of ICC from Auerbach’s plexus of adult small intestine. A: ICC fixed in situ had all major structural characteristics described previously (40). Nu, large euchromatic nucleus; G, perinuclear well-developed Golgi complexes; ce, centriole; SM, smooth muscle cell. Cytoplasm contained mitochondria (m), many free ribosomes, intermediate filaments (large circle), and thin filaments (small circle). Presence of caveolae (arrowheads) distinguished ICC from fibroblasts and macrophages. Magnification = ×20,230; bar represents 500 nm. B: ICC from dissected tissue were characterized by a dark, electron-dense cytoplasm and nucleus as a possible result of cell contraction. Note typical ICC characteristics: branched profile, perinuclear Golgi complex (G) and centriole (ce), numerous mitochondria (m), and ribosomes. Most caveolae (arrowheads) were internalized. Tissue ICC were surrounded by collagen fibers (co) and formed a close contact with a smooth muscle cell (SM). Magnification = ×15,000; bar represents 1 µm.
RESULTS

Identification of ICC Using Light Microscopy

ICC and smooth muscle cells derived from the isolation procedure appeared as contracted spindles. After they were plated in M199 medium, the cells became rounded. ICC attained their typical in situ morphology after ~2–5 days in cell culture. Identification of ICC by phase-contrast microscopy was based in part on their significantly darker appearance compared with other cells (Fig. 1). In addition, the cell body was generally triangular or stellar in shape and not flattened like smooth muscle cells. Under high magnification (×400), the cell body was shown to have a large nucleus with little surrounding cytoplasm. From the cell body, there were generally three to five primary processes that branched into secondary and tertiary processes. These processes were distinct in that they were of medium thickness (~0.1–0.2 µm), not as thick as branching smooth muscle processes (0.4–0.8 µm) and not as thin as neuronal processes (<0.1 µm). The branching pattern was distinctively different from both branching smooth muscle and neurons. The ICC branches formed a diffuse network tree with two or three secondary branches from a primary branch and often made contact with one or more smooth muscle cells. A distinguishing physical structure particular to ICC was the occurrence of large triangular bifurcations at junction points. These triangular bifurcations were sometimes quite large, up to almost one-fifth the size of a cell body. The ICC were frequently found in close proximity to smooth muscle cells, with processes terminating onto smooth muscle cells. The connections to smooth muscle cells averaged one to two connections per smooth muscle cell. However, ICC were also observed as completely isolated cells.

Fig. 3. Electron micrographs of freshly isolated cells. A: low-magnification micrograph of 2 isolated cells: a smooth muscle cell (SM) and an ICC, fixed 1 h after the dissociation procedure. Both cells were spherical and free of collagen fibers. Similar to an ICC from dissected tissue, this ICC was characterized by a dark, electron-dense cytoplasm and a heterochromatic nucleus (Nu). Its cytoplasm contained many membrane-bounded large empty spaces, probably as the result of extensive surface internalization due to cell contraction into a ball. Magnification = ×3,955; bar represents 2 µm. Inset: high-magnification micrograph of boxed area in A. Distinctive features of a 1-h-old dissociated ICC are shown: numerous intermediate filaments (large circle), thin filaments (small circle), free ribosomes. Most of the plasma membrane caveolae (arrowhead) were internalized. Magnification = ×42,600; bar represents 200 nm. B: this ICC after 1 day in culture was characterized by an oval, smooth-profile, large, partially folded euchromatic nucleus (Nu) and cytoplasm filled with mitochondria (m), small cisternae of rough endoplasmic reticulum (rER), and an increased number of free ribosomes. G, Golgi complex; arrowheads, caveolae. Magnification = ×10,930; bar represents 1 µm.
Isolated ICC Were Capable of Spontaneous Contractions

At temperatures between ~30 and 39°C, ICC demonstrated spontaneous rhythmic contractions with a frequency from 5 to 30 contractions/min as demonstrated previously (39), often with the processes contracting independently of the cell body. Sometimes second-order branches were contracting apparently independently of the rest of the cell. Contractions were most often seen in those ICC that contacted smooth muscle. Contraction patterns ranged from seemingly disorganized contractions to synchronized contractions with adjacent smooth muscle cells.

Electron Microscopy

The dissected muscle strips were composed of the longitudinal and outer circular muscle layer and Auerbach's plexus. The mucosa, submucosa, and innermost circular muscle layer including the deep muscular plexus were removed. Compared with ICC from intact tissue (Fig. 2A), ICC from dissected tissues were characterized by a high electron density of their nuclei and cytoplasm, although there were no structural signs of prominent cell injury (Fig. 2B). The ICC had all the major characteristics of intact tissue ICC: branched cell profiles, cytoplasm containing abundant ribosomes, mitochondria, thin and intermediate filaments, cisternae of endoplasmic reticulum, and numerous plasma membrane caveolae (Fig. 2B). ICC formed close contacts with neighboring smooth muscle cells and were surrounded by an extracellular matrix of collagen fibrils and elastic fibers (Fig. 2B).

One hour after isolation, ICC and smooth muscle cells were round and free from extracellular matrix (Fig. 3A). At this point, the freshly dissociated ICC had many ultrastructural features similar to ICC from dissected strips (Figs. 3B and 4A). Their cytoplasm was composed of thin and intermediate filaments and contained numerous mitochondria and free ribosomes (Fig. 3A, inset). The nuclei and cytoplasm had high electron densities. Most of the plasma membrane caveolae were internalized, the possible result of cell contraction. The presence of caveolae in the dissociated ICC excluded the possibility that these rounded cells were glial cells, macrophages, or fibroblasts. Freshly dispersed ICC lost cellular processes because of strong retraction into a ball shape (Fig. 3).

Electron microscopy analysis of isolated ICC after a few days in culture (1–4 days) showed structural
modification (Figs. 3B and 4). Two concurrent changes took place: 1) regression of the filament system and 2) proliferation of free ribosomes in the cytoplasm and dispersion of nuclear chromatin. After 1 day in culture, ICC were still round, but no membrane-bounded spaces were seen in the cytoplasm (Fig. 3B). Nuclei turned euchromatic, and numerous ribosomes appeared free in the cytoplasm, indicating an increase of synthetic activity. After 2–4 days in culture, ICC had started extending processes and making contacts with smooth muscle cells as seen by light microscopy (Fig. 4). Similar to day one, ICC after 2–4 days in culture displayed some characteristics of a synthetic phenotype. The ICC cytoplasm was dominated by a large number of free ribosomes, frequent, slightly distended cisternae of rough endoplasmic reticulum (ER) and smooth ER, numerous mitochondria, and few lysosomes. The nuclei were completely euchromatic. Lipid droplets were seen in some ICC processes. After 1 day in culture and in later days, no prominent bundles of regularly organized intermediate filaments were seen in the perinuclear cytoplasm or the large processes of ICC (Figs. 3B and 4B). The intermediate filaments were evident only in terminal ICC processes. In contrast with ICC, the cytoplasm of most of the smooth muscle cells after 4 days in culture was still dominated by numerous filaments (Fig. 4). After 1–4 days in culture, ICC could be distinguished from other cells by the presence of surface caveolae and occasional gap junctions with smooth muscle cells.

Electrophysiology

ICC were patched at the cell body with the nystatin perforated patch technique. Cells were chosen when they conformed to ICC criteria (see above) and showed spontaneous contractile activity when examined at 37°C. The patch-clamp experiments were carried out at room temperature, since pipette contact to an ICC or smooth muscle cell would induce a strong contraction at 37°C. Some intestinal tissue becomes electrically quiescent at room temperature (10). At room temperature, the mouse small intestine musculature generated slow wave activity (n = 6), as measured in tissue with intracellular microelectrodes, with a frequency reduced from 35.1 to 16.2 ± 1.5 cycles/min and membrane potential depolarized from −58.1 ± 1.5 to −47.6 ± 1.8 mV (Fig. 5).

Isolated ICC demonstrated spontaneous electrical activity. Single ICC demonstrated spontaneous oscillations in membrane potential at a frequency of 13.9 ± 11.2 cycles/min. These “slow waves” had an amplitude of 13.4 ± 11.2 mV and a duration of 1.2 ± 0.6 s, and the resting membrane potential of the ICC had an average value of −37.1 ± 7.9 mV at room temperature (n = 9) (Fig. 6). The spontaneous voltage oscillations observed in all ICC were resistant to L-type Ca2+ channel blockers, verapamil, D 600 (up to 10 µM), nifedipine (up to 5 µM), and diltiazem (up to 5 µM) (Fig. 6). Unlike action potentials in smooth muscle, the voltage oscilla-

![Fig. 5. At room temperature, slow wave activity in tissue from the small intestine occurs at low frequencies. With the use of microelectrodes, slow wave activity was recorded from circular muscle cells within tissue obtained from the mouse ileum. Top tracing (A) is recorded at 37°C and lower tracing (B) at 24°C. This tissue (3 × 3 mm, stretched and pinned to Sylgard) contains the network of ICC associated with Auerbach's plexus. Slow wave activity was very temperature sensitive, in particular with respect to the frequency, which slowed down markedly when the temperature was reduced from 37 to 22°C.](image-url)
Fig. 6. Spontaneous voltage oscillations recorded from an isolated ICC. High-amplitude voltage oscillations arose sharply from the resting membrane potential at regular intervals. Insets show striking resemblance to tissue slow waves; upstroke occurred independent of a prepotential component. Voltage oscillations were unaffected by the L-type Ca\(^{2+}\) channel blocker verapamil (1 µM; bottom). Top trace shows control. Recording was made from an ICC after 5 days in culture.

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Fig. 7. Spontaneous generation of action potentials recorded from an isolated smooth muscle cell. Generation of action potentials occurred at irregular frequencies. Inset shows that a prepotential depolarization is preceding each action potential. All action potential activity was abolished by the L-type Ca\(^{2+}\) channel blocker diltiazem (1 µM). Both recordings were from the same cell as part of a continuous recording. Diltiazem was applied at the beginning of bottom trace. Calibrations for inset: 10 mV and 2 s.
19.7 ± 7.6 mV (n = 4). The frequency of the oscillations (>5 mV) was 8.5 ± 5.6 cycles/min.

Effects of CPA. In whole tissue (30, 31), CPA, a specific inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase pump, caused abolishment of slow wave activity in a reversible manner. This inhibition can be preceded by a pattern whereby some slow waves are increased in duration (30). The effects of CPA were similar in isolated ICC (n = 3) (Fig. 9), although the membrane potential depolarized slightly (+5 mV). The spontaneous oscillations returned on washout of CPA, albeit with altered frequency and amplitude, similar to washout effects in tissue.

Frequency of spontaneous activity in ICC is not voltage sensitive. In the presence of 1 µM forskolin to promote regular stable oscillations and typical resting membrane potentials of −50 to −60 mV, depolarizing or hyperpolarizing current was injected into isolated ICC. Under such conditions, voltage oscillations occurred at all membrane potentials between −70 and −30 mV without significant differences in frequency. In contrast, spontaneous action potentials in smooth muscle cells were voltage sensitive. Hyperpolarizing current injection to achieve −60 mV abolished action potentials present at −40 mV (Fig. 10). When the hyperpolarizing current was switched off, the spontaneous action potentials reappeared (n = 3). At −60 mV, depolarizing (20 mV) square-current pulses evoked action potentials only when the tops of the square-current pulses had reached the voltage threshold for action potential activation (approximately −37 mV) (Fig. 11). Therefore, action potentials can be made to appear as bursts that are limited by the duration and frequency of the depolarizing pulses. Such pulses therefore mimic slow waves.

Profiles of whole cell currents in ICC and smooth muscle cells. Whole cell patch clamp was done with the singular objective of investigating if whole cell current profiles could distinguish ICC from smooth muscle cells. We have shown previously that ICC uniquely produce spontaneous inward currents that are not activated by voltage (39). We now show that voltage steps from −70 to +30 mV for 500 ms evoked whole cell currents, as shown in Fig. 12. The whole cell currents from ICC were predominantly outward (n = 7). The outward currents had a fast activation before proceeding to a steady state. The large tails confirmed the dominance of the outward currents to the total whole cell currents. The whole cell currents were not blocked by nifedipine (5 µM) nor by tetraethylammonium (TEA; 5 mM). The currents showed slight inactivation, especially at depolarized ranges (greater than +20 mV). Current-voltage (I-V) curves showed a sharp voltage activation takeoff at −40 mV. The slight voltage-dependent inactivation was confirmed by comparing the currents evoked after a prepulse of −30 mV to those evoked after a prepulse of −100 mV (n = 3). Currents elicited with the −30-mV prepulse were smaller in
amplitude only in more depolarized ranges, as shown in the I-V relationship.

For smooth muscle cells, the currents elicited with prepulses of \(-100\) and \(-30\) mV were distinctly different (Fig. 13). One of the prominent features of smooth muscle cells was the presence of transient outward currents that underwent voltage-dependent inactivation. With the \(-30\) mV prepulse, the transient outward currents did not develop. Another prominent feature of smooth muscle cells was the presence of spontaneous transient outward currents in response to depolarizing voltages. The I-V relationship shows the decrease in currents with the \(-30\) mV prepulse in comparison with the \(-100\) mV prepulse due to the inhibition of the transient outward currents. The outward currents from smooth muscle cells were sensitive to inhibition by TEA and L-type Ca\(^{2+}\) channel blockers (not shown).

Voltage activation of outward currents in ICC and smooth muscle cells. Normalizing the tail currents that occurred after the end of the voltage steps revealed the relative conductances of the ICC outward currents. The activation of the outward current followed a Boltzmann distribution and was fitted by the least-squares method (Fig. 14). The half-activation voltage \((V_h)\) was estimated to be \(-8.7\) mV with a slope factor \((V_s)\) of 9.9 mV. Because the Boltzmann relationship was asymptotic at both ends, the threshold and steady-state voltages were determined at 5\% and 95\% of activated relative currents and conductances, respectively. The threshold for activation (5\%) was calculated to be 49.6 mV, and the voltage at which the outward conductance goes to the chord conductance was calculated to be +14.2 mV \((n = 6)\).

For smooth muscle cells, the tail currents deactivated too quickly to be accurately measured. Instead, peak outward currents from each voltage step were normalized as conductances. The plot of normalized

Fig. 9. Cyclopiazonic acid (CPA) increased duration and thereafter abolished spontaneous voltage oscillations in ICC. In the presence of 5 \(\mu\)M D 600, 2 \(\mu\)M CPA was added, which caused a characteristic increase in duration before abolishing the voltage oscillations. After washout, effect was fully reversible. Parts of a continuous recording from a single ICC in primary culture are shown.

Fig. 10. Voltage sensitivity of spontaneous activity from an ICC (A) and a SMC (B). From a resting membrane potential of \(-60\) mV, regular 20-mV oscillations were observed from an ICC after 5 days in culture. Membrane potential was then depolarized to \(-40\) mV by current injection. Spontaneous activity was not affected by the depolarization. In contrast, action potential generation in a SMC was very voltage sensitive. Action potentials typically occurred at a membrane potential of \(-37\) mV. By current injection, membrane potential was hyperpolarized to \(-60\) mV. Action potentials were abolished by hyperpolarization below approximately \(-40\) mV.
conductances over the voltage range yielded the voltage activation curve, with $V_n$ of $+2.3$ mV and $V_s$ of $16.4$ mV. The threshold for activation (5%) was $-49.0$ mV, and the chord conductance voltage was $50.6$ mV. Thus the peak outward currents for smooth muscle cells activate and go to chord conductance at a range different from the outward currents for ICC.

**DISCUSSION**

**Main Findings**

A cell isolation procedure was developed for the mouse small intestine musculature that succeeded in isolating single ICC with normal ultrastructural characteristics. The ICC were grown in primary culture. Slow wave-like activity was observed in these ICC, which had many characteristics of slow waves recorded in tissue. Single ICC and single smooth muscle cells had distinctly different spontaneous electrical activity as well as different whole cell current profiles. While this paper was under review, Ward's laboratory (24) confirmed our observation (39) of the occurrence of a rhythmic inward current in isolated ICC and also convincingly demonstrated the generation of slow wave-like activity in small networks of ICC.

**Fig. 11.** Action potentials can be evoked and paced in a single SMC. Resting membrane potential was hyperpolarized below the voltage threshold to abolish action potential generation. Depolarizing current pulses (1 Hz) were given so that 20-mV depolarizations were superimposed on a slowly depolarizing ramp. Once the peaks of the depolarizing pulses reached threshold ($-37$ mV), action potentials occurred at the peak of each depolarizing pulse. Because action potentials only occurred at the peak of the depolarizing pulses, their occurrence was dependent on the duration and frequency of the depolarizing pulse.

**Fig. 12.** Whole cell current profiles from ICC. **A:** large outward whole cell currents were elicited by the voltage-clamp protocols shown in ICC from 4–7 days in culture ($n = 7$). Prepulses of either $-100$ (left) or $-30$ mV (right) were given before the test pulses in the same cells. Large outward currents were only slightly inactivated by the $-30$ mV prepulse. **B:** current-voltage relationships of the peak currents with either the $-100$ or $-30$ mV prepulse protocols (pp) were similar. In A, note the tail currents resulting from the slow deactivation of ICC outward currents.
Isolation and Identification of ICC

The ICC were difficult to isolate. In situ, these cells are intimately associated with several cell types such as smooth muscle cells and nerve fasciculi. Furthermore, in the adult intestine, ICC are embedded in a dense matrix of connective tissue, in particular collagen fibers (12). In addition, mechanical stress, especially stretching stress on the tissue while dissecting, is detrimental (41). This is not surprising considering the extensive branching pattern of the ICC and the delicacy of the branches seen in whole tissue. The isolation procedure produced isolated spherical ICC. These ICC needed 2–3 days in short-term culture to branch out and become identifiable by light microscopy. The "rounding up" of dissociated cells is not uncommon; most dissociation techniques that include trypsinization alter the appearance of isolated cells (14). Isolated ICC in short-term culture were frequently observed to contact each other as well as smooth muscle cells. This suggests that there may be a target-specific interaction that promotes the development of "mature" ICC. The presence of the Kit receptor on ICC, shown by other studies (20, 23, 43), suggests that specific differentiation signals are needed for the proper development of mature ICC. In short-term culture of adult ICC, de novo synthesis of steel factor may occur; however, the addition of Steel factor to the culture medium did not affect ICC (L. Farraway and J. D. Huizinga, unpublished observations).

ICC in primary culture showed a remarkable resemblance to in situ ICC. Although gap junction contacts between ICC from the Auerbach's plexus region and smooth muscle cells are rare in situ, they were observed in culture. All prominent ultrastructural features were preserved throughout the isolation procedure and short-term culture. However, ultrastructural changes did occur, in particular, the prominent appearance of a large number of free ribosomes and distended cisternae of rough ER and smooth ER with euchromatic nuclei. In accordance with this, the contractile filaments appeared at the periphery of the cell body. The prominence and central location of contractile filaments in cell processes were not affected. These ultrastructural changes reflect synthetic activity, likely associated with the restoration of ICC processes.

Because of our strict criteria in identifying ICC, there were probably many more ICC in culture than were positively identified. When we followed ICC in short-term culture using time-lapse video recording, we observed changes in ICC morphology over time (26). It is therefore important to use other criteria, such as the presence of the Kit receptor, in identifying ICC. It is now possible to identify isolated ICC after fixation. The
present study used electron microscopy, and Koh et al. (24) used immunohistochemistry after acetone fixation. Identification of living cells is still elusive. Our attempts to do this resulted in macrophages being stained positive for Kit because of nonspecific uptake (L. Farraway and J. D. Huizinga, unpublished results).

**Electrophysiology of ICC**

Isolated ICC generated voltage oscillations in a dramatic rhythmic fashion, showing a high similarity with tissue slow waves. The slow waves rose sharply from the resting membrane potential with a shape that was very similar to that of tissue slow waves. CPA abolished the voltage oscillations within 10 min. Before abolishment, it caused an increase in duration of some voltage oscillations, similar to the effect of CPA on slow waves in tissue (30, 32). CPA inhibits Ca$^{2+}$ cycling in the sarcoplasmic reticulum, which may be an essential step in the triggering of the rhythmic voltage oscillations (30). Cyclic Ca$^{2+}$ release may trigger the nonspecific cation channel that underlies the rhythmic currents spontaneously generated in ICC (39). Koh et al. (24) also came to the conclusion that intracellular Ca$^{2+}$ may regulate the mechanism that initiates slow waves. This was based on the observation that CPA in isolated ICC increased the frequency of slow waves. We have no explanation for the discrepancy between this result and that presented here. Koh et al. (24) also showed evidence for the occurrence of a Ba$^{2+}$-sensitive K$^{+}$ conductance that could contribute to the repolarization of slow waves in ICC.

It is controversial whether the intestinal pacemaker activity is driven by voltage-activated currents similar to the cardiac pacemaker (25). The alternative is that the slow waves are driven by currents initiated by intracellular metabolic events (30). In the present study, the voltage oscillations in the ICC occurred under current clamp and could not be triggered by depolarization. Furthermore, they occurred at a range of voltages, from $-20$ to $-70\,\text{mV}$, and were influenced by changes in intracellular Ca$^{2+}$ store status. Hence, this study is consistent with the hypothesis that ICC can generate metabolically driven voltage oscillations. This is in agreement with the observations that slow waves in tissue are relatively insensitive to voltage and are driven by metabolically sensitive events (10, 18, 30).

The activity seen in isolated ICC was irregular at times. This variability probably arose from instability of the pacemaker system in single cells. Koh et al. (24) noted that spontaneous activity was more stable in small networks of ICC compared with isolated cells. Similar observations have been made in isolated heart pacemaker cells (4). The stability of the frequency of cardiac pacemaker activity (15, 42) increases when the number of cells in a cluster increases (36). Establishment of cell-to-cell contact and electrical coupling is important for frequency stability (4). Indeed, ICC in tissue are extensively coupled among themselves by gap junctions forming a network structure. Therefore, the instability and variability seen in isolated ICC can be explained by the fact that they have little or no cell-to-cell communication by which to enhance their intrinsic pacemaker activity. Although ICC in tissue are always coupled to each other by gap junctions, their coupling to smooth muscle cells is usually devoid of gap.

**Fig. 14.** Voltage activation of the large outward currents in ICC and smooth muscle cells. Top: analysis of normalized tail currents ($I$) from ICC outward currents yielded the voltage activation plot shown ($n = 6$). Tail currents were taken $15–20\,\text{ms}$ after the end of the test pulse to be certain that the capacitance artifact would not contribute. Curve was fitted by the least-squares method. Large outward current in ICC has a half-activation voltage ($V_h$) of $-18.7\,\text{mV}$ with a slope factor ($V_s$) of 9.9 mV. With the use of 5% and 95% points as the threshold and steady-state points, respectively, this large outward current was found to activate at approximately $-49.6\,\text{mV}$ and reach steady state at $-42.4\,\text{mV}$. Bottom: similarly, the peak outward currents in SMC were normalized as conductances ($g$) and plotted as a voltage activation plot. Peak outward currents in SMC had a $V_h$ of $2.3\,\text{mV}$ and a $V_s$ of 16.4 mV. The 5% and 95% thresholds were $-49.0\,\text{mV}$ and $50.6\,\text{mV}$, respectively.
junctions but consists of close apposition contacts. Such contacts, however, do allow tracer spread (27). The gap junction contacts between ICC might be essential for metabolic coupling to synchronize metabolic products driving the slow wave activity.

Electrophysiology of ICC vs. Smooth Muscle Cells

Whole cell current profiles from ICC and smooth muscle cells were distinctly different. ICC had large outward currents that showed little to no inactivation. Smooth muscle cells had smaller but distinctive outward currents that included transient outward currents. Superimposed on the smooth muscle cell outward currents, spontaneous transient outward currents occurred from periodic activation of Ca$^{2+}$-activated K$^+$ current. The transient outward currents showed strong voltage-dependent inactivation. Current vs. voltage plots of peak currents following prepulses of −100 and −30 mV showed different voltage takeoffs, since the transient outward current activated at a more hyperpolarized range (approximately −50 mV) in contrast to the nonactivating outward current takeoff (approximately −20 mV). In comparison, ICC currents were larger in amplitude, showed a lesser degree of conductance noise, and showed only slight voltage-dependent inactivation.

Both ICC and smooth muscle cells were capable of generating spontaneous rhythmic oscillations. However, spontaneous membrane potential oscillations from ICC were distinct from the characteristic action potentials generated by smooth muscle cells. The action potentials had a relatively fast rate of rise and were sensitive to both L-type Ca$^{2+}$ channel blockers and hyperpolarization as noted previously in other smooth muscle cells (2, 34).

The experiment shown in Fig. 11 illustrates a model of the “slow wave-action potential complex” as it occurs in tissue but now seen in an isolated smooth muscle cell. The resting membrane potential of intestinal smooth muscle cells is in the nonexcitable voltage range. The occurrence of action potentials can be regulated by controlling the duration and frequency of depolarizing pulses (slow waves) that surpass the voltage threshold for action potential generation. It appears that ICC are needed to trigger slow wave activity in neighboring smooth muscle cells, since slow waves regeneratively propagate into the rest of the musculature (28, 45).

In summary, ICC from the small intestine can be isolated, cultured, and identified successfully by strict morphological criteria and electrophysiological characterization. The spontaneous membrane potential oscillations observed in ICC are insensitive to L-type Ca$^{2+}$ channel blockers, are not abolished by hyperpolarization to approximately −70 mV, and have a frequency similar to that of slow waves in tissue at room temperature. These data, together with those from two recent papers (24, 39), provide the strongest evidence to date that the generation of pacemaker activity (i.e., the nifedipine-insensitive part of intestinal slow waves) is an intrinsic property of the ICC.

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