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Spontaneous electrical activity of interstitial cells of Cajal isolated from canine proximal colon

(electrical rhythmicity/gastrointestinal motility/ionic channels/smooth muscle electrophysiology)

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ABSTRACT **Interstitial cells of Cajal (ICC) have been suggested as pacemaker cells in the gastrointestinal tract. A method was developed to isolate ICC from the slow-wave pacemaker region of the canine proximal colon. These cells were identified under phase-contrast microscopy, and their identity was verified by comparing their ultrastructure with the morphology of ICC *in situ*. Patch-clamp experiments demonstrated that these cells are excitable; voltage-dependent inward and outward currents were elicited by depolarization. Inward current transients were identified as calcium currents. A portion of the outward current appears to be due to Ca^{2+}-activated K channels commonly expressed in these cells. ICC were also spontaneously active, generating electrical depolarizations similar in waveform to slow-wave events of intact colonic muscles. These findings are consistent with the hypothesis that ICC initiate rhythmicity in the colon.**

Autorhythmicity is a fundamental property of gastrointestinal smooth muscles and is essential for normal gastrointestinal motility. The mechanism underlying rhythmicity has been elusive. Recently, the origin of electrical activity has been localized to discrete pacemaker regions within the bowel wall (1–5), and morphological studies have shown these pacemaker regions to be populated by a cell type referred to as interstitial cells of Cajal (ICC; refs. 5–7). ICC are interposed between nerves and muscle cells and form gap junctions with other ICC and with nearby smooth muscle cells (6, 8, 9–11). It has been suggested that these cells generate rhythmicity in the gut (5, 12–14), but support for this hypothesis has been largely limited to morphological evidence. Due to the difficulties in recording from these cells *in situ*, few physiological tests have been performed (5).

In the canine proximal colon, pacemaker activity is localized in two regions: (*i*) the submucosal surface of the circular muscle layer initiates electrical slow waves at 6 cycles per min (3), and (*ii*) the border between the circular and longitudinal muscle layers generates myenteric potential oscillations at 17 cycles per min (4). Morphological studies have shown that the submucosal pacemaker region is liberally populated with ICC (6, 7, 15). Coincidence of ICC and pacemaker function at the submucosal surface of the circular layer makes this a good preparation to test the hypothesis that these cells are capable of generating rhythmicity. In the present study, we attempted to (*i*) develop a method to isolate ICC from the submucosal region, (*ii*) identify isolated ICC by comparison of their ultrastructure with ICC *in situ*, (*iii*) determine whether ICC are electrically excitable, and (*iv*) determine whether ICC can generate spontaneous electrical rhythmicity.

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METHODS

Mongrel dogs of either sex were anesthetized and a segment of the proximal colon was removed and dissected as described (3). Thin strips of circular muscle along the submucosal border were dissected to isolate the region of the slow-wave pacemaker (3). These were incubated for 30 min in a Ca^{2+}-free Hanks' solution (16) and then digested in the Ca^{2+}-free Hanks' solution containing collagenase (1.5 mg/ml), Pronase (0.12 mg/ml), bovine serum albumin (4 mg/ml), trypsin inhibitor (1 mg/ml), ATP (Na^+ salt) (0.55 mg/ml) with gentle agitation at 37°C for 1 hr. After digestion, the muscle pieces were rinsed and resuspended in Ca^{2+}-free solution. Cells were plated in M199 (GIBCO) and incubated at 37°C in 95% O_2/5% CO_2 for 1 hr before use.

ICC-like cells were identified under phase-contrast microscopy and processed for transmission electron microscopy (TEM). Cells were fixed with 2.5% glutaraldehyde/2% paraformaldehyde, washed with sodium cacodylate (0.1 M), and postfixed with 1% osmium tetroxide. Embedded cells were sectioned and examined by TEM. The ultrastructure of isolated ICC-like cells was compared to (*i*) the ultrastructure of ICC *in situ* (6, 15) and (*ii*) isolated smooth muscle cells prepared for TEM in an identical manner.

Electrophysiological studies were performed at 20°C–24°C using the patch-clamp technique (16–18). Series resistance was compensated, and the experimental protocols were initiated after allowing 5 min for cell dialysis with the pipette solution. Collection and analysis of voltage-clamp data were performed as described (16, 17). Calcium currents were sampled at 3–5 kHz and filtered at 1 kHz. Data were corrected for a 10-mV liquid junction potential. Single-channel currents were recorded from "on-cell" and excised patches (17).

Solutions and Drugs. Macroscopic currents were recorded from cells in bathing medium containing 129.8 mM Na^+, 5.8 mM K^+, 1.8 mM Ca^{2+}, 0.9 mM Mg^{2+}, 135.0 mM Cl^-, 4.17 mM HCO_3^-, 0.34 mM HPO_4^{2-}, 0.44 mM $H_2PO_4^-$, 0.4 mM SO_4^{2-}, 10.0 mM dextrose, 2.9 mM sucrose, 10 mM Hepes (pH 7.4, with NaOH). Pipette solutions contained the following: 135 mM K^+, 110 mM gluconate$^-$, 21 mM Cl^-, 0.5 mM Mg^{2+}, 2.5 mM ATP (K^+ salt), 2.5 mM creatine phosphate (Na^+ salt), 1.0 mM EGTA, 5 mM hepes (pH 7.2). Calcium currents were recorded using either of the following pipette solutions: 70 mM potassium gluconate, 20 mM KCl, 0.5 mM $MgCl_2$, 40 mM tetraethylammonium (Cl^- salt), 5 mM K_2-ATP, 5 mM phosphocreatine, 5 mM Hepes, 1 mM EGTA or 40 mM potassium gluconate, 50 mM CsCl, 35 mM tetraethylammonium (OH^-), 5 mM K_2-ATP, 5 mM phosphocreatine, 1.5 mM $MgCl_2$, 10 mM Hepes, 20 mM EGTA. pH of these solutions was

Abbreviations: ICC, interstitial cells of Cajal; TEM, transmission electron microscopy.

adjusted to 7.2 with CsOH. Nifedipine was obtained from Boehringer Mannheim.

In single-channel experiments, the bath solution contained 140 mM K^+; 0.941, 0.922, 0.888, 0.796, or 0.61 mM Ca^{2+}; 140 mM Cl^-; 10 mM glucose; 1 mM EGTA; 10 mM Hepes (pH 7.4). Pipette solutions contained 140 mM K^+, 1 mM or less Ca^{2+}, 142 mM Cl^-, 10 mM glucose, 10 mM Hepes (pH 7.4). The pH of these solutions was adjusted with KOH. The concentration of Ca^{2+} in bath and pipette solutions was buffered by EGTA to obtain activities between 1.0 and 0.1 μM as described (17).

RESULTS

Morphology of Putative ICC. Dispersions of the pacemaker region yielded smooth muscle cells and a cell type with substantially different characteristics (Fig. 1*A*). Under phase contrast, these cells appeared darker than phase-bright smooth muscle cells and exhibited two to seven lateral projections and prominent nuclear regions. These characteristics are similar to those of ICC (13), but critical identification rests on ultrastructural criteria. Therefore, the putative ICC were examined by TEM.

Dispersed ICC-like cells and ICC *in situ* (6) had several common ultrastructural features: (*i*) clusters of mitochondria (Fig. 1 *B* and *C*); (*ii*) numerous caveolae along the plasma membrane (Fig. 1*C Inset*); (*iii*) cisternae of smooth endoplasmic reticulum (Fig. 1 *B* and *C*); (*iv*) free ribosomes (Fig. 1*C*); (*v*) ovoid nucleus with a sparse heterochromatin (Fig. 1*B*); (*vi*) abundance of thin filaments, but a lack of thick filaments and dense bodies (Fig. 1*C*). These similarities, shared with ICC *in situ* but not with smooth muscle cells (compare Fig.

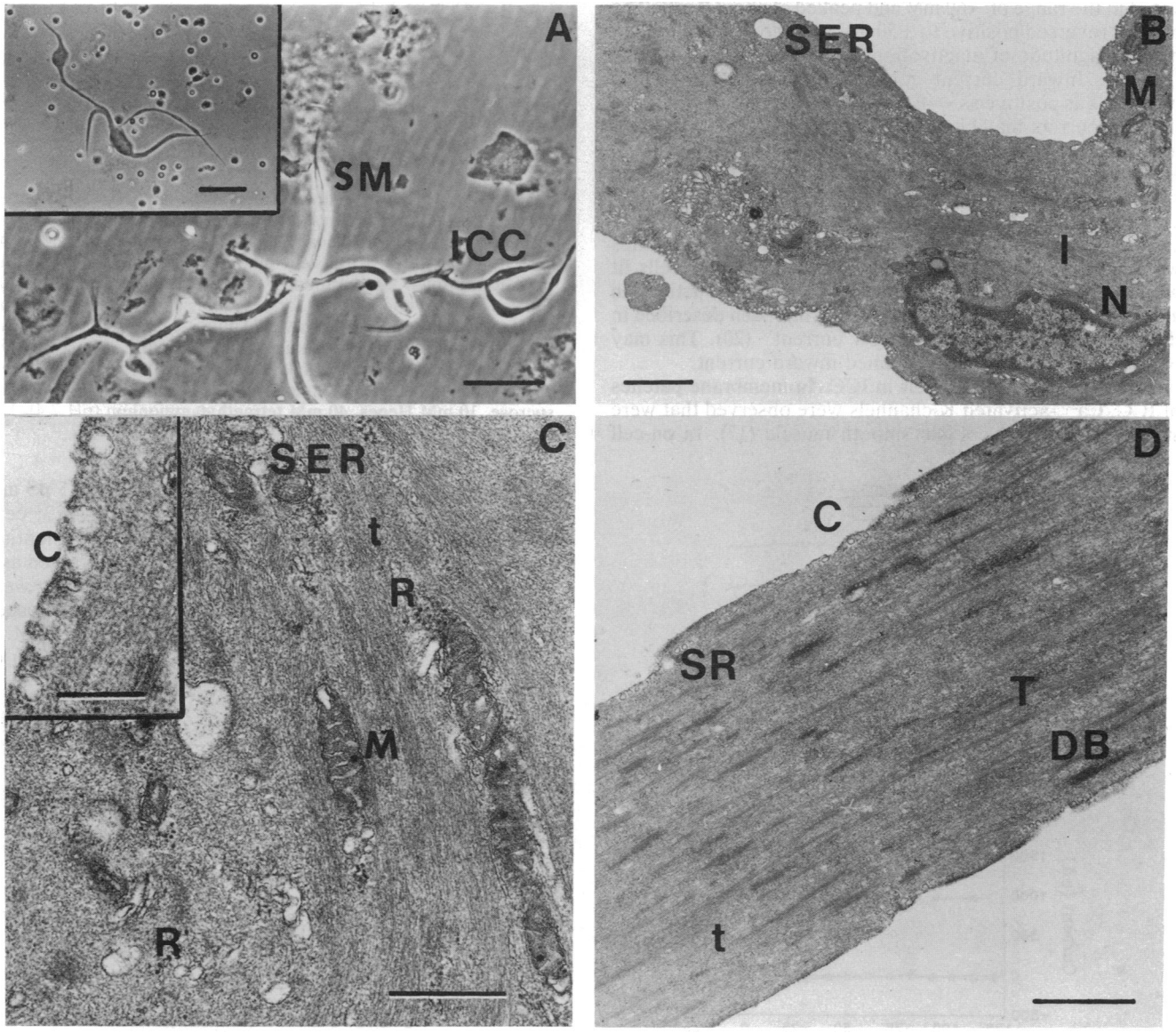

FIG. 1. Structure and ultrastructure of isolated ICC. (*A*) Typical ICC and a smooth muscle (SM) cell isolated from the submucosal pacemaker region. ICC were characterized by multiple lateral processes, a prominent nuclear region, and a dense-granular cytoplasm. Smooth muscle cells were more phase bright and lacked processes. Isolated ICC could be identified with phase-contrast microscopy and later were sectioned for TEM. (*Inset*) The ICC was prepared in such a manner. (*B* and *C*) Its ultrastructure is shown, and several morphological details support the notion that these cells are ICC (see text). Cells displayed numerous clustered mitochondria (M), surface caveolae (C; see *Inset* in *C*), ovoid nucleus with sparse chromatin (N), smooth endoplasmic reticulum (SER), thin filaments (t), and free ribosomes (R). ICC lacked thick filaments. (*D*) For comparison, a circular smooth muscle cell isolated, fixed, and sectioned in an identical manner is shown. These cells were distinctly different, displaying numerous dense bodies (DB), thin and thick filaments (t, T), caveolae (C), and sarcoplasmic reticulum (SR). Mitochondria were sparse in smooth muscle cells [*A*, bar = 50 μm (*Inset*, bar = 50 μm); *B*, bar = 1.0 μm; *C*, bar = 0.5 μm (*Inset*, bar = 0.1 μm); *D*, bar = 1 μm].

1 *B* and *C* with Fig. 1*D*), strongly suggest that the isolated ICC-like cells are ICC. The presence of caveolae in isolated ICC excludes the possibility that these cells are nerve cells, fibroblasts, or macrophages.

Voltage-Dependent Currents in ICC. Twenty-two isolated ICC were studied with the whole-cell patch-clamp technique. Depolarization from holding potentials of −50 to −80 mV elicited transient inward and sustained outward currents (Fig. 2). Expression of voltage-dependent currents supports the idea that ICC are excitable cells.

In 10 cells, outward currents were blocked by inclusion of 50 mM Cs^+ in the pipette and the addition 40 mM tetraethylammonium to the bath solution. The remaining inward current peaked within 5 ms and then decayed to sustained levels for the duration of test pulses (Fig. 3). The peak magnitude ranged from 55 to 505 pA (average, 274 pA) and was blocked by 1 μM nifedipine. The inward current activated in the range of −60 mV and peaked at about 0 mV. The current reversed positive to +50 mV and showed a reduction in the magnitude of inactivation near the reversal potential (Fig. 4). Inward current could be elicited from holding potentials as positive as −40 mV. These observations suggest that this current may be an "L-type" Ca^{2+} current (19). The magnitude of this current is consistent with the generation of active events and may be sufficient to depolarize neighboring smooth muscle cells *in situ* to which ICC are electrically coupled (6).

The steady-state activation and inactivation relationships were determined as described (16). Data from four cells fit with Boltzmann functions are shown in Fig. 4. The activation and inactivation curves overlap, which has been described in the cardiac literature as "window current" (20). This may provide a mechanism for sustained inward current.

Ca^{2+}-Activated K Channels in ICC. In membrane patches of ICC, Ca^{2+}-activated K channels were observed that were similar to those of colonic smooth muscle (17). In on-cell

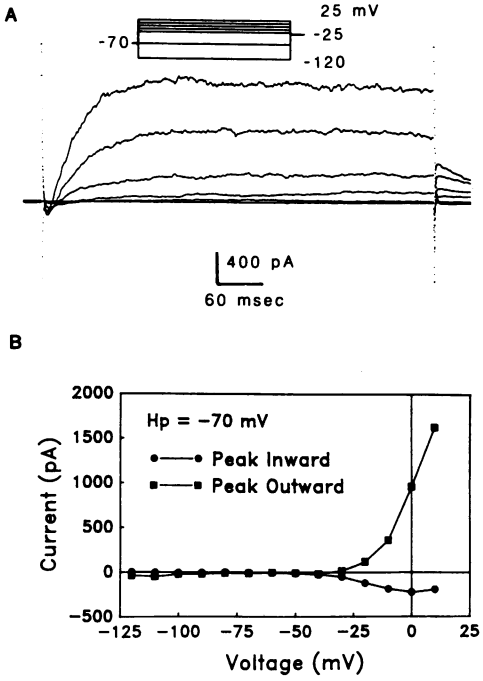

FIG. 2. Current responses of ICC under whole-cell voltage clamp. (*A*) Inward and outward currents elicited by a range of depolarizing test pulses from a holding potential (Hp) of −70 mV. Inward current was transient and gave way to a large sustained outward component. In this and other experiments, hyperpolarization did not elicit time-dependent currents. (*B*) Current–voltage relationship for net macroscopic currents.

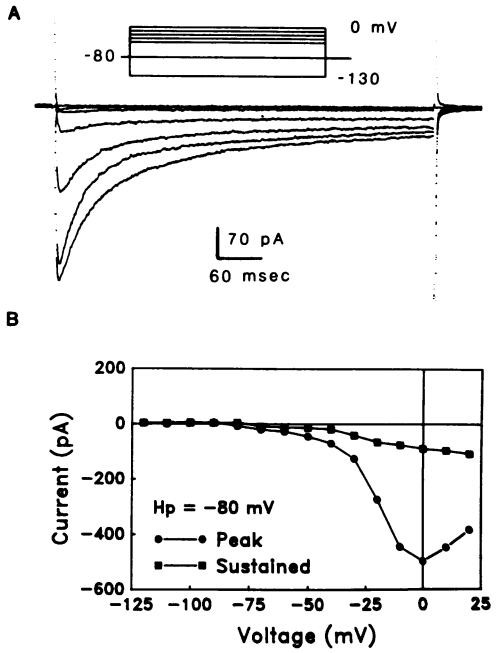

FIG. 3. (*A*) Inward current responses of ICC. Blockade of outward currents allowed resolution of substantial inward current in the presence of physiological (1.8 mM) Ca^{2+} concentration. This current consisted of a transient phase and a sustained phase that persisted through the duration of 500-ms test depolarizations. (*B*) Current–voltage relationships for peak and sustained currents. Note that inward current can be resolved at quite negative potentials. Bath solution in this experiment contained 89.8 mM Na^+, 135 mM Cl^-, 5.8 mM K^+, 1.8 mM Ca^{2+}, 0.9 mM Mg^{2+}, 4.17 mM HCO_3^-, 0.34 mM HPO_4^{2-}, 0.44 mM $H_2PO_4^-$, 0.4 mM SO_4^{2-}, 10 mM dextrose, 2.9 mM sucrose, 10 mM Hepes, 40 mM tetraethylammonium (pH 7.4). Hp, holding potential.

experiments, slope conductance averaged 218 ± 25 pS and the reversal potentials were −3.1 ± 3.1 mV (n = 3; cells bathed in 140 mM K^+). Openings occurred at patch potentials positive to +30 mV, and the open probability increased as a function of voltage. Four inside-out excised patches were studied. The open probability of these channels increased dramatically when Ca^{2+} was increased from 0.1 to 1.0 μM. A 10-fold change in free Ca^{2+} caused a greater activation of channels than a 100-mV change in potential (Fig. 5).

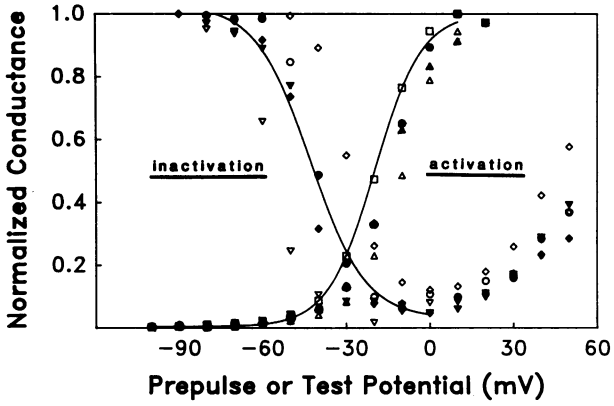

FIG. 4. Steady-state activation and inactivation of Ca^{2+} channels. The voltage dependence of activation and inactivation were determined by voltage-clamp experiments described in ref. 16. Data from four cells were fit by Boltzmann functions. Overlap of inactivation and activation curves reveals voltage range for "window current."

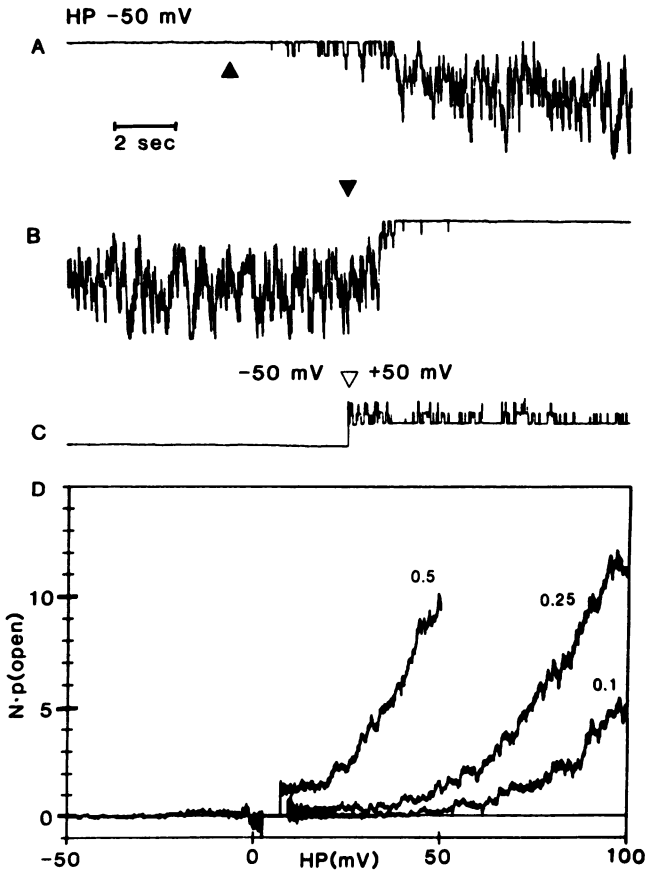

FIG. 5. Ca^{2+} activation of K channels. (*A*) An inside-out patch was held at −50 mV and bath Ca^{2+} concentration was changed from 0.1 to 1.0 μM (▲). This resulted in activation of multiple channels. (*B*) When Ca^{2+} was changed from 1.0 to 0.1 μM (▼), the probability of channel opening rapidly decreased. (*C*) Changing the holding potential (HP) from −50 to +50 mV at 0.1 μM Ca^{2+} (▽) resulted in an increase of open probability. (*D*) The number of channels in a single membrane patch times the open probability of channels [N·p(open)] as a function of voltage at three Ca^{2+} concentrations (0.1–0.5 μM) is shown. These curves were calculated by dividing the averaged current in response to voltage ramps by the single-channel amplitude (see ref. 6). Data show a significant shift in activation range of channels as a function of Ca^{2+} concentration.

The voltage and Ca^{2+} dependence of these K channels was further investigated by measuring current responses to potentials ramped between +100 and −50 mV at bath Ca^{2+} concentrations ranging from 0.1 to 0.5 μM. The open probability was calculated from averaged current responses as described (17). Increasing Ca^{2+} caused a shift in the activation curve to more negative potentials (Fig. 5*D*). These data show that the voltage and Ca^{2+} dependence of Ca^{2+}-activated K channels in ICC-like cells are not significantly different from those of colonic smooth muscle cells (17).

Autorhythmicity in ICC. Further experiments were performed on 12 ICC under current clamp to determine whether isolated ICC were capable of spontaneous rhythmicity. From resting potential or when small holding currents were applied to hyperpolarize cells to about −80 mV (resting potential of cells in pacemaker region; see ref. 3), spontaneous rhythmic depolarizations were observed (Fig. 6*A*). In most cases, these events were characterized by an upstroke depolarization to about −20 mV, partial repolarization to a plateau level that persisted several seconds, and eventual repolarization to the resting level. These events closely resembled colonic slow waves in terms of waveform shape, amplitude, and duration (see Fig. 6 *Inset*) and were unlike the action potentials previously recorded from isolated gastrointestinal muscle

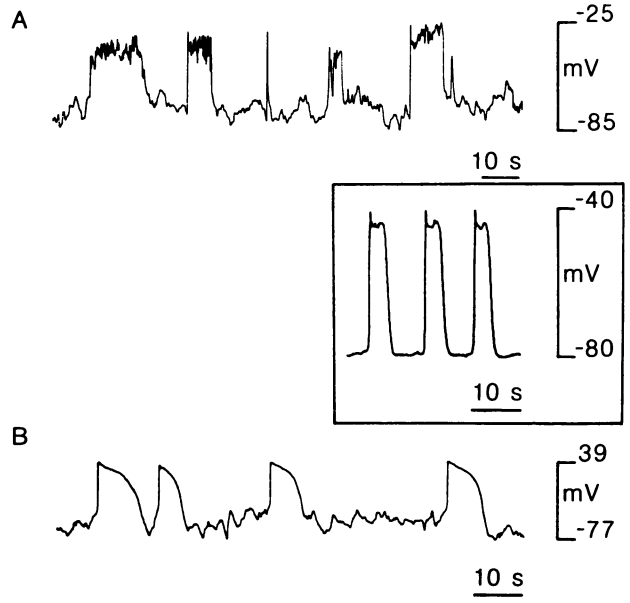

FIG. 6. Spontaneous electrical activity of isolated ICC. (*A*) Cells under current clamp displayed spontaneous depolarizations. These events resembled slow waves recorded from the pacemaker region of canine proximal colon (see *Inset* and ref. 20). Occasionally, spontaneous events were more spike-like, lacking a plateau phase. (*B*) Spontaneous events that occurred when outward currents were blocked with Cs^+ in the pipette and tetraethylammonium in the bath are shown. Application of a small holding current (5–20 pA) to hyperpolarize cells to normal resting potentials facilitated development of rhythmicity. These events overshot 0 mV and persisted for several seconds.

cells (21, 22). Cells in which outward currents were blocked were also spontaneously rhythmic (Fig. 6*B*). These events were greater in amplitude and duration, overshooting 0 mV and persisting for ≈10 s. Small applied currents (9–10 pA; duration, 250 ms) were capable of eliciting premature active events of similar waveform, further demonstrating the excitability of these cells.

Despite the fact that spontaneous rhythmic depolarizations appear to result from Ca^{2+} influx, these events were not associated with contraction of the cells. Moreover, contractions were not elicited by application of acetylcholine, norepinephrine, depolarizing concentrations of external K^+, or damage to the plasma membrane, all of which can elicit shortening of colonic smooth muscle cells (23). The observation that ICC were noncontractile is consistent with the absence of thick filaments in these cells (Fig. 1*B*; see also ref. 6).

DISCUSSION

According to Thuneberg's classification of ICC in the mouse small intestine (13), the ICC along the submucosal border of the circular layer in the canine colon would fall into the type III category (6). These cells are not stained by methylene blue (13). The lack of a specific stain makes identification of these cells difficult. Therefore, we chose to compare the ultrastructural features of isolated, putative ICC with ICC *in situ*. When isolated ICC-like cells were examined with TEM, many of the ultrastructural features common to ICC, most notably the high density of mitochondria and the presence of caveolae, were observed. These ultrastructural comparisons strongly suggest that the cells we have isolated are ICC.

The physiological role of ICC has been a mystery. Two physiological studies have used methylene blue to attempt to lesion ICC in cat and mouse small intestine (5, 14). In both preparations, methylene blue treatment blocked slow-wave

activity; however, in the mouse large depolarizations accompanied the disappearance of slow waves (14). Direct depolarization of smooth muscle cells could be responsible for the blockade of rhythmicity (24). Experiments on canine muscles have shown that methylene blue is not a specific antagonist of ICC function (24). Another problem in determining the physiological role of ICC is the difficulty in recording from these cells (5). Even if verified impalements could be made, it is unlikely that definitive results could be obtained about whether ICC are excitable or whether ICC generate electrical rhythmicity because ICC are electrically coupled to smooth muscle. Therefore, from intracellular recordings *in situ* it would be very difficult to discriminate between electrotonic spread of activity from neighboring smooth muscle cells and true pacemaker activity by ICC. Without specific drugs to lesion ICC and in light of the difficulties in studying these cells *in situ* with intracellular electrophysiological techniques, we reasoned that the electrical behavior of ICC would be best studied in isolated cells.

Our electrophysiological studies on isolated ICC support the notion that ICC are excitable, but noncontractile, cells. Significant voltage-dependent inward and outward currents were initiated upon depolarization. These events could provide a mechanism for the electrical events recorded under current clamp. The fact that the activation/inactivation relationships for the inward current overlap suggests that these cells may be capable of sustaining inward current for several seconds. A balance between sustained inward and outward currents might explain the plateau phase of the spontaneous electrical activity (see Fig. 6*A*) in which membrane potential lingers in the "window current" range for several seconds. To date it is unclear what initiates these events because it appears that activation of resolvable Ca^{2+} current occurs positive (at -60 mV) to the resting potential of cells in the pacemaker area *in situ* (approximately -80 mV; ref. 3). Repolarization is not understood either, but it is possible that Ca^{2+} entering during the plateau phase could lead to activation of Ca^{2+}-activated K channels. In support of this hypothesis, we have found that the activation curve of Ca^{2+}-activated K channels shifted into the voltage range experienced by these cells during spontaneous electrical events when Ca^{2+} increased from 0.1 to 1.0 μM.

A major question facing the ICC hypothesis is whether these cells can develop enough current to activate the adjacent smooth muscle syncytium. Electrically coupled smooth muscle would appear to present an enormous load on pacemaker cells. Recent characterization of circular smooth muscle cells shows that inward current can be resolved with test potentials positive to -50 mV (16). Since the circular cells adjacent to ICC maintain resting potentials in the -70- to -80-mV range, it would appear necessary for ICC to generate enough current to depolarize smooth muscle cells by 10–20 mV. Even though an individual ICC may be capable of producing hundreds of pA of inward current, this may not be enough current to bring circular muscle cells to threshold. In the canine proximal colon, ICC are organized into a two-dimensional network along the submucosal surface of the circular layer; frequent gap junctions occur between ICC (6). It is possible that pacemaker activity elicits regenerative events in the ICC network, and the abundant connections between ICC facilitate rapid spread of these events through the ICC network. Thus, regenerative spread of pacemaker activity within the ICC network may greatly amplify the current supplied to the electrically coupled smooth muscle syncytium, and the organization of ICC into an excitable surface may provide simultaneous activation of many smooth muscle cells. This hypothesis will probably require testing with modeling techniques.

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