Diverse Ionic Currents and Electrical Activity of Cultured Myenteric Neurons From the Guinea Pig Proximal Colon

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Vogalis, Fivos, Kirk Hillsley, and Terence K. Smith. Diverse ionic currents and electrical activity of cultured myenteric neurons from the guinea pig proximal colon. J. Neurophysiol. 83: 1253–1263, 2000. The aim of this study was to perform a patch-clamp analysis of myenteric neurons from the guinea pig proximal colon. Neurons were enzymatically dispersed, cultured for 2–7 days, and recorded from using whole cell patch clamp. The majority of cells fired phasically, whereas about one-quarter of the neurons fired in a tonic manner. Neurons were divided into three types based on the currents activated. The majority of tonically firing neurons lacked an A-type current, but generated a large fast transient outward current that was associated with the rapid repolarizing phase of an action potential. The fast transient outward current was dependent on calcium entry and was blocked by tetraethylammonium. Cells that expressed both an A-type current and a fast transient outward current were mostly phasic. Depolarization of these cells to suprathreshold potentials from less than −60 mV failed to trigger action potentials, or action potentials were only triggered after a delay of >50 ms. However, depolarizations from more positive potentials triggered action potentials with minimal latency. Neurons that expressed neither the A-type current or the fast transient outward current were all phasic. Sixteen percent of neurons were similar to AH/type II neurons in that they generated a prolonged afterhyperpolarization following an action potential. The current underlying the prolonged afterhyperpolarization showed weak inward rectification and had a reversal potential near the potassium equilibrium potential. Thus cultured isolated myenteric neurons of the guinea pig proximal colon retain many of the diverse properties of intact neurons. This preparation is suitable for further biophysical and molecular characterization of channels expressed in colonic myenteric neurons.

INTRODUCTION

Myenteric neurons comprise a heterogeneous population of cells, including sensory neurons, motor neurons, and interneurons (Costa et al. 1996; Furness et al. 1990), which coordinate the contractile activities of the muscle. Intracellular microelectrode recordings have revealed two broad electrophysiological classes of myenteric neuron in both the small and large intestine, called S/Type 1 and AH/Type 2 neurons (Bornstein et al. 1994). Fast excitatory postsynaptic potentials (fEPSPs) can be readily evoked in S neurons (S for synaptic), whereas they are rarely evoked in AH neurons (Hirst et al. 1974; Nishi and North 1973). AH neurons are named for their characteristically slow onset and long-lasting calcium-dependent afterhyperpolarization (3–20 s duration), which follows action potential firing in these neurons. S neurons comprise the interneurons and motor neurons within the reflex pathways (Brookes et al. 1997; Smith et al. 1992), whereas AH neurons include the primary afferent neurons (Kunze et al. 1995; Smith 1994).

In both the small and large intestine, most S neurons (80–90%) are phasic or rapidly adapting, with a minority of tonic or slowly adapting cells (Kunze et al. 1997; Wade and Wood 1988). Highly excitatory tonic S neurons can be more readily impaled in the corners of ganglia near the junction with internodal strands (Smith et al. 1999). Differences in the electrical activities and connections between myenteric neurons may well account for the different neurally mediated motility patterns that are generated in different regions of the gastrointestinal tract (Spencer et al. 1999; Stevens et al. 1999).

At the cellular level, the phasic firing pattern of AH neurons has been attributed to the expression of a Ca 2+ -activated K + conductance (G K-Ca ) (Hirst et al. 1985; Morita and North 1985). The slow afterhyperpolarization in AH neurons is abolished by blocking Ca 2+ entry (Hirst et al. 1974, 1985; Nishi and North 1973) and is generated by an as yet to be identified tetraethylammonium (TEA)-resistant (Hirst et al. 1985) and partially charybdotoxin-sensitive K + channel (Kunze et al. 1994). Neurons in which this conductance is inhibited, by stimulation of muscarinic (North and Tokimasa 1983) or tachykinin receptors (Morita and North 1985), can fire in a tonic manner. Although the expression of G K-Ca may account for the phasic firing pattern in AH neurons, in S neurons a phasic firing pattern probably depends on the differential expression of conductances other than G K-Ca, because a large proportion of S neurons also fires phasically (Bornstein et al. 1994; Kunze et al. 1997) but lack G K-Ca, and a subpopulation of S neurons that do express a G K-Ca (S K-type) are tonically active (Shuttleworth and Smith 1999; Smith et al. 1999).

In the rat and guinea pig sympathetic ganglia, the phasic firing pattern of neurons has been attributed to the higher expression of a large M-type K + channel current (Cassell et al. 1986; Wang and McKinnon 1995). Such M-type currents, however, have not been reported in myenteric neurons (Galligan et al. 1989; Morita and North 1985). In addition to the absence of an M-type current, repetitive firing pattern may be favored by the expression of an anomalously rectifying inward (I h ) current, as in A-type cells in the rat nodose ganglion (Doan and Kunze 1999).

In the present study the relationship between firing activity and the expression of ionic currents, principally K + -channel currents, in cultured myenteric neurons from the guinea pig proximal colon was investigated. Intact myenteric neurons from the proximal colon exhibit a diversity of firing patterns (Messenger et al. 1994). The aim of this study was to investi-
gate whether cultured neurons from this tissue retain their heterogeneity in culture, and whether the different firing patterns can be attributed to the expression of particular ionic conductances.

**METHODS**

**Dissociation of myenteric plexus**

Male guinea pigs (Simonsen Laboratories, Gilroy, CA; 200–300 g) were killed by asphyxiation with CO₂ in a specially constructed chamber and then exanguinated, in compliance with the requirements of the Animal Ethics Committee at the University of Nevada. A 3-cm length of proximal colon was removed and cleaned of its contents by flushing physiological saline through the lumen. The tissue was then sectioned longitudinally and pinned out flat in a dish containing sterile Ca²⁺-free Hanks solution. The mucosa and most of the circular muscle layer were dissected away using fine forceps and scissors under a binocular microscope. The myenteric plexus-longitudinal muscle preparation was cut into small pieces and transferred to a test tube containing 0.2% collagenase (Worthington) dissolved in Ca²⁺-free Hanks solution. The tissues were incubated in this solution for 12 min at 37°C and then washed four times with enzyme-free Ca²⁺-free Hanks solution and gently triturated through a fire-polished glass Pasteur pipette for 10–15 min. The suspension was then centrifuged at 800 rpm for 10 min after which the supernatant was discarded and the pellet resuspended in 2 ml of Ca²⁺-free Hanks solution. Aliquots of this solution were added to 35-mm plastic dishes fitted with glass coverslip bottoms that had been previously coated with polyornithine and laminin. The dishes contained 2.5 ml each of cell culture medium consisting of Dulbecco’s modified Eagle’s medium (DMEM no. 11885; GIBCO, Gaithersburg, MD) plus 10% fetal bovine serum. The muscle layer were dissected away using fine forceps and scissors and laminin. The dishes contained 2.5 ml each of cell culture medium consisting of Dulbecco’s modified Eagle’s medium (DMEM no. 11885; GIBCO, Gaithersburg, MD) plus 10% fetal bovine serum. The majority of neurons that were dissociated from the colon retained neuronal characteristics and were used for electrophysiological recordings. The remaining muscle preparations were cut into small pieces and transferred to a test tube containing 0.2% collagenase (Worthington) dissolved in Ca²⁺-free Hanks solution. The tissues were incubated in this solution for 12 min at 37°C and then washed four times with enzyme-free Ca²⁺-free Hanks solution and gently triturated through a fire-polished glass Pasteur pipette for 10–15 min. The suspension was then centrifuged at 800 rpm for 10 min after which the supernatant was discarded and the pellet resuspended in 2 ml of Ca²⁺-free Hanks solution. Aliquots of this solution were added to 35-mm plastic dishes fitted with glass coverslip bottoms that had been previously coated with polyornithine and laminin. The dishes contained 2.5 ml each of cell culture medium consisting of Dulbecco’s modified Eagle’s medium (DMEM no. 11885; GIBCO, Gaithersburg, MD) plus 10% fetal bovine serum. The cells were continuously bathed with a solution containing (in mM) 150 NaCl, 2 KCl, 1 MgCl₂, 5 CaCl₂, 10 HEPES, and 11 glucose, pH adjusted to 7.4 with NaOH. The bathing solution in the dish was maintained at 32–34°C. The majority of neurons that were patched lay isolated away from the clumps of cells, and the tip of the patch electrode was lowered onto the center of the cell body (Fig. 1). Membrane currents and membrane potentials were recorded using an EPC-9 patch-clamp amplifier (Heka Instruments, Lambrecht, Germany) and Pulse software running under Windows 95. Currents were usually filtered on-line at 2–5 kHz and digitized at 10 kHz. Current-clamp recordings were digitized at various frequencies (0.5–20 kHz). Patch pipettes were drawn from thin-walled fiber-filled capillary glass (Clark, GF-115011) to have resistances of 2–5 MΩ when filled with the following standard internal solution: 160 KCl, 1 MgCl₂, 2 Na₂ATP, 0.5 NaGTP, 10 HEPES, and 0.1 EGTA. The pH of this solution was adjusted to 7.2 with KOH. Where indicated, recordings were also obtained with pipettes containing high-EGTA (10 mM) intracellular solution and 1 mM CaCl₂. Series resistance compensation was usually employed (60–70%), but, as illustrated in Fig. 2, this was insufficient to faithfully space clamp the cell and resulted in unclamped action potentials being triggered. Capacitance transients were cancelled using a P/4 procedure. In the majority of recordings, the current-voltage (I-V) relationship was linear at potentials between −80 and −110 mV. In some cells, however, the “leak” current rectified inwardly (especially when [K⁺]o was raised to 5 mM) and the P/4 procedure was not employed, but the linear leak-current component was subtracted off-line.

All drugs used were bought from Sigma (St. Louis, MO). Stock solutions of the following drugs were made up: tetraethylammonium-Cl (TEA; 1 M), 4-aminopyridine (4-AP; 0.5 M, pH 7), and tetrodotoxin (TTX; 1 mM). All drugs used were bought from Sigma (St. Louis, MO). Stock solutions of the following drugs were made up: tetraethylammonium-Cl (TEA; 1 M), 4-aminopyridine (4-AP; 0.5 M, pH 7), and tetrodotoxin (TTX; 1 mM). All drugs used were bought from Sigma (St. Louis, MO). Stock solutions of the following drugs were made up: tetraethylammonium-Cl (TEA; 1 M), 4-aminopyridine (4-AP; 0.5 M, pH 7), and tetrodotoxin (TTX; 1 mM). The latter was stored at −20°C, whereas TEA and 4-AP were stored at 2–8°C.

**Analysis of data**

I-V plots were constructed for the current traces by averaging the current over the last 25 ms of 100- to 200-ms test pulses (I_{OUT-SUST}) and in the case of rapidly inactivating currents, measuring the maximum outward current generated within the initial 25 ms of the test pulse. Where illustrated, conductance-voltage (G-V) curves were constructed by dividing the current by driving force assuming a reversal potential close to −106 mV for our transmembrane [K⁺] gradient. For statistical comparison and averaging, currents were normalized to the peak current recorded at 0 mV.

Passive membrane resistance was determined from the slope of the I-V relationship of the nonleak subtracted currents in the region of −60 to −80 mV by fitting linear regression lines through the data points representing I_{OUT-SUST}. Usually this value closely matched the input resistance determined automatically by the amplifier in the whole cell mode. The resting membrane potential (RMP) was measured as the interpolated “zero current potential” at which the I-V of the end-of-pulse current crossed the voltage axis. The capacitance of the soma was usually read off the capacitance cancellation circuitry on the amplifier in the whole cell mode, or it was estimated by measuring the area under the capacitance current evoked by a 20-mV hyperpolarizing step and dividing by this value by 20 mV (Armstrong and Gilly 1992). Student’s t-test was used to test for statistical significance between means.

![FIG. 1. A bright-field image of colonic myenteric neurons in cell culture.](image-url)
RESULTS

Electrical characteristics of cultured myenteric neurons

Whole cell current recordings were obtained from a total of 132 cells that had been kept in culture for 2–7 days. Cells that were patch clamped had smooth agranular cell bodies with one or more axonal processes (Fig. 1). Neuronal cells were identified as those generating fast inward currents ($I_{IN-FAST}$) at the onset of a depolarizing voltage step. Based largely on the configuration of the outward currents that followed $I_{IN-FAST}$, neuronal cells were divided into three groups. In group I, cells generated a fast transient outward current ($I_{OUT-FAST}$), but they also expressed a subthreshold A-type transient outward current ($I_A$) at depolarizations positive of $-40$ mV. In group II, cells lacked a subthreshold $I_A$ but generated the $I_{IN-FAST}/I_{OUT-FAST}$ complex and an outward current that was sustained for the duration of test pulses ($I_{OUT-SUST}$), which was present in all cells. Neuronal cells that generated neither $I_{OUT-FAST}$ nor $I_A$, but only expressed $I_{OUT-SLOW}$ made up group III. Eight cells were inexcitable and lacked $I_{IN-FAST}$ and were assumed not to be neurons. Typical currents recorded from representative cells from each group are shown in Fig. 2. Also shown are the responses of these cells to depolarizing current pulses, recorded under current clamp.

Although these groups did not clearly distinguish between cells with different firing properties, a larger proportion of tonically firing cells were classified in group II. Some basic properties of cells in each group are given in Table 1. Notable differences between cell groups included the more negative “resting” potentials (or zero current voltages, $V_{I_50}$), the larger cell capacitance of group II cells, and the longer duration action potentials of cells in group III. Overall, the majority of neurons fired in a phasic manner (64%), but tonically firing neurons (20%) and AH neurons (16%) were also recorded. The outward currents generated by these cells were then analyzed in more detail.

![Figure 2](image_url)  FIG. 2. Electrical characteristics underlying the 3 groups of colonic neurons. A: group I neurons. A1: in response to a series of depolarizing steps (responses are shown to $-30$, $-50$, and $+10$ mV), these neurons exhibited a variety of outward currents in whole cell voltage-clamp recordings, distinguished by a subthreshold transient outward current ($I_A$). A2: typically fired in a phasic manner in current-clamp mode, but only after a characteristic delay and a creep of the electrotonic potential. B: group II neurons. B1: lacked the low-threshold $I_A$, but generated a fast transient outward current at suprathreshold potentials ($I_{OUT-FAST}$). B2: less likely to fire in a phasic manner. C: group III neurons. C1: only exhibited a slowly activating sustained outward current ($I_{OUT-SUST}$). C2: fired phasically.

### TABLE 1. Characteristics of three groups of cultured myenteric neurons

<table>
<thead>
<tr>
<th>Neuron Type</th>
<th>$V_{I_{50}}$, mV</th>
<th>$R_m$, MΩ</th>
<th>$C_m$, pF</th>
<th>Phasic, %</th>
<th>Tonic, %</th>
<th>AP Duration, ms</th>
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<tbody>
<tr>
<td><strong>Group I</strong></td>
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<tr>
<td>$I_A$ High EGTA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>$-42 \pm 36 (22)$</td>
<td>$910 \pm 240$</td>
<td>$32 \pm 4 (29)^* \pm 0.32$</td>
<td>$86 (18/21)$</td>
<td>$14 (3/21)$</td>
<td>$2.7 \pm 0.36$</td>
</tr>
<tr>
<td>$I_A$ Low EGTA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>$-39 \pm 5 (9)$</td>
<td>$1,035 \pm 527$</td>
<td>$24 \pm 2 (21)^* \pm 0.32$</td>
<td>$86 (18/21)$</td>
<td>$14 (3/21)$</td>
<td>$2.7 \pm 0.36$</td>
</tr>
<tr>
<td>$I_{OUT-FAST}$ Low EGTA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>$-44 \pm 4 (13)$</td>
<td>$823 \pm 204$</td>
<td>$52 \pm 4 (28)^* \pm 0.32$</td>
<td>$86 (18/21)$</td>
<td>$14 (3/21)$</td>
<td>$2.7 \pm 0.36$</td>
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<tr>
<td><strong>Group II</strong></td>
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<td></td>
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<tr>
<td>$I_A$ High EGTA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>$-39 \pm 5 (9)$</td>
<td>$1,035 \pm 527$</td>
<td>$32 \pm 4 (29)^* \pm 0.32$</td>
<td>$86 (18/21)$</td>
<td>$14 (3/21)$</td>
<td>$2.7 \pm 0.36$</td>
</tr>
<tr>
<td>$I_A$ Low EGTA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>$-44 \pm 4 (13)$</td>
<td>$823 \pm 204$</td>
<td>$52 \pm 4 (28)^* \pm 0.32$</td>
<td>$86 (18/21)$</td>
<td>$14 (3/21)$</td>
<td>$2.7 \pm 0.36$</td>
</tr>
<tr>
<td>$I_{OUT-FAST}$ Low EGTA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>$-44 \pm 4 (13)$</td>
<td>$823 \pm 204$</td>
<td>$52 \pm 4 (28)^* \pm 0.32$</td>
<td>$86 (18/21)$</td>
<td>$14 (3/21)$</td>
<td>$2.7 \pm 0.36$</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
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<td></td>
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<tr>
<td>$I_{OUT-SLOW}$ High EGTA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>$-42 \pm 7 (7)$</td>
<td>$574 \pm 145$</td>
<td>$26 \pm 7 (4)^* \pm 0.32$</td>
<td>$86 (18/21)$</td>
<td>$14 (3/21)$</td>
<td>$2.7 \pm 0.36$</td>
</tr>
<tr>
<td>$I_{OUT-SLOW}$ Low EGTA&lt;sub&gt;i&lt;/sub&gt;</td>
<td>$-44 \pm 4 (13)$</td>
<td>$823 \pm 204$</td>
<td>$52 \pm 4 (28)^* \pm 0.32$</td>
<td>$86 (18/21)$</td>
<td>$14 (3/21)$</td>
<td>$2.7 \pm 0.36$</td>
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</table>

Values are means ± SE with number of cells in parentheses. The table shows the electrical characteristics of cultured colonic myenteric neurons. Neurons were classified into 3 distinct groups.
Properties of \( I_{\text{OUT-FAST}} \) and role in the action potential

The fact that \( I_{\text{IN-FAST}} \) was activated over a narrow voltage range (−30 to −20 mV), in an all-or-none manner, suggested strongly that it was generated by action potentials that were triggered in a region of the cell that had escaped voltage control. Similarly, the close temporal relationship between \( I_{\text{OUT-FAST}} \) and \( I_{\text{IN-FAST}} \) suggested that \( I_{\text{OUT-FAST}} \) was action potential dependent and that it represented current flow during the repolarization/fast afterhyperpolarization. This conclusion is supported by the observation that \( I_{\text{OUT-FAST}} \) was absent in cells treated with TTX (1–3 μM, \( n = 3 \)), which blocked \( I_{\text{IN-FAST}} \).

The identity of the conductance that was responsible for \( I_{\text{OUT-FAST}} \) was investigated using known K⁺-channel blockers. External TEA ions (5 mM, \( n = 5 \)) abolished \( I_{\text{OUT-FAST}} \) (Fig. 3A1) and increased the duration of the action potential (Fig. 3A2). In two cells tested, addition of Cd²⁺ (0.1 mM) to the bathing solution also abolished \( I_{\text{OUT-FAST}} \) without affecting the \( I_{\text{IN-FAST}} \) (Fig. 3B1), and also broadened the action potential. These data indicate that \( I_{\text{OUT-FAST}} \) was dependent on Ca²⁺ influx through voltage-gated Ca²⁺ channels and that it is generated by the opening of TEA-sensitive Ca²⁺-dependent BK-type K⁺ channels that aid in action potential repolarization. This is further supported by the fact that the addition of 4-AP (1–4 mM, \( n = 4 \)) to the bathing solution had little effect on \( I_{\text{OUT-FAST}} \) (Fig. 3C1) or on action potential firing (Fig. 3C2). This suggests that \( I_{\text{OUT-FAST}} \) was not mediated by the opening of A-type or delayed-rectifier K⁺ channels.

Properties of A-type outward current and its role in AP firing

The voltage dependence of the subthreshold \( I_A \) was determined in five cells that had been treated with TTX to remove contamination by \( I_{\text{IN-FAST}} \) and the associated \( I_{\text{OUT-FAST}} \). As shown for one of these cells in Fig. 4A, \( I_A \) was typically activated by step depolarizations positive to −40 mV and was inactivated almost to completion at holding potentials positive of −40 mV (Fig. 4A2). The corresponding conductance-voltage \( (G-V) \) relationships of \( I_A \) and of the noninactivating \( I_{\text{OUT-SUST}} \) recorded from this cell are plotted in Fig. 3B to illustrate that the conductance underlying \( I_A \) activated more negative than that which generates \( I_{\text{OUT-SUST}} \). This suggests that these two currents are generated by different voltage-gated K⁺ channels.

The availability of \( I_A \) as a function of voltage suggests that

![Figure 3](http://jn.physiology.org/)

**Figure 3.** Effect of K⁺ and Ca²⁺ channel blockers on \( I_{\text{OUT-FAST}} \) and action potential duration. A: effect of tetraethylammonium-Cl (TEA; 5 mM). B: effect of Cd²⁺ (0.1 mM). C: effect of 4-aminopyridine (4-AP; 3 mM) on \( I_{\text{OUT-FAST}} \) (top traces) and on the action potential configuration. Note that both TEA (A1) and Cd²⁺ (B1) blocked \( I_{\text{OUT-FAST}} \) and broadened the action potential (A2 and B2), whereas 4-AP has no significant effect on \( I_{\text{OUT-FAST}} \) but decreased the sustained outward current (C1) and the amplitude of the fast afterhyperpolarization (C2).
20% of the current in the cell depicted in Fig. 4C is available for activation at -60 mV. In six cells analyzed by fitting a single Boltzmann function to plots of the available current versus conditioning (holding) potential (as in Fig. 4C), the mean voltage at which $I_A$ was half-maximally inactivated was -72.5 ± 4.8 mV, with an average slope factor of -7.3 ± 0.9 mV. The inactivating portion of the current, obtained by digital subtraction of the currents elicited from holding potentials of -65 and -40 mV (see Fig. 4A3), revealed that $I_A$ peaked within 5 ms at potentials positive of 0 mV. $I_A$ inactivated almost to completion, with a mean time constant of 24.3 ± 2.6 ms at 0 mV ($n = 5$).

Classic A-type $K^+$ channel currents are blocked by millimolar 4-AP. We therefore tested the effect of 4-AP (1–4 mM) on cells expressing $I_A$. Figure 5 shows that in a phasically firing cell, 4-AP (1 mM) had little effect either on the resting potential or on the action potential configuration triggered by a depolarizing current step (Fig. 5A1), although the afterhyperpolarization appeared to be blunted. The facilitation of action potential firing by 4-AP was only apparent when the membrane potential was held at potentials negative of about -60 mV, where a significant fraction of $I_A$ becomes available (see Fig. 4C). At these holding potentials, depolarizing current steps in the absence of 4-AP failed to trigger action potentials in the soma, and the resultant electrotonic potential displayed a characteristic creep. In the presence of 4-AP (1 mM), however, the
same depolarizing current step immediately triggered an action potential, and the “creep” was absent.

Under voltage clamp, it was revealed that 4-AP (1 mM) blocked a large fraction of the subthreshold $I_A$ (Fig. 5, B1 and B2). This concentration of 4-AP was sufficient to block $I_A$ by $\sim50\%$ (Fig. 5A2). A similar reduction in the magnitude of $I_A$ by 4-AP (1–4 mM) was seen in five other cells.

**Properties of $I_{OUT-SUST}$ and its role in electrical excitability**

The voltage dependence of the sustained outward current ($I_{OUT-SUST}$) recorded from cells lacking a net subthreshold $I_A$ (groups II and III) was determined by plotting the mean end-of-pulse currents as a function of test potential (pulse duration of 100–200 ms). The $I$-$V$ relationship constructed from averaged data from 29 cells is plotted in Fig. 6B (■) and shows that $I_{OUT-SUST}$ under net-current conditions activated at potentials positive to $-30\ mV$. The $I$-$V$ relationship of $I_{OUT-SUST}$ was also determined in eight cells treated with TTX (1–3 $\mu$M) to eliminate contamination by the $I_{IN-FAST}/I_{OUT-FAST}$ complex. Typical outward currents recorded under these conditions from one such cell are shown in Fig. 6A, whereas the averaged data are plotted in Fig. 6B (□). The $I$-$V$ relationship of $I_{OUT-SUST}$ in TTX indicates that this current was generally larger and activated at more negative potentials (approximately $-50\ mV$) in the presence of TTX. The corresponding $G$-$V$ relationship constructed from these data means was well described by a single Boltzmann function with a voltage of half-maximal activation of $-10.3\ mV$ and a voltage dependence of $+15\ mV$ (Fig. 6C). These values predict that $\sim5\%$ of the conductance generating $I_{SUST}$ is activated at $-60\ mV$.

On average, externally applied TEA (5 mM) inhibited $I_{OUT-SUST}$ by $66\pm8\%$ (mean $\pm SE$, $n=8$ cells from groups I and II; Fig. 7A). In a tonically firing cell with a subthreshold $I_A$, TEA (5 mM) reduced the firing frequency from 39 to 33 Hz and also decreased the peak afterhyperpolarization from $-44$ to $-34\ mV$ (Fig. 7B). In addition, TEA increased the peak of the action potential and almost doubled its duration (Fig. 7C). In two other cells, however, both lacking $I_A$, TEA (5 mM) had no effect on $I_{OUT-SUST}$ (see Fig. 3A), suggesting that this current may be generated by a heterogeneous population of delayed rectifier-type K$^+$ channels.

**Neurons with a slow afterhyperpolarization**

A characteristic feature of AH/type 2 myenteric neurons is the development of a slow afterhyperpolarization (AH) lasting several seconds following one or more action potentials (Hirst

![FIG. 5. Effect of 4-AP on $I_A$ expressed in a phasic neuron. A1: in current-clamp mode, 4-AP (1 mM) had no effect on the firing properties of the neuron at the resting membrane potential. A2: however, if the membrane potential was held negative of $-60\ mV$, the threshold for action potential activation was lowered by 1 mM 4-AP. B1: under voltage clamp, 4-AP blocked the subthreshold $I_A$ elicited by a test depolarization to $-30\ mV$. B2: 4-AP also reduced the magnitude of peak outward current (comprised mainly of $I_A$) at more positive potentials.](http://jn.physiology.org/doi/10.1152/jn.00509.2016)
et al. 1974; Nishi and North 1973). In the present study we found nine neurons that generated slow afterhyperpolarizations after firing one or several action potentials in response to short (50 ms) depolarizing current steps. Recordings from one such cell are shown in Fig. 8A and demonstrate that the excitability of the cell is decreased during the AH; injection of the supra-threshold current pulse during the recovery phase of the AH failed to trigger another action potential (pulse b in Fig. 8A).

The duration of action potentials in these cells averaged 3.2 ± 0.4 ms (n = 9), and in six cells a characteristic shoulder on the repolarization phase was evident.

Under whole cell voltage-clamp conditions, step depolarizations in AH neurons revealed the presence of either I_{OUT-FAST} (7/9 cells) or I_{A} (2/9 cells), and so the majority of AH neurons were classified into group II because they lacked an apparent subthreshold I_{A}. To determine the voltage dependence of the current underlying the slow AH, the current that was generated after a 50-ms stimulus step to +40 mV was recorded at several potentials (Fig. 8B). This current peaked within 1–2 s after the stimulus and was outward at potentials positive to −80 mV. To isolate the current underlying the AH (I_{AH}), the background current recorded at the same potentials was subtracted from the slowly developing poststimulus current (averaged over 1 s at its peak). All three currents, including I_{AH} are plotted in Fig. 8C, which shows that the I-V relationship of I_{AH} is linear between −30 and −110 mV and appears to weakly rectify inwardly. The extrapolated reversal potential (E_{rev}) of I_{AH} lies negative of −110 mV and close to −120 mV. A similarly negative extrapolated E_{rev} for I_{AH} was calculated for the other two cells.

Inward rectification in colonic myenteric neurons

The absence of any appreciable inwardly rectifying currents in the majority of neurons described in the preceding sections may have been due to the use of low (2 mM) [K+]_o in the bathing solution. In a series of experiments in which [K+]_o was raised to 5.4 mM, we found that inwardly rectifying current was present in 20 of 32 cells, at potentials negative of −60 mV (Fig. 9). In all but three of these cells, the inwardly rectifying current (I_{IR}) lacked time dependence (Fig. 9A1), whereas in 14 of 15 cells that expressed I_{IR} also expressed I_{A} (Fig. 9A1). The nature of I_{IR} was not investigated further in the present study.
However, we noted that cells expressing $I_{IR}$ fired phasically when depolarized (Fig. 9A2), and their resting potentials averaged $-41 \pm 4 \text{ mV}$ compared with $-49 \pm 4 \text{ mV} (n = 14)$ for cells lacking this current. A slow AH was not observed in any cell exhibiting $I_{IR}$. In eight cells that fired tonically or showed slow adaptation over 500-ms depolarizations, a time-dependent inwardly rectifying current was evident at potentials negative of $-60 \text{ mV}$ (Fig. 9, B1 and B2). In one cell tested, this current was inhibited by Cs$^+$ (1 mM) added to the bathing solution, suggesting that it was an $I_h$-like current.

**DISCUSSION**

**Retention of diverse firing properties of cultured myenteric neurons**

In the present study we have shown that myenteric neurons from the proximal colon of the guinea pig retain the same electrophysiological behaviors in culture that have previously been demonstrated in intact neurons from the same tissue (Messenger et al. 1994). Our findings contrast with those in cultured myenteric neurons from the rat small intestine (Nishi and Willard 1985), over 95% of which fired phasically, and none of which exhibited prolonged afterhyperpolarizations. At present, it is unclear whether any of these differences are due to varying culture conditions, or in the case of AH cells, the length of time in culture (Nishi and Willard 1985). However, differences in the proportion of neuronal phenotypes probably also reflects regional differences in neuronal excitability, because microelectrode studies have shown that the ratio of phasic to tonic S cells varies from 9:1 in the guinea pig ileum (Bornstein et al. 1994; Kunze et al. 1997) to 4:1 in the distal colon (Wade and Wood 1988).

Therefore the most meaningful comparison for the neurons in this study is to neurons from intact preparations of the guinea pig proximal colon (Messenger et al. 1994). The proportions of tonic and phasic S neurons and AH neurons in the present study were very similar to that found in intact preparations. Cultured neurons were comprised of 16% AH neurons, and of the remaining S neurons, 80% were phasic and 20% tonic. Fresh intact neurons were similarly comprised of 18% AH neurons, and of the remaining S neurons, 79% were phasic and 21% tonic (Messenger et al. 1994). Thus, although concerns about changes in phenotypic expression of cultured neurons cannot be completely allayed, in this preparation the proportion of different neurons demonstrates a high degree of correlation with intact neurons.

However, some minor differences were observed between cultured and intact myenteric colonic neurons, primarily, the RMP of cultured neurons was more positive than intact neurons. Because whole cell patch clamping causes less of a nonspecific leak current than using sharp intracellular electrodes, this may be reflected in the different RMPs. The differences in the electrode and/or extracellular solutions are also likely to reflect differences in the RMP. For example, in the present study, EGTA was present in varying concentrations in the electrode solution, thus altering the levels of free intracellular calcium and modulating calcium-activated conductances. The RMP was consistently more positive if higher concentrations of EGTA were present (see Table 1); therefore if no EGTA was present, then the RMP would probably be similar to the levels seen in intact neurons. In addition, the extracellular HEPES solution contained lower potassium and higher calcium concentrations than the Kreb’s solution used in intact preparations. This solution was used to maximize the driving force for the expression of $I_{AHP}$, to ensure that no AH neurons were missed.
Neuronal classification

The classification of neurons employed in the present study was based on the features of the outward current generated in cultured neurons to classify cells into three groups: 1) those with a subthreshold $I_A$-like current, 2) those with an action potential–associated fast transient outward current ($I_{OUT-FAST}$), and 3) a slowly activating outward current. This was not a complete characterization of channels expressed in these neurons, and it is likely that the firing properties of neurons are a reflection of a variety of channels active at different potentials. In light of this, it is perhaps not surprising that the firing properties of the cultured myenteric neurons in this study do not absolutely correlate with the classification scheme employed. However, certain trends were observed between the firing properties and channels expressed. Tonic S neurons were predominantly (73%) those cells that expressed $I_{OUT-FAST}$ but lacked $I_A$. Phasic neurons were found in all three groups, but all neurons that lacked both $I_{OUT-FAST}$ and $I_A$ were phasic. Interestingly, although all AH neurons expressed $I_{AH}$, the majority of AH neurons (78%) also expressed $I_{OUT-FAST}$ but lacked $I_A$.

Role of $I_{OUT-FAST}$ currents

The inhibition of $I_{OUT-FAST}$ and the consequent broadening of the action potential by external TEA and by Cd$^{2+}$ suggests that it was mediated by the opening Ca$^{2+}$-dependent K$^+$ channels of the BK-type that are involved in action potential repolarization (Davies et al. 1996). The fact that $I_{OUT-FAST}$ was also TTX sensitive suggests that the rapid upstroke of the Na$^+$-dependent action potential opens N-type voltage-gated Ca$^{2+}$ channels, which in turn leads to Ca$^{2+}$ entry and the opening of BK-type channels to aid in the termination of the action potential. The transient nature of this current is consistent with the Ca$^{2+}$-dependent K$^+$ channels being activated directly by Ca$^{2+}$ entering through co-localized voltage-gated Ca$^{2+}$ channels, as described in rat hippocampal neurons for N-type Ca$^{2+}$ channels and BK channels (Marrion and Tavalin 1998). Because this outward current was only seen when an unclamped action potential was elicited suggests that the Ca$^{2+}$-dependent K$^+$ channels are situated in a region of the cell where action potentials are initiated and that cannot be properly voltage clamped.
Role of \( I_A \) outward currents

The presence of \( I_{\text{OUT-FAST}} \) in many myenteric neurons may have underestimated the proportion of cells expressing \( I_A \). A-type currents have been reported previously in intact myenteric neurons, both in AH type neurons (Hirst et al. 1985; Hoffman et al. 1997) and also in tonically firing S-type neurons (Brookes et al. 1997; Smith et al. 1999) in the guinea pig ileum. Therefore the expression of \( I_A \) does not seem to determine the phasic/tonic firing pattern of myenteric neurons. The relatively negative activation range of \( I_A \) suggests that \( I_A \) may be important in inhibiting or delaying action potential firing at potentials slightly negative of the resting level and may influence the ability of these cells to integrate incoming signals.

Difference and roles of delayed rectifier-type outward currents

The slowly inactivating or sustained outward current \( (I_{\text{OUT-SUST}}) \) recorded in myenteric neurons is probably generated by a mixture of different delayed rectifier-type \( K^+ \) channels. For example, \( I_{\text{OUT-SUST}} \) in cells expressing \( I_A \) activated at more positive potentials (approximately \(-20 \) mV) than \( I_{\text{OUT-SUST}} \) in cells lacking \( I_A \). Thus cells may express different levels of at least two different delayed-rectifier type channels, and the pharmacological sensitivity and voltage dependence of \( I_{\text{OUT-SUST}} \) may depend on the relative contributions of the whole cell current.

Functionally, the negatively activating \( I_{\text{OUT-SUST}} \) in cells lacking \( I_A \) suggests that this current may contribute to the resting potential in these cells and in generating the late repolarization phase of the action potential. The low-voltage threshold of \( I_{\text{OUT-SUST}} \) is similar to the M-type current in phasic neurons in the superior mesenteric ganglion (Wade and Wood 1988), suggesting that \( I_{\text{OUT-SUST}} \) may contribute to the phasic firing pattern of myenteric neurons. The role of the more positively activating \( I_{\text{OUT-SUST}} \) in \( I_A \)-expressing cells may be to regulate excitability at more positive potentials and prevent excessive depolarization.

Myenteric AH neurons

Unlike Nishi and Willard (1985), we found that 16% of neurons were able to generate a slow AH after a single or a few action potentials. This slow AH had many properties in common with the slow AH in the intact myenteric neurons (Hirst et al. 1985). Examination of the underlying current revealed that it had a similar time course to the AH and showed weak inward rectification characteristic of the corresponding current in intact AH neurons (North and Tokimasa 1983). The current decreased in amplitude as membrane potential approached \( E_K \), suggesting that it was carried by \( K^+ \) (Hirst et al. 1985; North and Tokimasa 1983).

More recently, Zholos et al. (1999) have suggested that the slow AH in guinea pig myenteric neurons may be generated in part by an inwardly rectifying \( K^+ \) conductance. Such currents have been reported previously in intact AH neurons (Galligan et al. 1989; Hirst et al. 1985). In the present study, however, in cells that generated a slow AH, there was little evidence of inward rectification at potentials negative of \(-80 \) mV. Inwardly rectifying currents were evident, however, when external \( K^+ \) was raised to 5.4 mM. But in the majority of cells, this inwardly rectifying current showed no obvious time dependence and was inward at potentials negative of \(-60 \) mV.

In summary, this study has shown that cultured isolated myenteric neurons of the guinea pig proximal colon retain many of the electrophysiological properties, including diverse firing patterns, of intact neurons. Therefore this preparation is suitable for further characterization of the ion channels expressed in different functional classes of myenteric neurons, which together with single-cell polymerase chain reaction, will allow us to assign molecular entities to the ionic events underlying these different firing patterns.

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