Coordinated contractions and relaxations of the smooth muscle of the gut wall form the motility patterns that are responsible for mixing and propulsion of contents. In most of the gut, the muscularis externa consists of an outermost layer of longitudinal smooth muscle cells and a thicker inner layer of circular smooth muscle cells, which are innervated by separate populations of motor neurons (7, 8). Smooth muscle cells have an intrinsic electrical activity called “slow waves,” which are initiated by interstitial cells of Cajal (ICC) and spread into smooth muscle cells via gap junctions to activate ion channels and contractile mechanisms (28, 43). Input from axons of enteric motor neurons is mediated, at least partly, via nonpacemaker types of ICCs and, possibly, other nonmuscular cells. Enteric motor input combines with slow waves to generate the complex contractile activity patterns that underlie functional motility.

In many regions of the gut, smooth muscle cells are coupled to one another to form a functional syncytium. This coupling is mediated largely by gap junctions. Gap junctions are formed by pairs of hemichannels or connexons, which allow passage of ions and small (≤1,000 Da) molecules. Each connexon is made up of six subunits (connexins), which determine the properties of the channel. Gap junctions underlie electrical and metabolic coupling of cells in the smooth muscle apparatus and are the structures by which cells are dye-coupled (54). Their role in neuromuscular transmission and smooth muscle contractility has been debated (17, 19, 45). Several studies have identified specific connexins in the smooth muscle layers of the gut; Cx43 is the most abundant, and Cx40 and Cx45 have also been shown to be present (52). The connexins may be differentially located, with Cx45 being preferentially associated with junctions between ICCs (44).

In the present study, we identified postjunctional changes in neuromuscular transmission and gap junction coupling in the circular smooth muscle of the guinea pig ileum in the 1st min after the preparation was set up in vitro until consistent responses to electrical stimulation were obtained within 120 min. The inhibitory junction potential (IJP) described by Bülbring and Tomita (9) and the excitatory junction potential (EJP) described by Gillespie and Mack (23) were used as measures of motor neuron input to smooth muscle.

METHODS

Adult male guinea pigs (200–350 g body wt) (36) were stunned by a blow to the back of the head and exsanguinated in a manner approved by the Animal Welfare Committee of Flinders University. Specimens of small intestine, >10 cm proximal to the ileocecal junction or distal colon, were removed, flushed, and placed into cooled (~14°C) Krebs solution. The circular smooth muscle layer was removed from preparations in which recordings were made from S neurons. Unless otherwise specified, Krebs solution contained (in mM) 118 NaCl, 4.70 KCl, 1 NaH2PO4·2H2O, 25 NaHCO3, 1.2 MgCl2·6H2O, 11 d-glucose, and 2.5 CaCl2·2H2O, bubbled with 95% O2–5% CO2, with 1 μM hyoscine and 1 μM nicardipine to inhibit EJPs and muscle contractions. The specimen was cut open along the mesenteric border, and the mucosa and submucosa were removed using sharp dissection. A ~10-mm-long, full-circumference segment of tissue was isolated and pinned, using 50-μm tungsten pins, circular smooth muscle uppermost, into a Sylgard-lined (Dow Corning, Midland, MI) ~1-ml recording chamber.

Intracellular recording. The recording chamber was fixed onto the stage of an inverted microscope (model LH50A, Olympus) fitted with
flourescent optics. Krebs solution at 35°C was constantly superfused at a rate of 3 ml/min. The moment at which warmed Krebs solution first reached the preparation was designated time 0. From this time point, bath temperature increased to a stable 34–35°C within 5 min.

Smooth muscle cells or neurons were impaled using borosilicate glass capillary electrodes (1 mm OD, 0.58 mm ID; Harvard Apparatus) filled with 5% 5,6-carboxyfluorescein in 20 mM Tris buffer (pH 7.0) in 1 M KCl solution. Electrode resistance was 50–200 MΩ. Membrane potential was recorded using an Axoclamp 2A amplifier (Axon Instruments), viewed on an oscilloscope (model VP-5220A, Matsushita), digitized at 10 kHz, and stored via an analog-to-digital interface (MacLab SSP, ADInstruments, Sydney, Australia) using Chart 7 software (ADInstruments). Unless otherwise specified, a 0.4-ms single-pulse 15-V electrical focal stimulus was applied via paired insulated Pt-Ir wire stimulating electrodes placed 1 mm circumferential to the recording electrode. A Grass S48 stimulator and a Grass SIU5 stimulator isolation unit were used to generate the stimulus. With this stimulus, there was little contribution of nitric oxide or peptidergic transmitters to the large-amplitude fast IJP (fIJP) stimuli. With this stimulus, there was little contribution of nitric oxide or peptidergic transmitters to the large-amplitude fast IJP (fIJP) stimuli. With this stimulus, there was little contribution of nitric oxide or peptidergic transmitters to the large-amplitude fast IJP (fIJP) stimuli. With this stimulus, there was little contribution of nitric oxide or peptidergic transmitters to the large-amplitude fast IJP (fIJP) stimuli. With this stimulus, there was little contribution of nitric oxide or peptidergic transmitters to the large-amplitude fast IJP (fIJP) stimuli. With this stimulus, there was little contribution of nitric oxide or peptidergic transmitters to the large-amplitude fast IJP (fIJP) stimuli. With this stimulus, there was little contribution of nitric oxide or peptidergic transmitters to the large-amplitude fast IJP (fIJP) stimuli.

At the end of the recording period, cells were labeled with carboxyfluorescein by 0.2-s 0.5-nA hyperpolarizing current impulses at 2.5 Hz (50% duty cycle) for 2 min. The electrode was then withdrawn for measurement of resting membrane potential (RMP). Carboxyfluorescein-labeled cells were counted in situ 1 min later under a ×10 objective using an appropriate dichroic mirror and filter. ATP (10 mM, in Krebs solution) was pressure-ejected with nitrogen pulses (140 kPa, 20–50 ms) onto the recorded cell from a 1-mm borosilicate pipette that was broken to have a ~10-μm-diameter tip and placed 100 μm circumferential to the recording site. Inert food dye (Famous Queen; 0.01%) was used to visualize the trajectory of the ejection to ensure that it passed over the area of the electrode. Carbenoxolone (30, 50). Input resistance was calculated by injection of current impulses (0.1–0.5 nA) and measurement of related potential changes. Membrane potential was recorded using an Axoclamp 2A amplifier against time after preparation set-up. Reponses increased significantly within 120 min (c, P < 0.001). IJP amplitude plateaued after 120 min (a, P > 0.05). C: binned data (from B) shows mean fast IJP amplitude recorded in 10-min time intervals. D: mean fast IJP amplitude was significantly greater in cells impaled at ≤30 min than at >120 min (20 and 48 cells, respectively, n = 12). *P < 0.001.

RESULTS

Electrophysiological properties of circular smooth muscle cells following dissection. Single-shot electrical stimuli evoked fIJP that averaged −1.3 ± 1.0 mV in the first 30 min after the preparations were set up (Fig. 1D; 20 cells). However, their amplitude increased significantly after 120 min to −15.8 ± 0.9 mV (Fig. 1D; n = 12, 48 cells, P < 0.001). The averaged rate of increase of the fIJP, after commencement of superfusion of the preparation with warmed Krebs solution, is plotted in Fig. 1C. Linear regression over the first 120 min revealed a significant time-dependent increase in fIJP amplitude (P < 0.001, n = 12; Fig. 1B); however, it did not increase further after 120 min.

RMP depolarized significantly over the first 120 min (P < 0.001) and did not significantly change thereafter (P > 0.05, n = 12; Fig. 2A). RMP averaged −58.9 ± 1.3 mV in the first 30 min (20 cells) and −47.7 ± 0.6 mV after 120 min (48 cells, n = 12, P < 0.001; Fig. 2C). Apparent input resistance of circular smooth muscle cells was calculated from an current-voltage curve plotted for 10-ms-long current pulses (300–500 pA) after the bridge was balanced by the method of Martin et al. (36) (Fig. 3, A and B). Measurements showed a decreasing trend over the first 120 min, although this did not reach significance. Input resistance then stabilized (P > 0.05, n = 12; Fig. 3C). Input resistance was significantly greater in the first 30 min than after 120 min [14.4 ± 2.2 MΩ (16 cells) vs. 8.4 ± 0.7 MΩ (42 cells), P < 0.05, n = 12; Fig. 3E].

A standardized current-injection protocol was used to fill impaled muscle cells with 5% carboxyfluorescein in the recording microelectrode at the end of the recording. When viewed 1 min later, under standard illumination and magnification, the recorded cell could be clearly distinguished from coupled cells by its higher fluorescence intensity. Average dimensions were 476.6 ± 24.8 μm long, with a maximum diameter that averaged 3.3 ± 0.2 μm, in the spindle-shaped cells. In the first 30 min of the recording period, one to three recordings (Microsoft Excel), with the first 120 min and time after 120 min treated separately.

Data analysis. Values are means ± SE; n refers to the number of preparations. Statistical analysis was performed by Student’s two-tailed t-test for paired or unpaired samples using Microsoft Excel 2004. Where applicable, x-y plots were analyzed using linear regression (MacLab 8SP, ADInstruments, Sydney, Australia) using Chart 7 software (ADInstruments).
dye-filled cells were labeled after filling a single smooth muscle cell. More dye-coupled cells were distinguishable, extending beyond the ends of the impaled cell (Fig. 4D), as time after preparation set-up increased. Dye coupling increased significantly, in a time-dependent manner, within the first 120 min ($P < 0.001$; Fig. 4A) and stabilized thereafter. Dye coupling was significantly less in the first 30 min than after 120 min of the recording period ($2.0 \pm 0.3$ (20 cells) vs. $4.2 \pm 0.3$ (46 cells), $n = 12$, $P < 0.001$; Fig. 4C). These results suggest that gap junction coupling between circular smooth muscle cells increased significantly over the first 120 min after a preparation was set up, consistent with the trend toward decreased input resistance.

**Electrophysiological properties of longitudinal smooth muscle cells.** Immunohistochemical labeling of connexins shows that they are consistently less abundant in the longitudinal than circular smooth muscle layer (18). We compared the electrophysiological properties of circular and longitudinal smooth muscle cells. As previously reported (12, 13), fIJPs were smaller in longitudinal than circular smooth muscle cells, even after 120 min ($0.4 \pm 0.1$ vs. $14.7 \pm 0.9$ mV, $n = 6$; Fig. 5A). This probably reflects a sparser innervation by inhibitory motor neurons (7). RMP did not differ between longitudinal and circular smooth muscle cells ($-48.9 \pm 0.7$ and $-47.5 \pm 0.6$ mV, respectively, $n = 6$; Fig. 5B). The mean input resistance of longitudinal smooth muscle cells was significantly greater than that of circular smooth muscle cells recorded $> 2$ h after preparation set-up ($26.5 \pm 2.8$ vs. $8.9 \pm 0.9$ MΩ, $n = 5$, $P < 0.001$; Fig. 5C). Significantly less dye coupling was observed in longitudinal than circular smooth muscle cells ($1.2 \pm 0.1$ vs. $4.0 \pm 0.3$ cells, $n = 6$, $P < 0.001$; Fig. 5D). These results confirm previous findings and validate the methods used to measure dye coupling and input resistance.

**Effects of gap junction blockers on the excitability of circular smooth muscle cells.** The effects of gap junction blockers, carbenoxolone, 18β-glycyrrhetinic acid, and 2-APB, were tested on circular smooth muscle cells, after full responses had developed, to determine whether pharmacological reduction of gap junction coupling altered tissue excitability. In the presence of 100 μM carbenoxolone, fIJPs were reduced significantly from a control value of $-14.6 \pm 1.2$ to $-7.8 \pm 1.4$ mV ($P < 0.005$, $n = 6$). Carbenoxolone (100 μM) induced a small, but significant, hyperpolarization of circular smooth muscle cells from $-47.0 \pm 0.5$ mV in controls to $-49.3 \pm 0.5$ mV ($P < 0.05$, $n = 6$), and apparent input resistance increased from $8.9 \pm 1.7$ to $30.8 \pm 4.0$ MΩ ($P < 0.005$, $n = 6$). Correspondingly, dye coupling in equilibrated circular smooth muscle cells decreased from $4.4 \pm 0.5$ to $1.2 \pm 0.1$ cells ($P < 0.005$, $n = 6$; Table 1).
Similar results were obtained with 10 μM 18β-glycyrrhetinic acid. Mean fIJP amplitude was reduced from $14.0 \pm 1.8$ to $6.2 \pm 1.1$ mV ($P < 0.05, n = 6$). Cells were not hyperpolarized by 10 μM 18β-glycyrrhetinic acid ($-50.6 \pm 1.4$ vs. $-49.5 \pm 0.6$ mV for control, $n = 6$, not significant), but input resistance increased from $8.5 \pm 0.6$ MΩ in control to $111.5 \pm 40.4$ MΩ in the gap junction blocker ($P < 0.05, n = 6$). Correspondingly, dye coupling was reduced from $5.7 \pm 0.6$ to $1.2 \pm 0.07$ cells ($P < 0.001, n = 6$; Table 1).

2-APB has been shown to block gap junctions (1), including those consisting of Cx43 (55). 2-APB significantly reduced fIJP amplitude (from $-16.1 \pm 0.8$ to $-0.6 \pm 0.5$ mV, $P < 0.001, n = 4$). RMP of cells displayed a nonsignificant trend to hyperpolarization in the presence of 2-APB (from $-50.4 \pm 0.5$ to $-54.1 \pm 1.4$ mV, $n = 4, P = 0.08$). Input resistance increased in the presence of 2-APB, although this was also not significant (from $7.5 \pm 1.0$ to $140.3 \pm 52.0$ MΩ, $n = 4, P = 0.08$). 2-APB significantly decreased dye coupling from $9.7 \pm 0.2$ to $1.9 \pm 0.4$ cells ($P < 0.001, n = 4$; Table 1).

**Effects of exogenous ATP.** The fIJP in circular smooth muscle cells of the guinea pig ileum is mediated by ATP (16) or a related molecule, such as NADH (39), largely via P2Y1.
receptors (22, 50). We tested whether changes in IJP amplitude were due to a postjunctional mechanism by examining the effects of exogenous ATP applied by pressure microejection onto circular smooth muscle cells. In circular smooth muscle cells in which IJP amplitude had stabilized, pressure-ejected ATP typically evoked a biphasic hyperpolarization, in which the first, larger phase resembled the IJP in time course. This was followed by a later, slower hyperpolarization (Fig. 6A). In circular smooth muscle cells impaled after the first 120 min, the amplitude of the fast component was $-14.4 \pm 1.5$ mV and the amplitude of the slow component was $-8.8 \pm 1.1$ mV ($n = 5$; Fig. 6C). The first phase was blocked by 0.6 $\mu$M TTX, but the second phase persisted, with slightly reduced amplitude ($-6.0 \pm 0.6$ mV, $n = 6$; see Fig. 8A). This suggests that the first component was due to activation of inhibitory motor neurons (which then evoked a true IJP), whereas the second, TTX-resistant, phase was due to direct actions of ATP on the muscular apparatus. Fast and slow components were significantly smaller in cells during the first 30 min of recording ($0.0 \pm 0$ and $-0.6 \pm 0.6$ mV, respectively, $P < 0.05$, $n = 5$; Fig. 6B). In addition, carbenoxolone (100 $\mu$M) significantly reduced the amplitude of both hyperpolarizations (fast component from $-13.8 \pm 2.4$ to $-3.7 \pm 1.4$ mV and slow component from $-8.4 \pm 0$ to $-2.6 \pm 1.0$ mV, $P < 0.005$, $n = 6$; Fig. 7A). 2-APB (50 $\mu$M) also reduced the amplitudes of the ATP-evoked hyperpolarizations (fast component from $-13.1 \pm 1.3$ to $-0.3 \pm 0.3$ mV and slow component from $-8.1 \pm 0.4$ to $-0.3 \pm 1.1$ mV, $P < 0.005$, $n = 4$; Fig. 7B). In circular smooth muscle cells in the presence of TTX (0.6 $\mu$M), carbenoxolone significantly reduced the amplitude of the persistent ATP-evoked hyperpolarization from $-4.8 \pm 0.6$ to $0.2 \pm 1.0$ mV ($n = 4$, $P < 0.01$; Fig. 8B). The TTX-insensitive hyperpolarization was also reduced by MRS-2179 (10 $\mu$M), the P2Y, antagonist (50), from $-6.3 \pm 0.5$ to $-4.3 \pm 0.8$ mV ($n = 6$, $P < 0.05$; Fig. 8C).

Temperature as a cause for initial suppression of responses. Preparations were dissected and set up in Krebs solution at $-15^\circ C$, a $\approx 20$-min process, to maximize their longevity. We tested whether exposure to chilled Krebs solution might cause disruption of gap junction coupling by exposing full responsive preparations to Krebs solution at $15^\circ C$ for 5 min, then rapidly returning temperature to $35^\circ C$ following the 5 min. The interval at reduced temperature did not cause a significant change in IJP amplitude, RMP, input resistance, or dye coupling compared with the previously “equilibrated” conditions ($n = 6$; results not shown). We also tested whether exposure to

![Fig. 6. Responses to exogenous ATP were biphasic. A: application of 10 mM ATP (arrowhead) generated a biphasic response with a fast IJP-like component (a, arrow) followed by a slow hyperpolarization (b, arrow). B: fast (a) and slow (b) components showed time-dependent increases in the first 120 min ($P < 0.0001$, $n = 5$). C: fast (a) and slow (b) components were significantly greater in cells impaled at >120 min than ≤30 min ($n = 5$). *$P < 0.05$.](http://ajpgi.physiology.org/)

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**Table 1. Effects of gap junction blockers on circular smooth muscle cells impaled 120 min after dissection**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Carbenoxolone (100 $\mu$M)</th>
<th>Control</th>
<th>18β-Glycyrrhetinic Acid (10 $\mu$M)</th>
<th>Control</th>
<th>2-APB (50 $\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IJP amplitude, mV</td>
<td>$-14.6 \pm 1.2$</td>
<td>$-7.8 \pm 1.4^\dagger$</td>
<td>$-14.0 \pm 1.8$</td>
<td>$-6.2 \pm 1.1^$</td>
<td>$-16.1 \pm 0.8$</td>
<td>$-0.6 \pm 0.5^\dagger$</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>$-47.0 \pm 0.5$</td>
<td>$-49.3 \pm 0.5^$</td>
<td>$-49.5 \pm 0.5$</td>
<td>$-50.5 \pm 1.4$</td>
<td>$-50.4 \pm 0.5$</td>
<td>$-54.1 \pm 1.4$</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>8.9 ± 1.7</td>
<td>30.8 ± 4.0$^\dagger$</td>
<td>8.6 ± 0.6</td>
<td>111.5 ± 40.4$^$</td>
<td>7.5 ± 1.0</td>
<td>140.3 ± 52.0$^$</td>
</tr>
<tr>
<td>Dye coupling</td>
<td>4.4 ± 0.5</td>
<td>1.2 ± 0.1$^\dagger$</td>
<td>5.7 ± 0.6</td>
<td>1.2 ± 0.1$^\dagger$</td>
<td>9.8 ± 0.2</td>
<td>1.9 ± 0.4$^*$</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 6$ (carbenoxolone and 18β-glycyrrhetinic acid) and 4 (2-aminoethoxydiphenyl borate (2-APB)). IJP, fast inhibitory junction potential; RMP, resting membrane potential. *$P < 0.05$; †$P < 0.001$; $^\$P $< 0.05$; ‡$P < 0.005$; §$P < 0.05$ vs. respective control.
Krebs solution at 15°C for 30 min (the full duration of the initial dissection period) caused a loss of coupling. Rewarming the preparation to 35°C took 5 min. The longer period at reduced temperature did not cause an equivalent period of suppressed responses. 

![Graph](image1.png)

Effects on colonic circular smooth muscle cells. Similar initial suppression of responses followed by recovery was noted in colonic circular smooth muscle cells. fIJP amplitudes increased significantly with time within the first 120 min of recording ($P < 0.001$, 40 cells, $n = 5$). At >120 min, responses approached a plateau and did not significantly change (26 cells, $n = 5$). fIJP amplitudes within the first 30 min following dissection averaged $-20.4 \pm 1.2$ mV and significantly increased to $-27.6 \pm 2.5$ mV after 120 min ($P < 0.001$, $n = 5$). Hyperpolarizations averaged $10.1 \pm 0.7$ mV in control colonic smooth muscle cells and did not significantly change with 0.6 μM TTX ($9.3 \pm 1.0$ mV, $n = 4$). ATP-evoked responses were negligible within 30 min following dissection (0.0 ± 0.0 mV). Responses significantly increased over 120 min ($P < 0.001$, $n = 4$) and stabilized.
thereafter. Amplitudes averaged $-12.3 \pm 1.1$ mV after 120 min. RMP of cells impaled within 120 min displayed time-dependent depolarization ($P < 0.001$, $n = 5$). RMP stabilized for cells impaled after 120 min ($n = 5$). Cells impaled within 30 min of dissection had a hyperpolarized RMP compared with cells impaled after 120 min ($-57.4 \pm 3.9$ vs. $-43.3 \pm 0.7$ mV, $P < 0.05$). Over the 120 min following dissection, input resistance of colonic circular smooth muscle cells significantly decreased ($P < 0.05$, $n = 5$) and did not change thereafter. Cells impaled in 30 min tended to have a greater input resistance after 120 min ($10.9 \pm 1.1$ vs. $8.6 \pm 0.4$ MΩ); however, this was not significant ($P = 0.06$, $n = 5$). Dyecoupled profiles significantly increased over the first 120 min ($P < 0.0001$, $n = 5$); however, there were no significant differences for cells impaled thereafter. The number of dye-filled profiles was significantly less within 30 min of dissection than after 120 min ($4.5 \pm 0.7$ vs. $6.7 \pm 0.6$ cells, $n = 5$, $P < 0.05$).

The effects of the gap junction blocker carbenoxolone (100 μM) on colonic smooth muscle cells were measured. The amplitude of the sIJP was reduced from $-27.6 \pm 2.5$ to $-6.3 \pm 1$ mV by carbenoxolone ($P < 0.005$, $n = 5$), and ATP-evoked hyperpolarizations were reduced from $-12.0 \pm 1.3$ to $-2.4 \pm 0.8$ mV ($P < 0.005$, $n = 4$). Carbenoxolone caused the RMP of cells to significantly hyperpolarize from $-43.3 \pm 0.7$ to $-47.6 \pm 1.4$ mV ($P < 0.05$, $n = 5$). Apparent input resistance was increased from $8.6 \pm 0.4$ to $47.1 \pm 9.5$ MΩ by 100 μM carbenoxolone ($P < 0.05$, $n = 5$). Dye-filled profiles averaged $6.7 \pm 0.6$ cells in control conditions and decreased to $1.3 \pm 0.08$ cells in the presence of carbenoxolone ($P < 0.001$, $n = 5$).

Effects of dissection and gap junction blockers on nitrergic responses. The amplitude of slow, nitrergic IJPs (sIJPs) following dissection was measured from ileal smooth muscle cells. In the presence of apamin (0.25 μM), sIJPs were inhibited. Hyoscine (1 μM) was also added to reduce EJPs, and nicardipine (1 μM) was added to limit contractions. A high-frequency stimulus (3 pulses, 50-ms interval, 0.5-ms duration) was used to generate nitrergic junction potentials. sIJPs were not measurable in smooth muscle cells impaled within the first 30 min following dissection but were detected in cells impaled after 120 min ($-3.1 \pm 0.5$ mV, $P < 0.005$, $n = 5$; Fig. 9C). The amplitude of sIJPs showed a significant time-dependent increase over the 120 min following dissection ($P < 0.01$, $n = 5$), and amplitudes continued to increase after 120 min ($P < 0.05$, $n = 5$; Fig. 9B). Addition of 100 μM carbenoxolone significantly reduced mean sIJP amplitude from $-2.5 \pm 0.1$ to $-0.13 \pm 0.3$ mV ($P < 0.005$, $n = 4$; Fig. 9D). Local application of sodium nitroprusside (1 mM) by pressure ejection did not generate a hyperpolarization in responsive cells.

EJPs in the ileum and colon. EJPs could not be reliably recorded from circular smooth muscle cells in the guinea pig ileum (3). In colonic preparations, addition of apamin (0.25 μM) inhibited sIJPs, and nicardipine (1 μM) limited contractions. Under these conditions, EJPs could be evoked by electrical stimulation of intrinsic excitatory motor neurons with single stimuli applied 1 mm aboral to the impaled cell. EJP amplitude was $1.8 \pm 1.1$ mV in the first 30 min of dissection but increased to $5.6 \pm 1.2$ mV after 120 min ($P < 0.05$, $n = 5$; Fig. 10C). EJP amplitudes showed a significant time-dependent increase over 120 min following dissection ($P < 0.005$, $n = 5$; Fig. 10B); however, there was no further increase after 120 min (Fig. 10B). Addition of 100 μM carbenoxolone significantly reduced EJP amplitude from $8.9 \pm 3$ to $2.5 \pm 0.9$ mV ($P < 0.05$, $n = 5$; Fig. 10D).

Neurotransmission between enteric neurons was not blocked in the first 30 min. We tested directly whether all neurotransmission was suppressed during the first 30 min by recording from nerve cell bodies in myenteric ganglia in the presence of nicardipine and hyoscine (both at 1 μM). Six S neurons were recorded from six preparations within 30 min of superfusion with warmed Krebs solution. Mean RMP was $-53.1 \pm 7.4$ mV. A single electrical stimulus (0.4-ms duration) was applied 1 mm circumferential to the impaled nerve cell body. Fast excitatory postsynaptic potentials (EPSPs) were recorded in five of six cells with a mean amplitude (at a holding potential of $-90$ mV) of $9.6 \pm 1.6$ mV ($n = 5$); in the remaining cell, the fast EPSP could not be measured because of superimposed action potentials. Injection of constant current to shift membrane potential was used to calculate the reversal potential of fast EPSPs, which was $2–7$ mV between cells (average $3.4 \pm 1.2$ mV; Fig. 11A). Carboxyfluorescein dye injection revealed

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**Fig. 9.** Slow IJPs increase in amplitude with time following dissection. A: slow IJPs from cells impaled at 10.5 min (a), 64.5 min (b), and 145.6 min (c). B: slow IJP (sIJP) amplitude significantly increased 120 min following dissection ($\star$, $P < 0.001$, $n = 5$) and continued to increase thereafter ($\star\star$, $P < 0.005$, $n = 5$). C: measurable slow IJPs were not recorded within 30 min of dissection, and amplitudes were significantly greater at >120 min ($n = 5$), *$P < 0.005$. D: addition of 100 μM carbenoxolone significantly reduced the amplitude of slow IJPs ($n = 5$), *$P < 0.005$. 

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Dogiel type I morphology in all six cells (Fig. 11B). These results demonstrate that neurotransmission between enteric neurons is not blocked during the first 30 min of recording.

DISCUSSION

Postdissection "unresponsiveness." When preparations of circular smooth muscle from the guinea pig ileum were mounted in a recording chamber, they consistently lacked fIJPs, sIJPs, and EJPs, for at least the first 30 min. Over the ensuing 120 min, responses increased and then remained stable. It appears that the process of setting up the preparation caused a temporary and reversible loss of neuromuscular transmission. This suppression coincided with an initial loss of gap junction coupling between smooth muscle cells. Furthermore, pharmacological blockade of gap junctions mimicked the unresponsive state. This suggests that modulation of intercellular coupling between smooth muscle cells can potentely modify their physiology. This potentially represents a novel mechanism that may influence smooth muscle physiological responses.

Recovery following dissection varied greatly in time course: in some preparations, full-amplitude junction potentials and ATP responses were reached within 40 min in some preparations and in >120 min in other preparations. However, each preparation showed a similar pattern of recovery: over time, cells depolarized from an average RMP of −59 mV to a stable level of −47 mV, apparent input resistance decreased, junction potential amplitudes increased, and dye coupling increased significantly. It is not clear whether these four changes are independent or whether some may share common mechanisms. For example, as cells depolarize, the amplitude of the fIJPs would be expected to increase because of the larger driving force. However, the reversal potential for fIJPs in the guinea pig ileum is close to −90 mV (11). Thus a −16-mV IJP measured at a RMP of −47 mV would be expected to decrease to about −12 mV at a membrane potential of −59 mV. This does not come close to accounting for the fIJP amplitude of <1 mV during the initial equilibration period.

Loss of gap junction coupling. One of the main findings of this study was a major reduction in dye coupling between circular smooth muscle cells in the first 30 min after the preparation was set up for intracellular recording. One would expect then that electrical coupling between smooth muscle cells should be similarly suppressed. This expectation was supported by our observation that apparent input resistance fell significantly over the corresponding period, consistent with resumption of electrical coupling. The absolute values of input resistance were much smaller than those recorded from single isolated smooth muscle cells using patch-clamp techniques (33); nevertheless, the change in input resistance was significant. The same dye-injection protocol demonstrated significantly less dye coupling and significantly higher input resistance (in fully recovered preparations) in longitudinal than circular smooth muscle cells. These findings are compatible with previous reports that immunohistochemically characterized connexins and ultrastructurally identified gap junctions are less abundant in longitudinal than circular smooth muscle (18, 21, 38). Furthermore, known blockers of gap junctions, including carbenoxolone, 18β-glycyrrhetinic acid, and 2-APB, caused significant decreases in dye coupling between circular smooth muscle cells, as measured by dye-injection and current-injection protocols. Together, these findings suggest that the intracellular dye-injection method used here was a reliable method to measure gap junction coupling between smooth muscle cells. Similar methods have been used by others to demonstrate gap junction coupling between enteric glial cells (25, 37), between ICCs (4, 5), and between gut smooth muscle cells (20, 56).

Fig. 11. Fast excitatory postsynaptic potentials were recorded in S neurons impaled within 30 min of dissection. A: fast excitatory postsynaptic potentials over a range of membrane potentials. B: carboxyfluorescein was injected into the impaled cell and visualized in situ. Cell had Dogiel type I morphology.
Reduction of neuromuscular transmission. This study has shown an apparent suppression of neurotransmission between the enteric motor neurons and the smooth muscle cells during the reduction in dye coupling. In the presence of 1 μM hyoscine, electrical stimulation normally evokes prominent fIJPs (9), which include a fast component mediated by a purine (16), in circular smooth muscle cells. Depending on the frequency of stimulation, a slower IJP mediated by nitric oxide may also be present; in some preparations, an additional peptidergic component, mediated by VIP or a related peptide, can be recorded (27, 34). In the presence of 0.25 μM apamin without hyoscine, electrical stimulation can evoke an EJP mediated by acetylcholine acting on muscarinic receptors (14, 15) or by a noncholinergic mediator such as a tachykinin (2, 11, 12). In this study, we found that fIJPs, sIJP, and EJPs were profoundly depressed in the first 30 min. In many cells, junction potentials were not detectable. Strikingly, there was a comparable decrease in responses to exogenous ATP, applied by pressure ejection, suggesting that the deficit occurs at a postjunctional site. The observation that action potentials and fast EPSPs were robustly present in enteric neurons during the first 30 min discounts the possibility of a generalized impairment of neuronal function during suppression of neuromuscular transmission.

It is interesting to speculate on how changes in gap junction coupling may be related to the loss of junction potentials and responses to exogenous ATP. One possible explanation is that loss of coupling blocks the spread of junction potentials from a subset of smooth muscle cells that directly receive input from motor neuron axons (“responder cells”) to other cells. This seems unlikely. We recorded from >100 uncoupled smooth muscle cells during the study, and none had intact (full-amplitude) junction potentials or responses to ATP. Either responder cells are very rare, or motor neurons transmit via another type of cell from which we did not record and to which smooth muscle cells are coupled. This latter explanation is compatible with our observation that responses to exogenous ATP were also blocked in all uncoupled smooth muscle cells, suggesting that smooth muscle cells lack sufficient purine receptors and/or ion channels to support a full IJP.

ICCs and neuromuscular transmission. Immunohistochemistry and electron microscopy have demonstrated that, in the guinea pig ileum, ICCs of the deep muscular plexus are closely associated with axons of inhibitory and excitatory motor neurons, identified by nitric oxide synthase and substance P immunoreactivity, respectively (51). Nitricergic junction potentials in smooth muscle cells of the murine stomach are mediated indirectly, at least in part, by nitric oxide acting on muscarinic receptors in smooth muscle cells of the murine stomach are medi-

Fibroblast-like cells and neuromuscular transmission. We speculate that another class of cell within the muscle layer, “fibroblast-like cells,” which have been suggested to play a role in smooth muscle responses to purines released from axons of inhibitory motor neurons, may be involved. The purinergic fIJP is primarily mediated by opening of SK3, small conductance, calcium-dependent potassium channels (30, 50), and is blocked by the bee venom apamin (3). Fibroblast-like cells in the guinea pig colon are immunoreactive for SK3 channels (30). Electron microscopy has demonstrated gap junctions between fibroblast-like cells and outer circular smooth muscle cells; they also receive close contacts from motor neuron axons (57). Fibroblast-like cells are not immunoreactive for c-kit but are specifically labeled by antisera to platelet-derived growth factor receptor-α (29) and CD34 and SK3 (49), suggesting that they are not ICCs. In response to ATP, isolated fibroblast-like cells from the mouse colon generate large-amplitude outward currents, which are inhibited by apamin and the P2Y1 receptor antagonist MRS-2500. Current density in smooth muscle cells exposed to exogenous ATP was 1−2% of that in fibroblast-like cells (30, 50).

We suggest that purinergic responses in guinea pig ileum are generated primarily in fibroblast-like cells and then spread via gap junctions into the circular smooth muscle (32). This readily explains why a reduction in gap junction coupling in the first 30 min might be closely associated with temporary suppression of IJPs and loss of responses to exogenous ATP. When ATP was applied by pressure ejection to the recorded cell, it covered a ~200- to 400-μm-wide area, which would include ICCs and fibroblast-like cells. The routine use of carboxyfluorescein in recording electrodes indicated that fibroblast-like cells were not impaled during this study. One observation, however, suggests that intermediate cells may not be involved in neuromuscular transmission. Neither ICCs nor fibroblast-like cells were seen to be dye-coupled to smooth muscle cells in any of the dye fills. The reasons for this are not clear. It is possible that gap junctions between pairs of smooth muscle cells differ from those that connect smooth muscle cells to ICCs or to fibroblast-like cells (44). In a previous study, in which interconnected ICCs were intracellularly filled with dye in guinea pig small intestine, coupled smooth muscle cells were not labeled (4, 5).

RMP. The RMP of circular smooth muscle cells was significantly hyperpolarized in the first 30 min of recording. This may also be related to suppression of gap junction coupling, since carbenoxolone evoked a significant hyperpolarization of circular smooth muscle cells, and 18β-glycyrrhetinic acid and 2-APB caused a nonsignificant trend toward hyperpolarization. In canine colon (47) and feline jejunum (26), but not human colon (42), the RMP of circular smooth muscle cells varies throughout the circular smooth muscle layer. Whether such a gradient exists in the circular muscle of the guinea pig ileum is uncertain. If such a gradient does exist, blocking gap junctions could cause hyperpolarization, if distant cells with a depolarizing influence become uncoupled.

Mechanisms underlying the initial loss of gap junction coupling. The mechanisms underlying the reversible loss of gap junction coupling during the first 30 min of recording are not clear. Increases in intracellular calcium concentrations reduce gap junction permeability in other cellular systems, such as salivary gland and lens (40, 41). Phosphorylation by protein kinases can also modulate gap junction conductance in
a variety of cells (6), and changes in pH can alter coupling between ICCs of deep muscular plexus and smooth muscle cells in guinea pig ileum (31). It is feasible that uncoupling may result from mechanical distortion caused by stretching or cutting smooth muscle in the process of setting up the preparation. Consistent with this, flat-sheet preparations of circular smooth muscle of guinea pig ileum often lack slow-wave activity, but these slow waves are readily recorded in intact tubular preparations (46). Slow waves can be restored by indomethacin or nonselective cyclooxygenase blockers (35), possibly indicating that cyclooxygenase products suppress slow waves in flat-sheet preparations. We speculate that a combination of damage and damage-induced production of mediators could contribute to the uncoupling; this remains to be directly tested.

The present study showed that initial suppression of gap junction coupling occurs in circular smooth muscle cells of guinea pig ileum and colon. Changes in coupling may not affect inhibitory neuromuscular transmission equivalently in all species and regions of gut. IJP amplitude was reduced in circular smooth muscle cells of murine proximal colon in the presence of heptanol and octanol but was not affected by carbenoxolone or the Cx43 blocking peptide GAP-27, although gap junction permeability was not directly assessed (45). Similar findings have been reported for murine small intestine (19). It is possible that there are differences in gap junction pharmacology between species or regions of gut.

Conclusion. Immediately after dissection, circular smooth muscle cells show blunted responses to electrical stimulation of axons of enteric motor neurons or application of exogenous ATP. Responses develop over 30–120 min. Recovery of gap junction permeability correlates with an increase in junction potential amplitude and postjunctional ATP responses, depolarization of RMP, and decreased input resistance. Pharmacological blockade of gap junctions caused changes similar to those in the first 30 min of recording. The mechanism by which uncoupling takes place while tissues are being prepared for recording remains to be determined. Nevertheless, changes in gap junction coupling have a profound effect on smooth muscle responses and are subject to modulation. If such mechanisms operate under physiological conditions, they could represent a novel mechanism in the control of gut motility.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.E.C. and S.J.B. are responsible for conception and design of the research; S.E.C. performed the experiments; S.E.C., N.J.S., and S.J.B. analyzed the data; S.E.C., D.A.W., N.J.S., and S.J.B. interpreted the results of the experiments; S.E.C. prepared the figures; S.E.C. and S.J.B. drafted the manuscript; S.E.C., D.A.W., N.J.S., and S.J.B. edited and revised the manuscript; S.E.C., D.A.W., N.J.S., and S.J.B. approved the final version of the manuscript.

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