Effects of the gap junction blocker glycyrrhetinic acid on gastrointestinal smooth muscle cells

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Takeda, Yukari, Sean M. Ward, Kenton M. Sanders, and Sang Don Koh. Effects of the gap junction blocker glycyrrhetinic acid on gastrointestinal smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 288: G832–G841, 2005. First published November 4, 2004; doi:10.1152/ajpgi.00389.2004.—In the tunica muscularis of the gastrointestinal (GI) tract, gap junctions form low-resistance pathways between pacemaker cells known as interstitial cells of Cajal (ICCs) and between ICC and smooth muscle cells. Coupling via these junctions facilitates electrical slow-wave propagation and responses of smooth muscle to enteric motor nerves. Glycyrrhetinic acid (GA) has been shown to uncouple gap junctions, but previous studies have shown apparent nonspecific effects of GA in a variety of tissues. We tested the effects of GA using isometric force measurements, intracellular microelectrode recordings, the patch-clamp technique, and the spread of Lucifer yellow within cultured ICC networks. In murine small intestinal muscles, β-GA (10 μM) decreased phasic contractions and depolarized resting membrane potential. Preincubation of GA inhibited the spread of Lucifer yellow, increased input resistance, and decreased cell capacitance in ICC networks, suggesting that GA uncoupled ICCs. In patch-clamp experiments of isolated jejunal myocytes, GA significantly decreased L-type Ca²⁺ current in a dose-dependent manner without affecting the voltage dependence of this current. The IC₅₀ for Ca²⁺ currents was 1.9 μM, which is lower than the concentrations used to block gap junctions. GA also significantly increased large-conductance Ca²⁺-activated K⁺ currents but decreased net delayed rectifier K⁺ currents, including 4-aminopyridine and tetraethylammonium-resistant currents. In conclusion, the reduction of phasic contractile activity of GI muscles by GA is likely a consequence of its inhibitory effects on gap junctions and voltage-dependent Ca²⁺ currents. Membrane depolarization may be a consequence of uncoupling effects of GA on gap junctions between ICCs and smooth muscles and inhibition of K⁺ conductances in smooth muscle cells.

gastrointestinal motility; calcium ion currents; potassium ion currents; interstitial cells of Cajal; smooth muscle

Within the gastrointestinal (GI) tract, electrical slow waves are generated by a specialized population of cells known as interstitial cells of Cajal (ICCs) (28). Slow waves coordinate the phasic contractile activity of the muscle layers of the GI tract by spreading within ICC networks and into neighboring smooth muscle cells, causing membrane depolarization and activation of the contractile apparatus within smooth muscle cells (26, 29). Gap junction-mediated intercellular communication is important for the propagation of electrical slow waves through low-resistance pathways within GI muscles (7, 9, 10). Immunohistochemical studies have provided evidence for the existence of gap junctional proteins, including connexins 40, 43, and 45 (32, 33, 39). Physiological evidence for the role of gap junctions in the generation and propagation of slow-wave activity within the GI tract has also been studied by using a variety of gap junctional uncouplers, including octanol, heptanol, carbenevlole, oleamide, halothane, and 18-α and 18-β-glycyrrhetinic acid (GA). Although many of these compounds have nonspecific side effects, 18-α- and 18-β-GA are thought to be more specific at blocking gap junctional communication (11, 12).

Using isometric force measurements, carbenevlole derivatives at concentrations between 10⁻⁴ and 10⁻² M were shown to inhibit the amplitude of phasic contractions of isolated segments of longitudinal muscle, but had no effect on the frequency of spontaneous contractions (3, 8, 10, 31). With the use of intracellular microelectrode recordings, it was also shown that electrical slow waves in the guinea pig small intestine were abolished by octanol (1 mM) (13). Although octanol (0.5–1 mM) reduced the amplitude and frequency of slow waves in circular muscle in isolated canine ileum, it did not abolish them (10). Octanol caused depolarization of resting membrane potential (RMP) and reduced the nerve-evoked fast-inhibitory junction potentials in this tissue (10). These data suggest that the spread of slow waves between ICC networks and smooth muscle and neurally evoked inhibitory junction potentials may utilize low-resistance gap junctions, but that agents used to study this phenomenon may have indirect effects by directly inhibiting channels involved in slow-wave and inhibitory junction potential generation in ICC networks and smooth muscle cells.

The depolarization in membrane potential in GI muscles observed by using gap junctional uncouplers could be a consequence of the segregation of the ICC networks from neighboring smooth muscles cells. ICCs at the level of the myenteric plexus have been reported to have a more negative membrane potential than smooth muscle cells (21), and, in animal models when ICCs are absent, membrane potential in smooth muscle cells is more positive (17, 37, 41). An alternative explanation is that gap junctional uncouplers have nonspecific side effects on ionic currents on ICC or smooth muscle cells that could lead to membrane depolarization.

The decrease in the contractile activity of GI muscles in the presence of gap junctional uncouplers could also be interpreted in two ways. The inhibition of gap junctions between ICC and smooth muscle could lead to a decrease in the amplitude and coordinated spread of slow waves into the smooth muscle syncytium and subsequently depress phasic contractions. However, this disruption in coupling may cause an effect on the frequency of the phasic contractile activity. This was not the case.
dominant effect observed in contractility experiments (10). A second possibility is that gap junction uncouplers could have direct effects on Ca\(^{2+}\) currents that are essential for contractions of GI smooth muscles.

In the present study, we examined the effects of the commonly used gap junction uncoupler GA using a variety of physiological methods to determine specific or nonspecific effects of this compound on gap junctions and ionic currents in cultured ICC and freshly dispersed smooth muscle cells.

MATERIALS AND METHODS

Animals. BALB/c mice between the ages of 30 and 60 days were used for these studies. Animals were obtained from the Jackson Laboratory (Bar Harbor, MN). Mice were anesthetized with isoflurane (Baxter, Deerfield, IL) before cervical dislocation and decapitation.

The animals were maintained and the experiments were performed in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.” The Institutional Animal Use and Care Committee at the University of Nevada approved all procedures used.

Isometric force measurements. After animals were killed, jejunal tissues 10 cm from the ileocecal junction or the proximal colon 1–3 cm distal to the ileocecal junction were removed and placed in Krebs-Ringer bicarbonate solution (KRB). The jejunum was opened along the mesenteric border, and luminal contents were washed away with KRB. Mechanical responses were performed by using standard organ-bath techniques. Strips of muscle (2 × 5 mm) were cut from the tunica muscularis by sharp dissection. Muscles were attached at one end with sutures to a fixed mount within the organ bath and at the other end to an isometric strain gauge (World Precision Instruments, Sarasota, FL). The muscles were immersed in oxygenated KRB and maintained at 37.5 ± 0.5°C. The muscles were set at optimum length (2–4 MΩ). The measurements of the muscles were monitored, digitized, and stored with the use of AcqKnowledge software (Biopac Systems, Santa Barbara, CA) running on a personal computer-style computer.

Intracellular microelectrode recordings. The jejunal tunica muscularis (~10 mm × 4 mm) was isolated and placed in a recording chamber with the mucosal aspect of muscle facing upward. In some experiments, proximal colon tissues (1–3 cm from the ileocecal junction) were used for some experiments. Impalements of cells were made with glass microelectrodes that had resistances of 80–120 MΩ. Transmembrane potentials were recorded with a standard electrometer (Duo 773; World Precision Instruments). Data were recorded on a personal computer running Acknowledge data-acquisition software (Biopac Systems).

Solutions for isometric force measurements and intracellular recordings. The bath chamber was constantly perfused with oxygenated Krebs solution of the following composition (in mM): 118.5 NaCl, 4.5 KCl, 1.2 MgCl\(_2\), 23.8 NaHCO\(_3\), 1.2 KH\(_2\)PO\(_4\), 11.0 dextrose, and 2.4 CaCl\(_2\). The pH of the KRB was 7.3–7.4 when bubbled with 97% O\(_2\)–3% CO\(_2\) at 37°C. The muscles were set at optimum length (2–4 MΩ). To measure holding current, cell capacitance, and input resistance simultaneously from ICC networks, we used the built-in membrane capacitance tool in pClamp software (version 9.2; Axon Instruments, see Fig. 2). In most experiments on isolated smooth muscle cells, the uncompensated series resistance was between 2 and 4 MΩ, and voltage errors during the largest currents approached 12 mV. We compensated for at least 70% of the series resistance. Voltage errors were much smaller during steps to negative potentials.

Solutions for patch experiments. To measure outward currents, smooth muscle cells were bathed in a Ca\(^{2+}\)-containing physiological salt solution (CaPSS) containing (in mM) 5 KCl, 135 NaCl, 2 CaCl\(_2\), 10 glucose, 1.2 MgCl\(_2\), and 10 HEPES, adjusted to pH 7.4 with Tris. The pipette solution contained (in mM) 125 NaCl, 5.36 KCl, 15.5 NaOH, 0.336 NaHPO\(_4\), 0.44 KH\(_2\)PO\(_4\), 10 glucose, 2.9 sucrase, and 11 HEPES, adjusted to pH 7.4 with Tris. Muscosa and submucosa were removed. Most experiments were conducted by using myocytes isolated from bulk circular smooth muscle. Pieces of muscle were incubated in a Ca\(^{2+}\)-free solution supplemented with 2 mg fatty acid-free bovine serum albumin, 1.3 mg collagenase, and 2 mg trypsin inhibitor in 1 ml. Tissues were incubated at 37°C in enzyme solution for 8–12 min and then washed with Ca\(^{2+}\)-free solution. Tissue pieces were gently agitated to create a cell suspension. Dispersed cells were stored at 4°C in Ca\(^{2+}\)-free solution. Experiments were performed at room temperature within 6 h of dispersing cells.

Preparation of cultured ICC networks. ICC networks were cultured as previously described (21). Briefly, the mucosa was removed from small strips of intestinal muscle that were subsequently equilibrated in Ca\(^{2+}\)-free Hank’s solution for 30 min, and cells were dispersed with an enzyme solution containing 1 mg/ml collagenase (Worthington Type II), 2 mg/ml bovine serum albumin (Sigma), 2 mg/ml trypsin inhibitor (Sigma), and 0.7 mg/ml ATP. Cells were plated onto sterile glass coverslips coated with murine collagen (2 µg, Falcon/BDB) in 35-mm culture dishes. The cells were cultured at 37°C in a 95% O\(_2\)–5% CO\(_2\) incubator in smooth muscle growth medium (Clonetics, San Diego, CA) supplemented with 2% antibiotic/antimycotic (Gibco, Grand Island, NY) and murine stem cell factor (5 ng/ml, Sigma).

ICC were identified immunologically with a monoclonal antibody for kit protein (see Ref. 21) labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR). These studies were performed on small networks of ICCs (<10 cells). The morphologies of ICC and ICC networks were distinct from other cell types in the cultures, so it was possible to identify the cells with phase-contrast microscopy once the cells were verified with ACK2-Alexa Fluor 488 labeling. As previously reported, the pacemaker currents from small clusters of ICCs were more robust and more regular than from isolated ICCs (21).

Voltage patch-clamp experiments. The whole cell patch-clamp technique was used to record membrane currents from dissociated murine colonic smooth muscle cells. Currents were amplified with a list EPC-7 (List Electronic, Darmstadt, Germany) or Axopatch 200B (Axon Instruments, Foster City, CA). Data were digitized with a 16-bit analog-to-digital converter (Digitida 1322A, Axon Instruments). Data were stored directly and digitized online using pClamp software (version 9.2, Axon Instruments). Data were sampled at 4 kHz and filtered at 2 kHz by using an eight-pole Bessel filter and analyzed by using pClamp (version 9.2; Axon Instruments), Graphpad Prism (version 3.0, Graphpad Software, San Diego, CA), and Origin software (version 5.0, MicroCal Software, Northampton, MA). Pipette resistances were 1–4 MΩ. To measure holding current, cell capacitance, and input resistance simultaneously from ICC networks, we used the built-in membrane capacitance tool in pClamp software (version 9.2; Axon Instrument, see Fig. 2). In most experiments on isolated smooth muscle cells, the uncompensated series resistance was between 2 and 4 MΩ, and voltage errors during the largest currents approached 12 mV. We compensated for at least 70% of the series resistance. Voltage errors were much smaller during steps to negative potentials.
For studies of inward currents, we performed the perforated-patch technique. Amphoterin B (Sigma) was dissolved in dimethyl sulphoxide (Sigma) as a stock solution (0.06 mg/µl) and added to the pipette solution (0.3 mg/ml). Membrane potential was held at −60 mV during the formation of membrane pores. External solution was CaPSS. Internal solution for voltage-dependent inward currents contained (in mM) 120 CsCl, 20 TEA-Cl, 0.1 EGTA, and 10 HEPES, adjusted to pH 7.2 with Tris.

**Analysis of electrophysiological data.** Data are expressed as means ± SE. The Student’s t-test was used where appropriate to evaluate differences in the data. P values < 0.05 were taken as statistically significant differences. For intracellular recordings, several electrical parameters were analyzed: 1) RMP, 2) slow-wave amplitude, and 3) slow-wave frequency. For patch-clamp experiments, we analyzed the peak currents before and after drug treatments.

**RESULTS**

**Effects of GA on isometric contractions and slow-wave activity.** The effects of β-GA were tested on the spontaneous mechanical activity of jejunal muscle strips. β-GA (10⁻⁵ M) reversibly decreased phasic contractions (area under contractions decreased from 2.2 ± 0.6 g/min under control conditions to 0.9 ± 0.5 g/min after β-GA, P < 0.05, n = 4; Fig. 1A). The reduction in the area observed in β-GA was due to the decrease in the amplitude and frequency of the phasic contractions. In the presence of β-GA, contractions developed an irregular pattern compared with control activity (Fig. 1, Aa and Ab).

Conventional microelectrode recordings were performed to examine the effects of α-GA and β-GA on membrane potential of the circular muscle layer from the jejunum. α-GA (3 × 10⁻⁵ M) depolarized RMP from −63.0 ± 3.1 to −50.7 ± 3.4 mV (P < 0.01, n = 4). Slow-wave amplitude decreased from 34.3 ± 1.7 to 10.8 ± 1.2 mV (P < 0.001), but slow-wave frequency remained unchanged (from 33.3 ± 1.5 to 36.8 ± 3.0 cycles/min, P > 0.05; Fig. 1B). The application of β-GA (10⁻⁵ M) showed similar effects as that of α-GA. β-GA depolarized jejunal muscles from −65.9 ± 1.1 to −55.6 ± 0.7 mV (n = 5, P < 0.001), decreased slow-wave amplitude from 35.5 ± 2.6 to 14.9 ± 1.1 mV, but did not change slow-wave frequency.

**Effects of GA on Lucifer dye spread and passive electrical properties of ICC cultures.** To determine the effects of GA on ICC networks, we used 3- to 4-day primary cultures of ICC networks (21). Connected ICCs had low-input resistances (62 MΩ) and high capacitances (13.5 pF). ICC networks possessed spontaneous inward currents averaging −316 pA at a frequency of 17 cycles/min. The slow-wave activity of these networks was similar to that previously reported (21). After treatment of α-GA (3 × 10⁻⁵ M), input resistance increased to 348 MΩ, and membrane capacitance decreased to 6.4 pF. GA also abolished the spontaneous inward currents that were observed under control conditions (Fig. 2, A–C). We also performed current-clamp (I = 0) experiments to characterize the effects of GA on freshly dispersed jejunal myocytes (Fig. 2D). The membrane potential under normal physiological condition averaged −54 ± 7 mV. The application of β-GA (10⁻⁵ M) did not affect the membrane potentials (−55 ± 7 mV, n = 5; P > 0.05). This data suggest that depolarization observed in tissue experiments and ICC networks was caused by the uncoupling effects of GA between ICCs and ICCs to smooth muscle cells.

ICC networks were also dialyzed by using Lucifer yellow dye using patch pipettes (2 mg/ml added to the internal solution in the pipette) to examine the spread of dye through junctions.

![Fig. 1](http://ajpgi.physiology.org/) Effect of glycyrrhetinic acid (GA) on isometric contractility and electrical activity in jejunal muscle. A: effect of β-GA on isometric contraction. Aa: expanded time scale for control; Ab: expanded time scale for maximal effect after β-GA. B: effect of α-GA on membrane potentials from conventional microelectrode recordings. Ba: expanded time scale for control; Bb: expanded time scale for maximal effect after α-GA.
that formed between individual ICCs. Under control conditions, Lucifer yellow dye normally spread to four or five connected ICCs (Fig. 3, A and B, n = 5). After preincubation of β-GA (10^-5 M) for 15 min, Lucifer yellow dye was never observed to spread to adjacent ICCs when dialyzed (Fig. 3, C and D). The increase in input resistance and decrease in cell capacitance with the decrease in the spread of Lucifer yellow dye between cells suggest that GA uncoupled the cell junction between neighboring ICCs.

**Effects of GA on inward currents in smooth muscle cells.** The depolarization produced by α-GA and β-GA could produce an increase in the force of contractions of muscle strips. However, in the presence of α-GA or β-GA, there was a marked decrease in phasic contractions (see Fig. 1A). These data lead us to investigate the effects of α-GA and β-GA on inward currents in murine jejunal smooth muscle cells. To isolate inward currents, cells were bathed in CaPSS (see MATERIALS AND METHODS), and pipettes were filled with Cs-TEA solution (see MATERIALS AND METHODS). To prevent run-down of Ca^2+ currents, the perforated whole cell technique was used. First, we examined the dose-dependent responses of β-GA from 10^-3 M to 5 × 10^-5 M on inward currents. Circular muscle cells were stepped from a holding potential of -80 to 0 mV every 30 s. The peak currents were plotted as a function of time shown in Fig. 4, A and B. IC_{50} for β-GA was 1.9 ± 0.3 μM with a -0.5 of slope factor (n = 6), calculated by using the Hill dose-response curve fit (Fig. 4C). Current-voltage (I-V) relationships were plotted for the peak inward currents generated by voltage steps from a holding potential of -80 mV and stepping to test potentials ranging from -80 to +50 mV in 10-mV increments in murine jejunal myocytes (Fig. 5, A-C). Peak inward currents were -451 ± 55 and -427 ± 43 pA at -10 and 0 mV under control conditions, respectively. β-GA decreased peak inward currents to -199 ± 19 and -184 ± 25 pA (P < 0.05 at -10 and 0 mV, n = 4), respectively. A summarized I-V relationship for inward currents before and after β-GA is shown in Fig. 5C. We, therefore, examined whether β-GA affected the voltage dependence of activation and inactivation of inward currents. To evaluate voltage dependency of steady-state activation, the peak conductance at each test potential was calculated with the use of the equation: \( I_{Ca} = G_{Ca} (V - E_{rev}) \), where \( I_{Ca} \) is calcium current, \( G_{Ca} \) is peak conductance, V is test potential, and \( E_{rev} \) is reversal potential. The results were plotted and fitted with a Boltzmann equation. β-GA did not change the half-activation voltage in jejunal myocytes compared with control (-20.3 ± 0.2 mV in control vs. -23.6 ± 0.4 mV in β-GA, n = 5, Fig. 5, D-F). To examine steady-state inactivation, colonic myocytes were stepped to conditioning potentials between -100 and +50 mV for 5–6 s from a holding potential of -80 mV before a test step to 0 mV. Conditioning potential for 5–6 s was needed to reach steady-state inactivation. Resulting currents were normalized to the maximum current obtained and plotted as a function of the conditioning potential. The data were fitted by a Boltzmann equation. Half-inactivation voltage also was not shifted by β-GA (-41.0 ± 1.7 mV in control vs. -42.3 ± 1.4 mV in β-GA, n = 5, Fig. 5, D-F). These data suggest that the decrease in the mechanical activity is likely caused by a direct...
inhibition of inward currents without a change in the voltage dependence of activation and inactivation.

Effects of GA on outward currents. To determine the effects of GA on voltage-dependent K⁺ currents, we performed conventional whole-cell recordings on jejunal and colonic myocytes using CaPSS as the external solution and high K⁺ (140 mM) with a low concentration of EGTA (0.1 mM) in the internal pipette solution (see MATERIALS AND METHODS). First, we

Fig. 3. Effect of β-GA on dye (Lucifer yellow) spread in cultured ICC. A and C: contrast microscope image under control condition in different cultured cells. B: spread of Lucifer yellow under control condition in culture shown in A. D: block of Lucifer yellow dye to spread to adjacent ICC in culture shown in C.

Fig. 4. Effect of β-GA on peak inward currents in perforated whole-cell configuration. The currents were measured at 0 mV from −80 mV of holding potentials. A: changes in peak currents of different concentrations of β-GA as a function of time. B: peak inward currents as denoted in A (a–e). C: summarized data of dose-response curve fitted with Hill equation (n = 6).
measured cell capacitance before and after GA in freshly dispersed jejunal myocytes. GA did not change the cell capacitance in isolated myocytes (e.g., 41 ± 2 pF for control and 40 ± 2 pF after GA, n = 6; P > 0.05). The colonic myocytes were held at −80 mV and stepped to test potentials ranging from −80 to +70 mV in 10-mV increments (Fig. 6, A–C). Peak outward currents at 0 and +40 mV were 1,324 ± 242 and 2,953 ± 299 pA, respectively, under control conditions (n = 4). The application of α-GA (3 × 10−5 M) decreased peak outward currents at 0 and +40 mV to 912 ± 210 and 2,131 ± 255 pA, respectively (P < 0.05, Fig. 6, A and B). An I-V relationship plotted for peak outward currents recorded from murine colonic myocytes is shown in Fig. 6C. We also tested the effects of β-GA on outward currents in murine jejunal myocytes. Peak outward currents at 0 and +40 mV were 959 ± 74 and 2,143 ± 122 pA, respectively, under control conditions (n = 4). The application of β-GA (10−5 M) significantly decreased the peak outward currents at 0 and +40 mV to 697 ± 18 and 1,664 ± 91 pA, respectively (Fig. 6, D–F, P < 0.05 compared with control values). However, at more positive potentials than +50 mV, peak currents after α-GA and β-GA were not different from control values. These results led us to examine the effects of GA on Ca2+−activated K+ conductance and delayed rectifier K+ conductance (K_{DR}).

To examine the effects of GA on Ca2+−activated K+ [large-conductance (BK) and small-conductance (SK)] currents, we performed two different experimental protocols. Murine colonic myocytes were held at −30 mV to induce inactivation of voltage-dependent K_{DR} currents and to reveal BK and SK currents. BK and SK were isolated pharmacologically. First, 300 nM apamin were added to isolate BK currents, and currents were measured at test potential from −80 to +70 mV. The application of α-GA (3 × 10−5 M) increased BK currents significantly (Fig. 7, A–D). The average outward current was 245 ± 32 pA under control conditions and 449 ± 66 pA after α-GA at +50 mV (P < 0.05, n = 5). The I-V relationship of subtracted currents of α-GA is shown in Fig. 7E. β-GA (10−5 M) also had similar effects on BK currents (data not shown).

To isolate SK currents, we used 10 mM TEA in the bathing solution. TEA-resistant currents were activated at negative potentials and measured from 

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**Fig. 5.** Current-voltage (I-V) relationship and voltage dependence before and after β-GA. The cells were held at −80 mV and stepped to test potentials ranging from −80 to +50 mV in 10-mV increments. A and B: representative traces from murine jejunal myocytes before and after β-GA. C: I-V relationship from 4 cells. D and E: voltage-dependence of inactivation with 6-s period of test potential (up to +10 mV) to induce steady-state inactivation in murine jejunal myocytes before and after β-GA. F: voltage dependence of activation and inactivation fitted with Boltzmann equation from 5 cells. The values were normalized from maximal conductance (G_{max}) for activation and maximal currents (I_{max}) for inactivation. Fitted lines for inactivation were completely overlapped.

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**Fig. 6.** Effect of GA on outward currents. External solution was Ca2+-containing physiological salt solution and dialyzed with high K+ (140 mM) with low concentration of EGTA (0.1 mM) (see MATERIALS AND METHODS). The cells were held at −80 mV and stepped to test potentials ranging from −80 to +70 mV in 10-mV increments. A and B: representative traces before and after α-GA in murine colonic myocytes. C: summarized I-V relationship from 4 cells. D and E: representative traces before and after β-GA in murine jejunal myocytes. F: summarized I-V relationship from 4 cells.
The application of α-GA (3 × 10⁻⁵ M) did not affect TEA-resistant currents at all tested potentials significantly (n = 4, Fig. 7, F–H), suggesting that α-GA may not affect SK or ATP-sensitive K⁺ channels.

To isolate IᵥDR currents, cells were bathed in MnPSS (see MATERIALS AND METHODS), and patch pipettes contained BAPTA (10 mM) to minimize any contamination of BK channels. The application of β-GA (10⁻⁵ M) significantly decreased IᵥDR currents of jejunal myocytes under these conditions. Peak outward currents were 614 ± 53 and 1,581 ± 110 pA under control conditions at 0 and +30 mV, respectively (P < 0.05, n = 5). To examine the voltage dependence of β-GA, we used a double-pulse protocol with long 3- to 5-s prepulses. The treatment of 4-AP (5 mM) revealed a slowly activating and inactivating current (IᵥA) of β-GA in jejunal myocytes. The application of TEA (10 mM) revealed a rapidly activating and inactivating current (IᵥB). β-GA (10⁻⁵ M) significantly decreased this outward current in the presence of TEA from 1,103 ± 137 pA under control conditions to 871 ± 88 pA after β-GA at +30 mV (n = 4, P < 0.05, Fig. 8, G–I). β-GA have no effect on the voltage dependence of activation and inactivation (e.g., −3 ± 1 mV in control vs. −4 ± 1 mV after β-GA for activation; −44 ± 1 mV in control vs. −45 ± 1 mV after β-GA for inactivation; n = 4). These data suggest that the decrease of net outward currents by GA is caused by direct inhibition of IᵥA.

**DISCUSSION**

In the GI tract, pacemaking activity is generated by a specialized population of cells known as ICC (see Ref. 25). These cells form discrete networks along the intermuscular plane known as the myenteric plexus region that lies between the circular and longitudinal muscle layers. A second network of these cells lies along the submucosal interface of the circular muscle layer (37). In some regions of the GI tract, smooth muscle cells form muscle bundles and are surrounded by a second population of ICC within septal structures and are known as septal ICC. These cells form propagation pathways for the spread of electrical excitability into the adjacent smooth muscle layers (42). A third population of intramuscular ICC is
located within the muscle layers, form close anatomical relationships between nerve terminals and smooth muscle cells, and are critical for enteric motor neurotransmission (40).

Slow waves coordinate the phasic contractile activity of the smooth muscle cells by causing membrane depolarization via L-type calcium channels that leads to an increase in intracellular calcium and the subsequent activation of the contractile apparatus of the cells (16). The exact role of how ICCs coordinate the spread of electrical excitability within GI muscles is controversial. Ultrastructural analysis (9, 23) and immunohistochemical (33) studies have provided morphological evidence for the existence of gap junctions between ICCs, between ICCs and smooth muscle cells, and between smooth muscle cells of the circular muscle layer. Little ultrastructural or immunohistochemical evidence has been provided to demonstrate the presence of gap junctions within the longitudinal muscle layers throughout the GI tract. However, one immunohistochemical report has shown the presence of low levels of connexins that were localized to the longitudinal muscle layer of the murine stomach but not small intestine (14).

Connexin 43 was the highest expressed connexin protein in GI tissues and was expressed in all regions where gap junctions occur, and connexin 40 is also widely distributed in the circular muscle of the lower esophageal sphincter, stomach, and ileum. Other investigations examining the coupling between smooth muscle cells in the GI tract have utilized dye spread with Lucifer yellow (19). Although gap junctions have been observed in the circular but not the longitudinal muscle layer of the small intestine, dye coupling between smooth muscle cells was observed in both muscle layers. The spread of dye between muscle cells in the circular and longitudinal layers suggests the presence of gap junctions in both muscle layers and is inconsistent with previous ultrastructural studies that failed to demonstrate the presence of gap junctions in the longitudinal muscle layer (45).

Numerous functional studies have been performed to examine the role of gap junctional communication between smooth muscle cells in the GI tract (3, 10, 31) and other visceral and vascular systems (5, 6, 24, 25, 30, 38). GA in either the α- or β-isof orm has been widely used to examine the functional role of gap junctions in smooth muscles using several assays, including electrical measurements, dye coupling, or intracellular Ca^{2+} measurements. 18α-GA was shown to abolish coupling through gap junctions using these assays in a variety of smooth muscle preparations (15, 27, 35, 36, 44).

In the GI tract, mechanical experiments using GA have been performed to examine the role of gap junctions in coupling of

Fig. 8. Effects of GA on delayed-rectifier K⁺ currents. To minimize the contamination of BK currents, cells were bathed in Mn containing physiological salt solution (see MATERIALS AND METHODS) dialyzed with BAPTA (10 mM). A and B: representative traces before and after β-GA, respectively, under these conditions. C: summarized I-V relationship from 5 cells. D and E: representative traces obtained from double-pulse protocol (see RESULTS) before and after β-GA in the presence of 4-aminopyridine (4-AP). F: summarized I-V relationship from 5 cells. G and H: representative traces obtained from double-pulse protocols before and after β-GA in the presence of TEA. I: summarized I-V relationship from 4 cells. *Statistical significance (P < 0.05) by paired Student’s t-test.
smooth muscle cells (8, 10). In these experiments, GA was reported to decrease both the amplitude and frequency of spontaneous contractions in canine ileum and colon. These findings were consistent with the hypothesis that GA uncouples gap junctions between ICCs and between ICCs and smooth muscle, which leads to a reduction in the mechanical activity of this tissue (10, 23).

To confirm the uncoupling effects of GA on gap junctions, we performed a series of patch-clamp and dye-spread experiments using this compound on ICC networks that were maintained in primary culture for 3–4 days. In these experiments, the application of GA increased input cellular resistance and decreased capacitance. Furthermore, the spread of Lucifer yellow within ICC networks was greatly reduced after application of GA. Taken together, these observations suggested that the uncoupling effects of GA on gap junctions are consistent with previous reports.

In the present study, we also examined the nonspecificity of GA. Using isometric force measurements, intracellular microelectrode recordings and the patch-clamp technique examined the effects of GA on mechanical activity, membrane potential, and voltage-dependent ionic currents from jejunal and colonic tissues and cells. In mechanical experiments, GA decreased the amplitude and frequency of spontaneous contractions from jejunal tissues. This finding is consistent with a previous report in canine ileal muscle (10, 23) and could be interpreted as an uncoupling effect of GA on gap junctions between ICCs and smooth muscle cells, leading to a reduction in mechanical activity. However, microelectrode recordings revealed that GA also induced a significant membrane depolarization with an associated decrease in slow-wave amplitude. In contrast to the contractility experiments, these findings could not be explained by the uncoupling effects of GA alone. It has been previously reported that gap junction blockers may have nonspecific effects in addition to the actions of uncoupling gap junctions (4, 8, 18, 34). Although 18β-GA is considered more specific than 18α-GA and heptanol or octanol (11, 12), a systematic study on the nonspecific effects of GA on ionic channels in the GI tract has not been performed.

The decrease in mechanical activity observed in the presence of GA could have resulted from the inhibition of an inward calcium conductance in smooth muscle cells. Therefore, we examined net inward currents using perforated patches in murine jejunal and colonic myocytes. The IC_{50} of β-GA was 1.9 μM. This concentration of GA was significantly lower than the concentrations that have been used to uncouple gap junctions, which often range between 20 and 100 μM. At a concentration of 30 μM, GA inhibited 90% of Ca^{2+} currents in murine jejunal myocytes. Although the inhibitory mechanism of GA on Ca^{2+} currents is not clear, it was observed to decrease net calcium currents at all potentials tested without a change in the voltage-dependent activation and inactivation of these currents. The inhibitory potency of α-GA on inward currents was also not different than that observed with the β-isof orm of GA. These data suggest that decrease in the spontaneous mechanical activity resulted from inhibition of Ca^{2+} currents in murine jejunum and colon.

The membrane depolarization produced by GA could have resulted from the inhibition of an outward, or activation of an inward, ionic conductance in smooth muscle cells. GA has been shown to inhibit a chloride membrane conductance in confluent primary cultures of rat hepatocytes (2). Inhibition of a chloride conductance is unlikely in the present study, as this would have caused membrane hyperpolarization rather than the observed depolarization. We, therefore, examined the effects of GA on outward potassium currents.

To examine the effect of GA on outward K^+ currents, we performed conventional dialyzed whole-cell experiments. Under these conditions, net outward currents were decreased by external application of both isoforms of GA. To examine the effects of GA on specific K^+ currents, we isolated three types of voltage-dependent K^+ currents using voltage protocols and pharmacological approaches. First, we isolated BK currents using a holding potential of −30 mV to induce full inactivation of voltage-dependent delayed rectifier K^+ currents (22). Under these conditions, GA increased BK currents. In vascular smooth muscles, BK channel is one of major conductances that regulate RMP. The activation of BK channels by GA may induce hyperpolarization of vascular tissues. Indeed, a previous report suggested that gap junction blockers attenuate myogenic tone through membrane hyperpolarization (2, 24). In the circular muscle layer of the murine small intestine and colon, BK channels do not appear to act as an active conductance for the regulation of RMPs in these tissues (1, 2, 22, 24). The expression of SK channels in murine colonic myocytes has been reported, and these channels appear to be involved in setting RMP in this tissue (20). Using the same voltage protocol described above, incubation of TEA reveals a small amplitude of outward currents. α-GA had no effect on the remaining outward current in the presence of TEA. These data would suggest that the observed depolarization produced by GA was not through the inhibition of SK channels.

To examine the effects of GA on voltage-dependent I_{AS}, we isolated these currents using MnPSS (see MATERIALS AND METHODS) and dialyzed smooth muscle cells with BAPTA (10 mM) to reduce intracellular concentration to −10 nM. Under these conditions, when smooth muscle cells were exposed with 4-AP (5 mM), a slow-activating and inactivating delayed rectifier current remained (I_{DK}) (22). GA significantly inhibited the K_{DR} currents, suggesting that decrease in net outward currents resulted from inhibition of K_{DR} currents. Therefore, the membrane depolarization produced by GA could be a consequence of its blocking effects on delayed rectifier K^+ currents in smooth muscle cells.

Finally, using TEA (10 mM), we isolated a fast-activating and inactivating delayed rectifier K^+ current (I_{A}) (22). GA (30 μM) decreased I_{A} current without a change in the voltage dependence of activation and inactivation.

In conclusion, we have performed a systematic investigation of the effects of GA on networks of ICC cultures and ionic currents in smooth muscle cells. The effects of GA on membrane properties and dye spread are consistent with this compound inhibiting coupling between individual ICCs. However, GA also has several nonspecific side effects on calcium and potassium currents that make the use of this agent to specifically inhibit gap junctional coupling in GI tissues unreliable.

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REFERENCES


