Small-conductance Ca\(^{2+}\)-dependent K\(^+\) channels activated by ATP in murine colonic smooth muscle

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Koh, S. D., G. M. Dick, and K. M. Sanders. Small-conductance Ca\(^{2+}\)-dependent K\(^+\) channels activated by ATP in murine colonic smooth muscle. Am. J. Physiol. 237 (Cell Physiol. 42): C2010–C2021, 1997.—The patch-clamp technique was used to determine the ionic conductances activated by ATP in murine colonic smooth muscle cells. Extracellular ATP, UTP, and 2-methylthioadenosine 5'-triphosphate (2-MeS-ATP) increased outward currents in cells with amphotericin B-perforated patches. ATP (0.5–1 mM) did not affect whole cell currents of cells dialyzed with solutions containing ethylene glycol-bis(s-aminoethyl ether)-N,N,N',N'-tetraacetic acid. Apamin (3 × 10\(^{-7}\) M) reduced the outward current activated by ATP by 32 ± 5%. Single channel recordings from cell-attached patches showed that ATP, UTP, and 2-MeS-ATP increased the open probability of small-conductance, Ca\(^{2+}\)-dependent K\(^+\) channels with a slope conductance of 5.3 ± 0.2 pS. Caffeine (500 µM) enhanced the open probability of the small-conductance K\(^+\) channels, and ATP had no effect after caffeine. Pyridoxal phosphate 6-azophenyl-2',4'-disulfonic acid tetrasodium (PPADS, 10\(^{-4}\) M), a nonselective P\(_2\) receptor antagonist, prevented the increase in open probability caused by ATP and 2-MeS-ATP. PPADS had no effect on the response to caffeine. ATP-induced hyperpolarization in the murine colon may be mediated by P\(_2\)-induced release of Ca\(^{2+}\) from intracellular stores and activation of the 5.3-pS Ca\(^{2+}\)-activated K\(^+\) channels.

MANY VISCERAL TISSUES are innervated by motoneurons that do not utilize adrenergic or cholinergic transmitters (nonadrenergic noncholinergic neurotransmission) (for reviews see Refs. 20 and 35). Recent evidence suggests that nitric oxide (NO) is a major neurotransmitter mediating nonadrenergic noncholinergic inhibition in many autonomically innervated tissues (9, 28, 34); however, other transmitters, such as ATP, vasoactive intestinal polypeptide (VIP), and pituitary adenylyl cyclase-activating peptide (PACAP), are coexpressed in inhibitory neurons and may be coreleased (10, 18, 29, 31). Many studies of gastrointestinal (GI) muscles have described at least two components of hyperpolarization [i.e., inhibitory junction potentials (IJPs)] in postjunctional cells in response to inhibitory nerve stimulation (19, 23, 36, 38). Typically, IJPs consist of an initial fast component of hyperpolarization that is usually attributed to the release of ATP. The fast component of the IJP is variably sensitive to apamin (20). In some preparations the fast component is totally blocked by 100 nM apamin, but in other preparations the fast component is far less sensitive (23). Superimposed on, and extending beyond, the fast component is a slower component that is attributed to release of NO and, possibly, VIP (19, 23, 38). Different species and tissues manifest different degrees of the slow and fast components, suggesting considerable heterogeneity in the postjunctional receptors and mechanisms linked to neurally evoked inhibition. For example, in the canine colon, inhibitory components are mediated primarily by NO (13, 39) via activation of a variety of K\(^+\) channels (24), and apamin-sensitive responses are not present (37). In mouse and human GI muscles, apamin-sensitive and -insensitive components are present (23, 36). Although there has been speculation that the apamin-sensitive responses are mediated by ATP, the conductance(s) responsible for the fast component of IJPs has not been described. Therefore, it is unclear how ATP causes smooth muscle hyperpolarization.

It is thought that IJPs are due to the activation of K\(^+\) channels (40), and many authors have speculated that ATP activates small-conductance Ca\(^{2+}\)-dependent K\(^+\) (SK) channels similar to those expressed in skeletal muscles (7), brain (25), and hepatocytes (11), because IJPs and responses to ATP are reduced by apamin (1), an inhibitor of SK channels. Apamin-sensitive and -insensitive isoforms of SK channels have been identified (25), and therefore it is possible that the apamin-sensitive portion of ATP responses and fast IJPs could be mediated via apamin-insensitive SK channels. The molecular structure of SK channels has recently been determined, and functional channels have been cloned from mammalian brain (25). SK channels are Ca\(^{2+}\) sensitive and voltage independent and have unitary conductances of 5–20 pS. These are characteristic properties that could be used to identify SK channels in GI smooth muscles.

In the present study we examined the effects of ATP on outward currents of murine colonic muscle cells to identify the conductance(s) that may mediate ATP-dependent inhibitory responses in the GI tract. We have used the patch-clamp technique and compared responses in dialyzed and “perforated-patch” cells to test the involvement of intracellular signaling mechanisms. Single channel recordings were also performed to identify specific K\(^+\) channels activated by ATP.

METHODS

Cell preparation. Colonic smooth muscle cells were prepared from 20- to 30-day-old Balb/C mice of either sex. Mice were anesthetized with chloroform and killed by cervical dislocation, and the proximal colon was quickly removed. Colon pieces were cut open along the longitudinal axis, pinned out in a Sylgard-lined dish, and washed with Ca\(^{2+}\)-free Hanks’ solution containing (in mM) 125 NaCl, 5.36 KCl, 15.5 NaHCO\(_3\), 0.336 Na\(_2\)HPO\(_4\), 0.44 KH\(_2\)PO\(_4\), 10 glucose, 2.9
sucrose, and 11 N-2-hydroxyethylpiperazine-N' -2-ethanesulfonic acid (HEPES). After removal of the mucosa and submucosa, pieces of muscle were incubated in a Ca\(^{2+}\)-free Hanks' solution containing 4 mg/ml fatty acid-free bovine serum albumin (Sigma Chemical), 14 U/ml papain (Sigma Chemical), 230 U/ml collagenase (Worthington), and 1 mM dithiothreitol (Sigma Chemical). Tissues were incubated at 37°C in enzyme solution for 8–12 min and then washed with Ca\(^{2+}\)-free Hanks' solution. Tissue pieces were gently triturated to create a cell suspension. Dispersed cells were stored at 4°C in Ca\(^{2+}\)-free Hanks' solution supplemented with minimum essential medium for suspension culture (Sigma Chemical) and (in mM) 0.5 CaCl\(_2\), 0.5 MgCl\(_2\), 4.17 NaHCO\(_3\), and 10 HEPES. The cells were allowed to adhere to the bottom of a recording chamber on an inverted microscope for 5 min before commencement of experiments. All experiments were performed within 6 h of dispersion of cells.

Voltage-clamp experiments. Dialyzed whole cell, perforated-patch whole cell, and single channel voltage-clamp experiments were performed on murine colonic smooth muscle cells. Currents were amplified with a List EPC-7 amplifier and digitized with a 12-bit analog-to-digital converter (model TL-1, DMA interface, Axon Instruments). Data were stored on videotape or digitized on-line using pCLAMP software (version 5.5.1 or 6.03, Axon Instruments). Data were sampled at 1–5 kHz and low-pass filtered at 0.2–1 kHz using an eight-pole Bessel filter. Probability density plots were obtained by scaling the amplitude histograms so that the total area under the curve was equal to 1.0. In the experiments where the Ca\(^{2+}\) sensitivity of the small-conductance K\(^+\) channels was studied in excised patches, a sampling rate of 1 kHz and a filtration rate of 0.2 kHz were used. Variations in the baseline were corrected with ASCD software (Dr. G. Droogmans, KU, Louvain, Belgium). After baseline adjustment, all-points amplitude histograms were constructed and open probability was determined. A sampling period of 2 min was used for these analyses.

Solutions. For the recordings of K\(^+\) currents with the dialyzed whole cell technique, the external Mn\(^{2+}\)-containing physiological saline solution (MnPSS) consisted of (in mM) 5 KCl, 135 NaCl, 2 MnCl\(_2\), 10 glucose, 1.2 MgCl\(_2\), and 1 HEPES adjusted to pH 7.4 with tris(hydroxymethyl)amino methane (Tris). In some experiments, MnCl\(_2\) was replaced with CaCl\(_2\) [Ca\(^{2+}\)-containing physiological saline solution (CaPSS)]. Composition of the internal solution for dialyzed cells was (in mM) 110 potassium gluconate, 20 KCl, 5 MgCl\(_2\), 2.7 K\(_2\)ATP, 0.1 Na\(_2\)GTP, 2.5 creatine phosphate disodium, 5 HEPES, and 1 ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA) adjusted to pH 7.2 with Tris. For perforated whole cell patch-clamp experiments, composition of the pipette solution was (in mM) 110 potassium gluconate, 20 KCl, 0.5 EGTA, and 5 HEPES adjusted to pH 7.2 with Tris. Amphotericin B (90 mg/ml, Sigma Chemical) was dissolved in dimethyl sulfoxide, sonicated, and diluted in the pipette solution to give a final concentration of 270 µg/ml. External solution for these experiments was the same as for the dialyzed whole cell patch-clamp experiments. In some experiments, 3 × 10\(^{-7}\) M aminophylline (Sigma Chemical) was added to the external solution. Freshly prepared K\(_2\)ATP, UTP, and 2-methylthioadenosine 5′-triphosphate (2-MeS-ATP) were applied to cells via addition to the bath solution and superfusion.

For recording K\(^+\) channel currents in cell-attached or excised patches, the bath solution contained (in mM) 140 KCl, 1 EGTA, 0.61 CaCl\(_2\), and 10 HEPES adjusted to pH 7.4 with Tris. Various concentrations of Ca\(^{2+}\) were added to bath solutions buffered by 1 mM EGTA to create Ca\(^{2+}\) activities from 10\(^{-5}\) to 10\(^{-9}\) M. Activities were calculated with a program developed by C.-M. Hai (University of Virginia, Charlottesville, VA). In these experiments the pipette solution was identical to the bath solution, except 200 mM charybdotoxin was added to the pipette to inhibit large-conductance K\(^+\) (BK) channels in most on-cell experiments. In some experiments, KCl was replaced with NaCl (equimolar) to study ion selectivity. K\(^+\) concentration ([K\(^+\)]) gradients are given as external [K\(^+\)] divided by internal [K\(^+\)]. Caffeine (500 µM, Sigma Chemical) and 10\(^{-5}\)–10\(^{-4}\) M pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid tetrasodium (PPADS; Research Biochemicals) were added to bath solutions in some experiments.

RESULTS

ATP effects in dialyzed whole cell configuration. The effects of ATP on outward current were first tested by external application of 1 mM ATP to cells voltage clamped with the conventional, dialyzed, whole cell configuration of the patch-clamp technique. EGTA (1 mM) was added to the pipette solution to buffer intracellular Ca\(^{2+}\). In the presence of external MnPSS (see METHODS), outward currents were generated by depolarizing test potentials from a holding potential of ~80 mV. The outward currents reached a peak and inactivated during 150-ms test depolarizations. Data generated from inactivation protocols were fitted with a Boltzmann equation: 

\[
y = \frac{1}{1 + \exp\left(\frac{V - V_i}{k}\right)}
\]

where \(k\) represents the slope factor and \(V_i\) is voltage of 50% inactivation, which averaged ~44 ± 1 mV (\(n = 5\), data not shown). The time-dependent decay of the outward current was fit with a single time constant (\(\tau = 62 ± 2\) ms at +20 mV; data not shown). At the end of 150-ms test potentials, the outward current had inactivated to 30% of the peak current. Application of external ATP had no effect on the outward currents elicited in dialyzed cells. Similar observations were made when CaPSS was used as the external solution: 1) inactivating outward currents were elicited by step depolarizations from ~80 mV (Fig. 1, A and B); 2) the sustained current was 26% of the peak current at the end of 150-ms test pulses; and 3) ATP did not alter the amplitude of the peak or the sustained current (\(n = 4\); Fig. 1, B–D).

Effects of ATP on cells using the perforated-patch, whole cell configuration. In the presence of MnPSS, cells voltage clamped with the perforated-patch technique (34) showed a pattern of current responses similar to the currents observed in experiments on cells using the dialyzed, whole cell configuration (i.e., peak and sustained currents). Application of 1 mM ATP activated outward currents in cells in which the perforated-patch technique was used (Fig. 2, A and B). ATP produced a sustained outward component of current, as shown by difference currents (Fig. 2B). For example, at +40 mV the outward current was enhanced 18 ± 4% at the end of a 500-ms test depolarization (\(n = 6\)). The difference currents elicited by ATP were well fit by the Goldman-Hodgkin-Katz (GHK) equation for K\(^+\): 

\[
x = \frac{1}{1 + \exp\left(\frac{V - V_i}{k}\right)}
\]

where \(r^2 = 0.9726, n = 6\); Fig. 2C). ATP induced a K\(^+\) permeability of 8.3 × 10\(^{-15}\) cm\(^{-2}\)·s\(^{-1}\)·cm\(^{-2}\).
Application of 100 µM 2-MeS-ATP, a P2y receptor agonist, activated outward currents with characteristics similar to the currents elicited by ATP (Fig. 2D). 2-MeS-ATP evoked a sustained outward current (Fig. 2E). For example, at +40 mV the outward current was enhanced 24.5 ± 2% at the end of a 500-ms test depolarization (n = 4). The difference currents elicited by 2-MeS-ATP were well fit by the GHK equation for K⁺ (r² = 0.9716; Fig. 2F), suggesting that the current induced by 2-MeS-ATP was due to a K⁺-selective conductance. UTP (1 mM) elicited a current similar to the currents elicited by ATP and 2-MeS-ATP; however, lower concentrations of UTP (i.e., 100 µM) had no effect (n = 2 each dose; data not shown). The currents activated by ATP and 2-MeS-ATP were not affected by 5 mM 4-aminopyridine (4-AP) or the combination of 5 mM 4-AP and 10 mM tetraethylammonium (data not shown).

In the presence of CaPSS, the pattern of the outward currents elicited by depolarization of cells using the perforated-patch technique changed. Stepping from −80 to +50 mV in 10-mV increments induced outward currents that reached a peak early in the test pulse, partially relaxed, and then increased during the remainder of 500-ms test depolarizations. Thus the inactivating trend observed when cells were bathed in MnPSS was masked by the development of an additional, slowly activating component of current in CaPSS (Fig. 3A). ATP significantly increased the amplitude of the outward currents elicited in these cells (e.g., 52 ± 6% at +40 mV, n = 5; Fig. 3, B and C). We also noted that significant inward tail currents developed on repolarization from test potentials in the presence of ATP (Fig. 3B), suggesting that, in CaPSS, inward currents, possibly due to Cl⁻ or nonselective cation conductances, were also activated. Under these conditions the current-voltage (I-V) relationship of the difference current elicited by ATP was not well fit by the GHK equation for K⁺, suggesting that ATP activated more than a single ionic conductance (Fig. 3D).

Effect of apamin on ATP-activated currents. Apamin (3 × 10⁻⁷ M), an inhibitor of SK channels, was applied to the bath solution to test the pharmacological sensitivity of the current induced by ATP. Apamin itself had no effect on the outward current elicited in dialyzed cells (data not shown). Cells with permeabilized patches were stepped from a holding potential of −80 to +80 mV in 20-mV steps (Fig. 4). ATP (1 mM) increased outward currents, and apamin partially attenuated the response to ATP (32 ± 5%, n = 5; Fig. 4, A and B). Under control conditions, peak outward current averaged 760 ± 2 pA and ATP increased current amplitude to 889 ± 2 pA. In a series of time control experiments the outward current activated by ATP declined very little during 11 min of exposure (i.e., to 862 ± 4 pA, n = 5). In another group of five cells with average outward currents of 756 ± 4 pA, ATP increased the outward current to 884 ± 4 pA. Apamin decreased the outward current in these cells to an average of 827 ± 3 pA (n = 5).

K⁺ channels activated by ATP. Single channel studies were performed on 198 patches to identify K⁺ channels activated by ATP. Application of 1 mM ATP to the external solution increased the open probability of a small-conductance channel in cell-attached patches (32 of 52 patches tested with this protocol). At a patch potential of −60 mV in the presence of 10⁻⁷ M extracellular free Ca²⁺, few spontaneous channel openings were noted. However, brief openings of a small-conductance channel (mean open time = 2 ± 0.5 ms) were occasionally observed. Because of the brief open times of these channels, we could not fully resolve the amplitude of the currents (Fig. 5A). ATP increased open channel probability of the small-conductance channels. The amplitude of the unitary conductance was 0.35 pA at −60 mV. After 3 min of exposure to ATP, open probability was 0.14 ± 0.05 (Fig. 5, B and F), and, after 7 min, open probability reached a maximum level of 0.26 ± 0.07 (Fig. 5C). The increase in open probability resulted from an increase in mean open time (i.e., to 29 ± 3 ms), and multiple channel openings were also observed. Open probability of the channels decreased
when ATP was removed from the external solution. Within the 1st min after removal of ATP, open probability decreased to $0.13 \pm 0.06$ (Fig. 5D). Several minutes of washout were required to return open probability to the control level (Fig. 5E). Figure 5F shows the changes in open probability elicited by ATP as a function of time ($n = 7$ patches in which the entire exposure and washout of ATP were accomplished).

I-V relationship of ATP-induced current. To further characterize the small-conductance channels activated by ATP, we performed steady-state recordings and step protocols on inside-out excised patches. Ionic conditions included symmetrical (140 mM K⁺ in pipette and bath solution) and asymmetrical (5 mM K⁺ in pipette and 140 mM K⁺ in bath solution) K⁺ gradients with $10^{-7}$ M extracellular free Ca²⁺. With the inner surface of the patch exposed to this concentration of Ca²⁺, spontaneous openings of the small-conductance channels were observed. Amplitudes of the currents were plotted as a function of potential (Fig. 6C), and the slope conductance in symmetrical K⁺ solutions was determined by linear regression to be $5.3 \pm 0.02$ pS ($n = 4$). Because of the small amplitude of the currents, it was difficult to resolve single channel openings at potentials positive to $-240$ mV in symmetrical K⁺ solutions (Fig. 6A).

To confirm that the currents were due to activation of a K⁺ conductance, step depolarizations were also performed in asymmetrical K⁺ solutions (i.e., 5 mM K⁺ in pipette and 140 mM K⁺ in bath solution). In these experiments we noted three unitary conductances in excised patches: 1) a large-conductance channel (identified as BK channels by biophysical and pharmacological properties; data not shown), 2) a 13-pS channel that showed inactivation and sensitivity to 4-AP (delayed
Ca\(^{2+}\) the number of channel openings increased additionally (Fig. 7, A and B, trace e). The effects of increased Ca\(^{2+}\) concentration were fully reversible, and reducing Ca\(^{2+}\) back to very low concentrations (10\(^{-9}\) M) decreased open probability in a concentration-dependent manner. Figure 7C shows a plot of open probability vs. Ca\(^{2+}\) concentration, and the 50% effective concentration, calculated from the averaged data, was 4.9 \times 10^{-7} M Ca\(^{2+}\).

Activation of 5-pS K\(^{+}\) channels by caffeine. The fact that open probability of the 5-pS K\(^{+}\) channels was increased by intracellular Ca\(^{2+}\) suggests that entry of Ca\(^{2+}\) or release of Ca\(^{2+}\) from intracellular stores could mediate the activation of these channels by ATP in the cell-attached configuration. Therefore, we tested the effects of caffeine to determine whether release of stored Ca\(^{2+}\) might activate the 5.3-pS channels. Caffeine (500 \(\mu\)M) was applied in the external bath solution, and single channel activity was monitored while stepping from −80 to 0 mV in the cell-attached configuration with an asymmetrical K\(^{+}\) gradient (i.e., 5 mM K\(^{+}\) in the pipette and 140 mM K\(^{+}\) in the bath solution). Three K\(^{+}\) channels, with conductances of 250, 13, and 5.3 pS (Fig. 8A), were observed on step depolarizations. The open probability of 13-pS channels, likely to be rectifier channels; data not shown), and 3) small-conductance channels that corresponded to the 5.3-pS channels observed in symmetrical K\(^{+}\) solutions (Fig. 6B). The currents resulting from these channels were fitted with the GHK equation and had a chord conductance of 2.9 pS at 0 mV (Fig. 6, B and C). The calculated equilibrium potential for Cl\(^{-}\) and the theoretical reversal potential for nonselective cation conductance that may also have been activated by ATP. D: summary plot of I-V relationship of difference currents obtained from 5 cells. Data were fit with GHK equation for K\(^{+}\). Data were not fit well by GHK equation, suggesting that ATP activated more than a single conductance in Ca\(^{2+}\)-containing PSS.

Fig. 3. Effects of ATP on perforated-patch cells in presence of extracellular Ca\(^{2+}\). A: control currents generated by depolarization of an amphotericin-permeabilized cell bathed in Ca\(^{2+}\)-containing PSS. B: current records in same cell after addition of 1 mM ATP. C: difference currents; ATP increased outward current during depolarization steps. Small tail currents noted on repolarization are suggestive of a Cl\(^{-}\) or nonselective cation conductance that may also have been activated by ATP. D: summary plot of I-V relationship of difference currents obtained from 5 cells. Data were fit with GHK equation for K\(^{+}\). Data were not fit well by GHK equation, suggesting that ATP activated more than a single conductance in Ca\(^{2+}\)-containing PSS.

Fig. 4. Apamin attenuated outward current activated by ATP. A: currents generated by depolarization of a cell bathed in Mn\(^{2+}\)-containing PSS using permeabilized-patch technique. Trace a: control record; trace b, increase in outward current produced by ATP; trace c: reduction in outward current after addition of 3 \times 10^{-7} M apamin in continued presence of ATP in another cell. Apamin partially blocked ATP-induced current. B: peak currents in response to repetitive steps from −80 to 0 mV. *o, Time course of average responses to sustained exposure to ATP; ○, reduction in current caused by application of 3 \times 10^{-7} M apamin. Points are averages ± SE from 5 cells exposed continuously to ATP (for at least 800 s) and 5 cells in which apamin was added during continuous exposure to ATP.
delayed rectifier channels (see above), was unaffected by ATP or caffeine. The 250-pS channels, identified as BK channels, were activated by caffeine treatment (open probability $p = 0.01$ and $0.12$ for control and caffeine treatment, respectively, $n = 4$). The increase in BK open probability was taken as an indication that the caffeine treatment released intracellular Ca$^{2+}$. Caffeine also increased open probability of the 5.3-pS channels (Fig. 8, B and B'). After application of caffeine, there was an initial increase in the openings of the 5.3-pS channels; however, this effect decreased within 3 min. After exposure to caffeine (8 min), addition of ATP had no effect on 5.3-pS channels (data not shown).

Effects of 2-MeS-ATP and UTP on 5.3-pS K$^+$ channels. To observe the effects of more specific agonists of P$_2$Y receptors, we also tested whether 2-MeS-ATP and UTP enhanced the open probability of the 5.3-pS channels. 2-MeS-ATP at 10 µM ($n = 3$) and 100 µM ($n = 4$) increased the open probability of the 5.3-pS channel in cell-attached patches. Figure 9 also shows all-points amplitude histograms for control conditions and the response to 2-MeS-ATP stimulation (open probability $p = 0.55$ and $0.11$, $n = 4$) at $-60$ mV. UTP (1 mM; $n = 3$) also increased the open probability of the 5.3-pS channels; however, 100 µM UTP was without effect ($n = 3$; data not shown).

Effects of PPADS on activation of 5.3-pS K$^+$ channels. Because 2-MeS-ATP increased the open probability of the 5.3-pS channel, we tested whether PPADS, a P$_2$ receptor blocker (27), inhibited this effect. PPADS (10$^{-5}$ or 10$^{-4}$ M) was added to the external solution, and single channel activity was recorded in the cell-attached configuration. PPADS itself did not change the activity of the 5.3-pS channels under control conditions (Fig. 10, A and B). In the presence of PPADS, application of 1 mM ATP or 100 µM 2-MeS-ATP did not increase the open probability of 5.3-pS K$^+$ channels (Fig. 10C). Caffeine was able to activate 5.3-pS channels in the presence of PPADS (open probability $p = 0.18$ and 0.07, $n = 4$; Fig. 10D).

Fig. 5. ATP activated a small-conductance channel in cell-attached patches. A–E: representative excerpts of a continuous on-cell patch-clamp recording before and during exposure to ATP. A: current under control conditions, i.e., symmetrical 140 mM K$^+$ and holding potential of $-60$ mV. Very brief openings of a small-conductance channel were observed (downward deflections). B: 1 mM ATP increased open probability, and a peak level was reached in C. D and E: effect of ATP on channel activity was reversible on washout. F: plot of channel activity, expressed as open channel probability ($NP_o$), as a function of time ($n = 7$); letters (A–E) indicate times at which traces in A–E were recorded.

Fig. 6. I-V relationship of small-conductance K$^+$ channel in excised membrane patches. A: steady-state recordings from an inside-out patch with symmetrical 140 mM K$^+$ and Ca$^{2+}$ buffered to $10^{-7}$ M. Solid line and c indicate 0 current when channels were closed; channel openings are represented by downward deflections. B: recordings made in asymmetrical K$^+$ (5 mM in pipette and 140 mM in bath solution at inside surface of patch). c, Closed; o, open. Data were sampled at 1 kHz and filtered at 0.2 kHz. C: I-V relationship for currents in experiments depicted in A (c) and B (o). Data points were fitted with GHK equation. Note shift in extrapolated reversal potentials that would be predicted if channels were K$^+$ selective.
DISCUSSION

Stimulation of intrinsic enteric inhibitory nerves leads to hyperpolarization and relaxation of GI muscles (6, 20). There has been considerable work to determine the transmitters that mediate enteric inhibitory responses in these muscles, and ATP, VIP, PACAP, and NO have been considered candidates. In fact, enteric inhibitory neurotransmission appears to be accomplished by at least two transmitters released in parallel from the same population of inhibitory motoneurons. The postjunctional mechanisms that mediate the inhibitory effects are not well understood, but this question has importance, because the postjunctional receptors and effectors (i.e., ion channels or other cellular mechanisms) may be excellent targets for therapeutic agents to regulate GI motility. Recent work has described several K⁺ channels in canine colonic muscles that are activated by NO (24), and the present study has addressed the conductance activated by purinergic agonists. ATP and the P₂₇₇ receptor agonist 2-MeS-ATP activated 5.3-pS Ca²⁺-activated K⁺ channels. These channels had properties similar to SK channels, the small-conductance Ca²⁺-activated K⁺ channels.

Fig. 7. Ca²⁺ sensitivity of small-conductance channel in inside-out excised patches. Intracellular surface of an inside-out membrane patch was exposed to 10⁻⁹–10⁻⁵ M free Ca²⁺. A: excerpts of current records while free Ca²⁺ concentration in bath solution was changed to levels indicated above and below current traces. Open probability of 5.3-pS channel was increased as free Ca²⁺ increased. B: parts of trace in A are expanded (as indicated by a–h in A), and unitary conductance levels are estimated by dotted lines (solid line and c indicate closed state). At 10⁻⁶ and 10⁻⁵ M Ca²⁺, openings of at least 2 channels were evident. C: plot from 4 experiments of open probability vs. logarithm of free Ca²⁺ concentration ([Ca²⁺]); 50% effective concentration (EC₅₀) was calculated to be 4.9 × 10⁻⁷ M. D: all-points histogram obtained from records taken in presence of 10⁻⁶ M free Ca²⁺; opening of at least 2 channels is demonstrated.

Fig. 8. Caffeine activated 5.3-pS and large-conductance K⁺ (BK) channels in on-cell patches. A: control recordings from an on-cell patch. Patch was stepped from −80 to 0 mV. Three unitary conductances were noted in this patch. Trace a: opening of 5.3-pS channel; trace b: opening of 13-pS channel (o, open state; c, closed state). Openings of BK channels (large transitions) can also be observed. B: 500 µM caffeine, which releases Ca²⁺ from intracellular stores, increased open probability of BK channels (confirming that caffeine increased intracellular Ca²⁺) and 5.3-pS channels. Caffeine did not affect 13-pS channels. A' and B': all-points amplitude histograms before and after caffeine treatment, respectively. In A', histogram was well fit with a Gaussian distribution (solid line); histogram in B' was not well fit by same function as a result of activation of 5.3-pS channels.
It is quite clear that the BK channel, another type of Ca\(^{2+}\)-activated K\(^{+}\) conductance channel, is not responsible for ATP-dependent hyperpolarization, because blockers of this channel do not affect IJPs (S. Ward, personal communication). Ca\(^{2+}\)-activated K\(^{+}\) channels with conductances smaller than BK channels have been found in the rabbit portal vein (22), taenia coli (21), and jejunum, guinea pig mesenteric artery (4), and canine colon (24), but the channel conductances (63–150 pS) are much greater than the conductance activated by ATP in murine colonic smooth muscles (i.e., 5.3 pS). The medium-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels have been reported to be insensitive to apamin (8, 47). Apamin blocks some isoforms of SK channels (25) and reduces responses to inhibitory transmitters and ATP in a variety of GI muscles, including guinea pig stomach (26, 43), taenia coli (30, 43, 46), and ileum (48). This suggests that a portion of the responses to enteric inhibitory transmitters may be due to activation of SK channels. The present study documents the expression of a small-conductance, Ca\(^{2+}\)-activated K\(^{+}\) channel with properties consistent with SK channels in GI muscles.

ATP released from nerves could activate different types of purinergic receptors expressed by smooth muscle cells. Others have documented excitatory effects of ATP released from extrinsic sympathetic neurons in colonic muscle, and these effects are mediated by P\(_{2y}\) receptors (41). P\(_{2y}\) receptors are ligand-gated nonselective cation channels (5, 16, 44). Therefore, it is unlikely that P\(_{2y}\) receptors could mediate IJP responses, because activation of nonselective cation channels would result in inward current and depolarization (14, 32). P\(_{2x}\) and P\(_{2u}\) receptors are G protein-coupled and mediate inositol trisphosphate (IP\(_3\))-induced release of Ca\(^{2+}\) from internal stores (2). By initiating the release of small amounts of Ca\(^{2+}\) near the plasma membrane, P\(_{2y}\) and P\(_{2u}\) occupation could lead to the activation of Ca\(^{2+}\)-dependent outward currents. In the present study we found that ATP and the P\(_{2y}\) agonist 2-MeS-ATP activated small-conductance K\(^{+}\) channels. This observation suggests that P\(_{2y}\) receptors could mediate ATP-dependent hyperpolarization. We cannot entirely rule out involvement of P\(_{2u}\) receptors, but we found UTP to be less effective than 2-MeS-ATP in activating SK channels. Nothing is known about the expression of P\(_{2u}\) receptors in colonic muscles, and the effects of UTP could have been mediated by nonspecific activation of P\(_{2y}\) receptors. We also found that the P\(_2\) receptor antagonist PPADS (27) blocked responses to ATP and 2-MeS-ATP, but we view this observation with caution, because a recent study has suggested that PPADS may nonselectively inhibit IP\(_3\) receptors, rather than block P\(_{2y}\) receptors (42). It should be clearly stated that the exact nature of the receptor(s) that mediates the ATP responses we have observed is difficult to determine, since highly selective antagonists for these receptors are not available.

ATP had no effect on currents in cells with use of the standard, dialyzing configuration of whole cell recording. This finding suggests that intracellular second...
messengers, such as Ca\(^{2+}\) or other factors, are required for ATP-induced activation of outward current. The cells were dialyzed with solutions containing EGTA in these experiments, and it is possible that changes in intracellular Ca\(^{2+}\) are important in mediating the response to ATP. When the perforated-patch technique, which preserves intracellular signaling mechanisms (34), was used, ATP activated a K\(^{+}\) conductance (i.e., the current was outward and the I-V relationship of the conductance was well fit by the GHK equation) when an extracellular solution containing Mn\(^{2+}\) (to replace Ca\(^{2+}\)) was used. When standard Ca\(^{2+}\)-containing extracellular solution was used, the currents generated by ATP were not well described by the GHK equation, and inward tail currents were observed on repolarization. Thus ATP may generate a mixed current consisting of the K\(^{+}\) current elicited in Mn\(^{2+}\) solution plus Ca\(^{2+}\)-dependent Cl\(^{-}\) or nonselective cation conductances. The characteristics of these inward conductances are not a subject of this study. The fact that a mixed current is generated on perfusion of cells with ATP suggests that release of this substance from nerves in situ must be directed at specialized populations of receptors [i.e., specialized motor end plate regions, as demonstrated morphologically in some purinergically innervated tissues (17)]. Otherwise it would be difficult to explain how release of ATP from extrinsic sympathetic nerves results in excitation (41) whereas release of ATP from intrinsic inhibitory neurons results in hyperpolarization and inhibition (20).

Binding of P\(_{2Y}\) receptors is coupled via G proteins to activation of phospholipase C, IP\(_{3}\) formation, and release of Ca\(^{2+}\) from internal stores (15). We suggest that this may be the major pathway of coupling between ATP binding and activation of SK channels, because 1) ATP had no effect on cells dialyzed with EGTA to buffer intracellular Ca\(^{2+}\), 2) ATP activated an outward current in cells in which influx of Ca\(^{2+}\) was blocked by replacement of external Ca\(^{2+}\) with Mn\(^{2+}\), 3) caffeine treatment also activated SK independent of purine receptors, and 4) after an initial activation of SK with caffeine, open probability waned, possibly from depletion of sarcoplasmic reticulum Ca\(^{2+}\) stores. During this refractoriness, further addition of ATP failed to activate...
SK channels. The fact that PPADS also blocked SK channel activation in response to ATP and 2-MeS-ATP may also support the notion that IP$_3$-dependent release of Ca$^{2+}$ is responsible for activation of SK channels, since PPADS may block this mechanism (42).

It might seem odd that the effects of a compound reputed to be an inhibitory neurotransmitter are mediated by the release of Ca$^{2+}$ in smooth muscle cells. However, recent studies have shown that Ca$^{2+}$ release from internal stores can be highly directional and cause local increases in subsarcolemmal Ca$^{2+}$ concentration without affecting global Ca$^{2+}$ (33). A number of Ca$^{2+}$-dependent conductances are expressed in smooth muscle cells (12), and the issue of how Ca$^{2+}$-dependent inhibitory or excitatory responses might be preferentially activated should be considered. One level of selectivity may result from the relative Ca$^{2+}$ sensitivity and voltage dependence of inward and outward currents. For example, SK channels are more sensitive to Ca$^{2+}$ (7) than most of the other conductances present. We found that SK channels were activated at <100 nM Ca$^{2+}$, even at negative membrane potentials. Therefore, small increases in Ca$^{2+}$ near the membrane may selectively activate SK channels, and agonists that release focalized, small amounts of Ca$^{2+}$ may preferentially cause activation of SK channels. Higher concentrations of Ca$^{2+}$, perhaps supplemented by entry of Ca$^{2+}$ or more potent stimulation of Ca$^{2+}$ release, may facilitate nonselective cation channels and activate Ca$^{2+}$-dependent Cl$^-$ conductances and BK channels (for review see Ref. 12). Opening of BK channels tends to be constrained by the negative membrane potential range over which smooth muscle cells operate, but this conductance appears to provide important feedback regulation of membrane potential in some smooth muscles (8). Activation of Cl$^-$ and nonselective cation channels provides a depolarizing influence that is reinforced by positive feedback as Ca$^{2+}$ enters via voltage-dependent Ca$^{2+}$ channels. Ca$^{2+}$-dependent conductances could also be selectively regulated by the physical proximity of receptors and channels. For example, the location of P$_{2Y}$ receptors near SK channels may facilitate Ca$^{2+}$-dependent coupling, because local production of IP$_3$ might produce a “sparklike” event that might affect nearby channels without significantly affecting Ca$^{2+}$ levels near more distant channels. We were able to activate SK channels within the pipette in the “on-cell” configuration by adding purines to the external bath solution. This observation does not support an extremely close obligatory association between P$_{2Y}$ receptors and SK channels and suggests that IP$_3$ production resulting from P$_{2Y}$ binding may raise local Ca$^{2+}$ within a diameter of ≥1 μm.

Responses to ATP were sustained for several minutes in whole cell and single channel experiments. Sustained hyperpolarization and inhibition of spontaneous electrical activity have also been noted when murine colonic muscles are exposed to exogenous ATP (45). If ATP-dependent activation of SK channels is due to release of Ca$^{2+}$ from the sarcoplasmic reticulum, then the sustained nature of these responses suggests that ATP stimulation increases the rate of Ca$^{2+}$ release rather than causes a massive dumping of the Ca$^{2+}$ stores. A graded increase in the open probability of Ca$^{2+}$ release channels may facilitate local increases in Ca$^{2+}$ in the vicinity of SK channels without having an impact on global Ca$^{2+}$. Graded release of Ca$^{2+}$ from intracellular stores has been previously demonstrated by responses to caffeine. In the first reported measurements of spontaneous transient outward currents (STOCs), now considered a hallmark of Ca$^{2+}$ release from stores in smooth muscles, caffeine had a differential response depending on dose: a high concentration of caffeine caused a burst of STOCs and then quiescence; a lower concentration caused a sustained increase in the frequency of STOCs (3). These observations are now known to be due to the effects of caffeine on ryanodine receptors (33). A high concentration of caffeine greatly increases the open probability of ryanodine receptors and “dumps” the Ca$^{2+}$ stores. After the stores are dumped, there are no release events and no STOCs. A lower caffeine concentration increases the open probability of ryanodine receptors to a lesser extent, and the stores are not rapidly emptied. Thus the increase in the frequency of STOCs is sustained. Something of this nature (i.e., graded Ca$^{2+}$ release via an IP$_3$-dependent mechanism) may explain the sustained effects of ATP on SK channels in murine colonic cells.

We are unable to say with certainty that the 5.3-pS single channel currents activated by ATP and 2-MeS-ATP are the basis for the whole cell currents and hyperpolarization responses in intact muscles activated by these agonists. We could not make clear recordings of such small channels in “outside-out” patches, and therefore we could not adequately test the effects of apamin on these channels. Even if this experiment were practical, it may not be definitive, because apamin reduced peak whole cell current by only about one-third. ATP may activate more than a single type of K$^+$ conductance in whole cell experiments, and it is also possible that apamin-sensitive and -insensitive isoforms of SK channels could contribute to the whole cell response to ATP. It would be very difficult to demonstrate the latter simply by addition of apamin to the pipette solution. A tentative link between the single channels and whole cell currents can be made inferentially on the basis of the following observations: 1) the current activated by ATP was partially blocked by apamin; 2) apamin blocks some isoforms of SK channels (25); 3) whole cell currents were not resolved when cell interiors were dialyzed and buffered for Ca$^{2+}$. This further supports the hypothesis that apamin-sensitive and -insensitive isoforms of SK channels are expressed by GI muscles using Northern analysis and isoform-specific cDNA probes.
NOTE ADDED IN PROOF

After acceptance of this paper, another paper showing activation of small-conductance Ca\(^{2+}\)-activated K\(^+\) channels in response to purinergic agonists appeared in the literature [F. Vogalis and R. K. Goyal. Activation of small conductance Ca\(^{2+}\)-dependent K\(^+\) channels by purinergic agonists in smooth muscle cells of the mouse ileum. J. Physiol. (Lond.) 502: 497–508, 1977].

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