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Vasoactive Intestinal Peptide-stimulated Cl^- Secretion: Activation of cAMP-dependent K^+ Channels

L.T. Izu*, S.L. McCulle*, M.T. Ferreri-Jacobia†, D.C. Devor[2], M.E. Duffey[1]

[1]Department of Physiology and Biophysics, State University of New York, 124 Sherman Hall, University at Buffalo, Buffalo, NY 14214, USA

[2]Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA, 15261, USA

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Abstract. Vasoactive intestinal peptide (VIP) stimulates active Cl^- secretion by the intestinal epithelium, a process that depends upon the maintenance of a favorable electrical driving force established by a basolateral membrane K^+ conductance. To demonstrate the role of this K^+ conductance, we measured short-circuit current (I_{sc}) across monolayers of the human colonic secretory cell line, T84. The serosal application of VIP (50 nM) increased I_{sc} from 3 ± 0.4 μA/cm^2 to 75 ± 11 μA/cm^2 (n = 4), which was reduced to a near zero value by serosal applications of Ba^{2+} (5 mM). The chromanol, 293B (100 μM), reduced I_{sc} by 74%, but charybdotoxin (CTX, 50 nM) had no effect. We used the whole-cell voltage-clamp technique to determine whether the K^+ conductance is regulated by cAMP-dependent phosphorylation in isolated cells. VIP (300 nM) activated K^+ current (131 ± 26 pA, n = 15) when membrane potential was held at the Cl^- equilibrium potential (E_{Cl^-} = −2 mV), and activated inward current (179 ± 28 pA, n = 15) when membrane potential was held at the K^+ equilibrium potential (E_{K^+} = −80 mV); however, when the cAMP-dependent kinase (PKA) inhibitor, PKI (100 nM), was added to patch pipettes, VIP failed to stimulate these currents. Barium (Ba^{2+}, 5 mM), but not 293B, blocked this K^+ conductance in single cells. We used the cell-attached membrane patch under conditions that favor K^+ current flow to demonstrate the channels that underlie this K^+ conductance. VIP activated inwardly rectifying channel currents in this configuration. Additionally, we used fura-2AM to show that VIP does not alter the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. Caffeine (5 mM), a phosphodiesterase inhibitor, also stimulated K^+ current (185 ± 56 pA, n = 8) without altering $[Ca^{2+}]_i$. These results demonstrate that VIP activates a basolateral membrane K^+ conductance in T84 cells that is regulated by cAMP-dependent phosphorylation.

Key words: Cl^- secretion — K^+ conductance — cAMP-dependent protein kinase (PKA) — Intestine — Colon

Introduction

The intestinal secretion of water and electrolytes contributes to the physiological process of digestion by mixing digestive enzymes into the undigested contents of the lumen and diluting the products of digestion to facilitate absorption of nutrients by the intestinal epithelium. If this secretion does not overwhelm the absorptive capacity of the intestinal epithelium, the secreted fluid is reabsorbed along with the products of digestion. The principal physiological regulator of secretion in the intestine is vasoactive intestinal peptide (VIP) [9, 59], a neurocrine that is released by autonomic nerves in the wall of the intestine [22]. Parasympathetic autonomic nerves also regulate secretion via the release of acetylcholine (ACH); however, ACH produces a transient secretory response [35, 52]. A wide variety of stimuli can produce excess intestinal secretion capable of overwhelming the absorptive capacity of the intestinal epithelium and resulting in a net loss of

**Present address:* Departments of Cardiology and Physiology, University of Maryland, School of Medicine, Baltimore, MD 21201, USA.

†*Present address:* Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104, USA.

Correspondence to: M.E. Duffey; email: duffey@buffalo.edu

body water and electrolytes, a condition known as secretory diarrhea [50]. These stimuli include enterotoxin-producing bacteria (e.g., *Vibrio cholerae* and *Escherichia coli*), parasites, neuroendocrine tumors, and secreted organic compounds (e.g., bile acids). Secretory diarrhea also results from intestinal disorders of complex etiology, such as inflammatory bowel disease [23].

Originating from the crypts of Lieberkühn [27, 62], intestinal fluid secretion is osmotically coupled to electrogenic Cl^- secretion by secretory crypt cells [4, 26]. In the secretory cell model, an apical membrane Cl^- conductance provides the pathway for the movement of Cl^- into the lumen. On the basolateral membrane, Cl^- enters through a bumetanide-sensitive, electroneutral Na^+-2Cl-K^+ cotransporter. Concurrent with an agonist-regulated increase in apical membrane Cl^- conductance, an increase in basolateral membrane K^+ efflux maintains the electrical driving force for Cl^- exit across the apical membrane by hyperpolarizing the membrane potential [3, 13, 61]. At least two separate signaling pathways appear to coordinate the basolateral membrane K^+ conductance with the apical membrane Cl^- conductance. The parasympathetic neurocrine, ACH, increases intestinal secretion through an IP_3-mediated Ca^{2+} signaling pathway [12, 16, 19, 29]. Along with the underlying K^+ channels, this basolateral membrane K^+ conductance activated by ACH has been well characterized. Insensitive to the chromanol, 293B [33], and external Ba^{2+} [12, 55], this Ca^{2+}-mediated K^+ conductance is sensitive to charybdotoxin (CTX) [12, 34, 55], chlotrimazole (CLT) [17], levamisole [41], and quinidine [39]. The neurocrine, VIP, stimulates intestinal secretion through a cAMP-dependent phosphorylation pathway [4, 9]. The elevation of cAMP activates a CTX-insensitive K^+ conductance in intestinal epithelia [34] that is sensitive to 293B [33, 34], external Ba^{2+} [34], CLT [17], and levamisole [41]. The VIP-activated signaling pathway and the underlying K^+ channels responsible for this basolateral membrane conductance are not well understood.

We report the results of experiments designed to determine whether VIP activates a basolateral membrane K^+ conductance in cells of the human colonic secretory cell-line, T84; and, if so, whether Ca^{2+} activation or cAMP-dependent phosphorylation regulates this conductance. Our results demonstrate that VIP activates a K^+ conductance, which appears to involve cAMP-dependent phosphorylation catalyzed by the catalytic subunit of protein kinase A (PKA). Furthermore, activation of this conductance does not require an elevation of intracellular Ca^{2+}($[Ca^{2+}]_i$). These findings suggest that the basolateral membrane K^+ channels activated by VIP are separate from the K^+ channels activated by Ca^{2+}-mediated agonists.

Materials and Methods

Cell Culture

T84 cells were grown in DMEM and F-12 (1:1) supplemented with 20 mM HEPES, 10 mM glucose, 5% newborn calf serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Experiments were performed on cells at passages 56–75. Whole-cell and patch-clamp current measurements were made on isolated cells plated onto glass coverslips the previous day. For short-circuit current measurements (I_{sc}) T84 cells were seeded onto Costar transwell cell culture inserts (0.33 cm^2), and the culture medium was changed every 48 hr. I_{sc} measurements were made on a filter after 10–16 days in culture. Tissue-culture media were obtained from GIBCO Laboratories.

Solutions

For measurements of I_{sc}, the bath solution contained (in mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, and 10 glucose. The pH of this solution was 7.4 when gassed with a mixture of 95% O_2-5% CO_2 at 37°C. For current measurements on isolated cells, coverslips containing cells were placed in a Plexiglas chamber and mounted on the stage of an inverted microscope. Bath solution entered the chamber by gravity feed and was removed by aspiration, such that a complete solution exchange was accomplished in 30–60 sec. The standard bath solution contained (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES; pH was adjusted to 7.4 with NaOH. All measurements were made at room temperature (22–25°C), except as described in the text. When needed, solutions were heated to temperatures above room temperature using a water-filled heat exchanger or thermoelectric heater (model TC-202, Medical Systems, Great Neck, NY) to heat the bath solution entering the cell chamber.

The methods for fabrication of whole-cell patch pipettes were similar to those previously described [15, 16]. The whole-cell patch-pipette solution contained (in mM): 130 KCl, 5 NaCl, 41 $MgCl_2$, 0.12 $CaCl_2$, 10 HEPES, 2 Mg-ATP, 0.5 GTP, and 0.2 EGTA; pH was adjusted to 7.4. The calculated free Ca^{2+} concentration was 100 nM (Maxchelator, Chris Patton, Pacific Grove, CA). The perforated-patch pipette solution contained (in mM): 60 K_2SO_4, 40 KCl, 5 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES; pH was adjusted to 7.4 with KOH. Before use, a nystatin stock solution (50 mg/ml) was diluted 1:300 into the pipette solution and sonicated for 30 sec. A small column of nystatin-free pipette solution was drawn into the tip of a pipette to facilitate patch formation, and the rest of the pipette was completely filled with the nystatin-containing solution. The cell-attached patch-pipette solution contained (in mM): 145 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES; pH was adjusted to 7.4 with KOH. For whole-cell, perforated and cell-attached patch measurements, cells were bathed in the standard bath solution.

I_{sc} Measurements

Costar transwell cell culture inserts were mounted in Ussing chambers (Jim's Instruments, Iowa City, IA), and tissues were continuously short-circuited (model 558C-5, Bioengineering, the University of Iowa, Iowa City, IA). Transepithelial resistance was measured by applying a 5-mV pulse at 30 to 60 sec intervals, and resistance was calculated using Ohm's law. Monolayers had resistances of 500–1500 Ωcm^2. VIP, CTX and 293B were added to the serosal solution at concentrations indicated in the text.

Whole-Cell Current Measurements

Membrane electrical properties were recorded using a patch-clamp amplifier (model EPC-7, Medical Systems-List, Great Neck, NY or model Axopatch 200, Axon Instruments, Foster City, CA). Membrane potential and current were referenced to the bath using an $Ag\text{-}Ag_2Cl_2$ electrode connected to the bath. An outward current was defined as a cation flowing from the cell to the bath across the cell membrane. During whole-cell voltage-clamp experiments using the standard pipette and bath solutions the cells were alternately voltage-clamped (model VCC600, Physiologic Instruments, San Diego, CA, or P-Clamp, Axon Instruments between the K^+ equilibrium potential ($E_{K^+} = -80$ mV) and the Cl^- equilibrium potential ($E_{Cl^-} = -2$ mV).

Voltage steps were made at 2-sec intervals. During perforated-patch voltage-clamp experiments cells were alternately voltage-clamped between the K^+ equilibrium potential ($E_{K^+} = -88$ mV) and the Cl^- equilibrium potential ($E_{Cl^-} = -29$ mV) at 2-sec intervals. Recordings were not begun until the access resistance of the patch had decreased below 20 MΩ. Peak current values were determined by measuring the difference between the peak current recorded during stimulation of a cell and the current recorded before stimulation. During whole-cell and perforated patch recordings, we assumed there was no significant change in seal resistance if inward and outward currents returned to near the initial values after agonist removal. Whole-cell and perforated-patch currents were sampled using a digital data recorder (model VR-10, Instrutech, Great Neck, NY) and recorded onto VCR tape. Currents were also filtered at 1 kHz using an eight-pole low-pass filter (model 902, Frequency Devices, Haverhill, MA) and recorded directly onto a strip chart recorder.

Single-Channel Analysis

Single-channel currents were recorded using a patch-clamp amplifier (model Axopatch 200, Axon Instruments), sampled using a digital data recorder (model VR-10B, Instrutech) and stored on videotape for analysis. For analysis of single-channel currents, records were sampled using the digital data recorder, filtered at 1 kHz using an eight-pole low-pass filter (model 902, Frequency Devices, Haverhill, MA) and digitized at 2 kHz using a data acquisition program (Axotape, Axon Instruments). Amplitude characteristics were determined using software based upon the segmental k-means algorithm (SKM) [46]. A Markov model was developed based on the number of channels in the patch. A state was specified for the closed conductance level and each open conductance. The analysis software determined the amplitude characteristics based upon likelihood functions performed on the amplitude data. Taken from regions where single channels could be resolved, such as the trailing edges of agonist application and washout, traces totaling a minimum of 5 sec in duration were analyzed using this method.

Intracellular Ca^{2+} Measurements

Cells on glass coverslips were loaded at room temperature with fura-2AM (4 μM) for 20 min in Ca^{2+}-free solution followed by incubation in a 5% CO_2 atmosphere for 1 hr in the standard, Ca^{2+}-containing culture medium for T84 cells. Coverslips containing the fura-2AM loaded cells were placed in a Plexiglas chamber and mounted on the stage of an inverted microscope (Diaphot, Nikon, Japan) equipped for epifluorescence using a 40× oil-immersion lens, as previously described [14]. Fura-2AM fluorescence images at 340 nm and 380 nm excitation wavelengths were captured with a SIT video camera (model C2400-08, Hamamatsu, Japan) and analyzed using imaging software (Image 1/FL, Universal Imaging Corp., West Chester, PA). Average whole-cell ratio values from single cells were determined.

Chemicals

293B was a generous gift from Dr. Rainer Greger (University of Freiburg, Freiburg, Germany). Nystatin was a generous gift from Dr. S. J. Lucania (Bristol Meyers-Squibb, Princeton, NJ). Carbachol and caffeine were obtained from Sigma (St. Louis, MO). VIP and CTX were obtained from Bachem California (Torrence, CA). PKI was obtained from Calbiochem-Novabiochem, Int. (La Jolla, CA). Fura-2AM was obtained from Molecular Probes (Eugene, OR).

Data Analysis

T-tests were performed for differences in peak membrane currents using a computer program (NCSS 60, Number Cruncher Statistical Systems, Kaysville, UT). In all cases, a value of $P < 0.05$ is considered statistically significant. The data are presented as the mean ± SE.

Results

VIP Activates I_{sc} across T84 Monolayers

Polarized monolayers of T84 cells secrete Cl^- in response to many of the same neurotransmitters and hormones as intestinal tissue [19]. To determine the effects of VIP, we grew T84 monolayers on Costar transwell permeable membrane supports and measured I_{sc} across these monolayers. The tissues were mounted in Ussing chambers and bathed in standard NaCl electrolyte solutions at 37°C. Figure 1 shows the results of 2 experiments in which tissues were exposed to VIP. Before VIP was added I_{sc} was near zero (3 ± 0.4 μA/cm^2, $n = 4$). Panel *A* shows that addition of VIP (50 nM) to the serosal bath increased I_{sc} to a plateau (75 ± 11 μA/cm^2, $n = 4$). Previous isotopic flux measurements confirmed that the VIP-stimulated I_{sc} is equal to the rate of Cl^- secretion [8, 36]. Addition of CTX (50 nM) to the serosal bath did not affect the current ($n = 4$). This result suggests that a Ca^{2+}-activated K^+ conductance is not involved in the generation of I_{sc}, since CTX predominantly inhibits Ca^{2+}-activated K^+ conductances [24]. However, Fig. 1*A* shows that serosal addition of Ba^{2+} (5 mM) reduced I_{sc} to near the basal value (8 ± 1 μA/cm^2, $n = 4$). Since Ba^{2+} is an inhibitor of K^+ conductance in T84 cells [12, 16, 19, 36], these results suggest that a basolateral membrane K^+ conductance is involved in Cl^- secretion. Since VIP causes an elevation of intracellular cAMP concentration in T84 cells [36], we then determined whether the basolateral membrane K^+ conductance is sensitive to the chromanol, 293B, which inhibits the

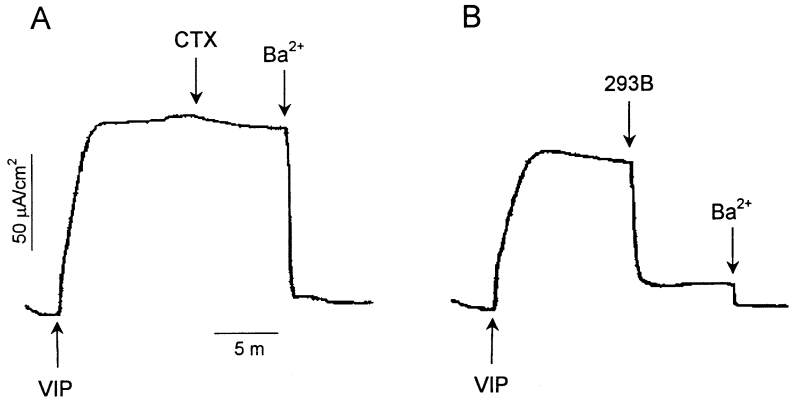

Fig. 1. Short-circuit current (I_{sc}) across T84 monolayers exposed to vasoactive intestinal peptide (VIP, 50 nM). Two confluent monolayers (*A* and *B*) were grown on Costar transwell filters. (*A*) Charybdotoxin (CTX, 50 nM) was added to the serosal bath, followed by Ba^{2+} (5 mM). (*B*) The chromanol 293B (100 nM) was added to the serosal bath, followed by Ba^{2+} (5 mM).

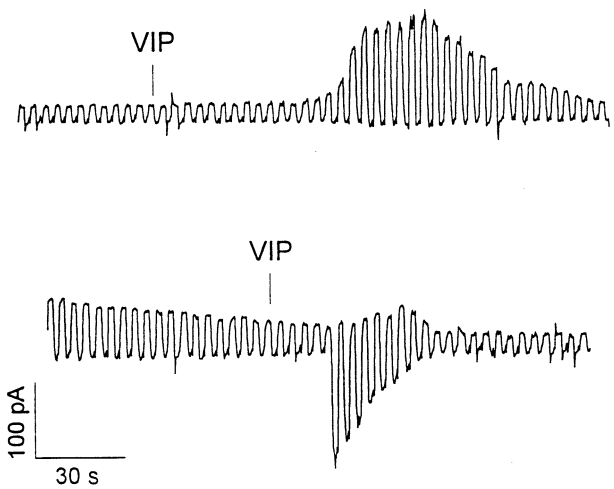

Fig. 2. Membrane current as a function of time for cells exposed to 300 nM VIP. Two cells were whole-cell voltage-clamped. The bar (|) indicates onset of VIP exposure that continued for the duration of the recording. In this and all subsequent figures an outward current is represented as an upward deflection from baseline.

forskolin-induced I_{sc} across rabbit colon [33], human colon [35], and T84 monolayers [13]; forskolin is a cAMP-mobilizing agent that increases the activity of adenylate cyclase. Panel *B* shows I_{sc}, activated by VIP in another monolayer (78 ± 10 μA/cm², $n = 4$). When 293B (100 μM) was added to the serosal bath, I_{sc} was dramatically reduced by 74% (20 ± 2 μA/cm², $n = 4$). Ba^{2+} further reduced I_{sc} to near the basal value (5 ± 0.3 μA/cm², $n = 4$). These results suggest that VIP activates a basolateral membrane K^+ conductance that is separate from the K^+ conductance activated by Ca^{2+}-mediated agonists.

VIP Activates K^+ and Inward Currents in Isolated T84 Cells

We measured whole-cell membrane currents in isolated T84 cells to characterize the K^+ conductance activated by VIP. Figure 2 illustrates the effect of VIP on membrane currents recorded from 2 isolated cells. In these experiments, the pipette contained standard high-K^+ Ringer solution; while the bath contained standard high-Na^+ Ringer solution. The cells were alternately voltage-clamped between E_{K^+}(= −80 mV) and E_{Cl^-}(= −2 mV) at 2-sec intervals. VIP exposure (300 nM) began approximately 90 sec after achieving the whole-cell configuration. The top trace of Fig. 2 demonstrates that, after a brief delay, VIP activated a rapid increase in outward current (seen as upward deflections). This outward current was measured during the intervals when the cell was voltage-clamped to E_{Cl^-}. This condition produced an outwardly directed electrochemical gradient for K^+, an inwardly directed electrochemical gradient for Na^+ and no gradient for Cl^-. Thus, the outward current is principally a K^+ current. This K^+ current gradually declined despite the presence of VIP. Although a large electrochemical gradient exists for Na^+ and Cl^-, no increase in inward current is observed during the interval when the cell was voltage-clamped to E_{K^+}. The bottom trace shows a recording from a different cell, in which VIP activated an inward current (seen as downward deflections). This inward current could result from either Na^+ influx and/or Cl^- efflux created by the large electrochemical gradients that existed across the cell membrane for Na^+ and Cl^-. Since T84 cells express the cystic fibrosis transmembrane conductance regulator (CFTR) [58], which may be activated by VIP, a portion of this current is likely to be a Cl^- current. Overall, 15 of 28 cells (54%) responded to VIP with an increase in membrane current. The peak K^+ current averaged 131 ± 26 pA and the peak inward current was 179 ± 28 pA ($n = 15$). Seven of the 15 responding cells had both K^+ current (peak current, 144 ± 42 pA) and inward current (peak current, 185 ± 35 pA). Six cells responded only with K^+ current (116 ± 37 pA), and the remaining 2 cells had only an inward current (155 ± 35 pA). Since VIP causes an elevation of intracellular cAMP concentration in T84 cells [36], it follows that a cAMP-dependent phosphorylation pathway increases both the K^+ and inward currents.

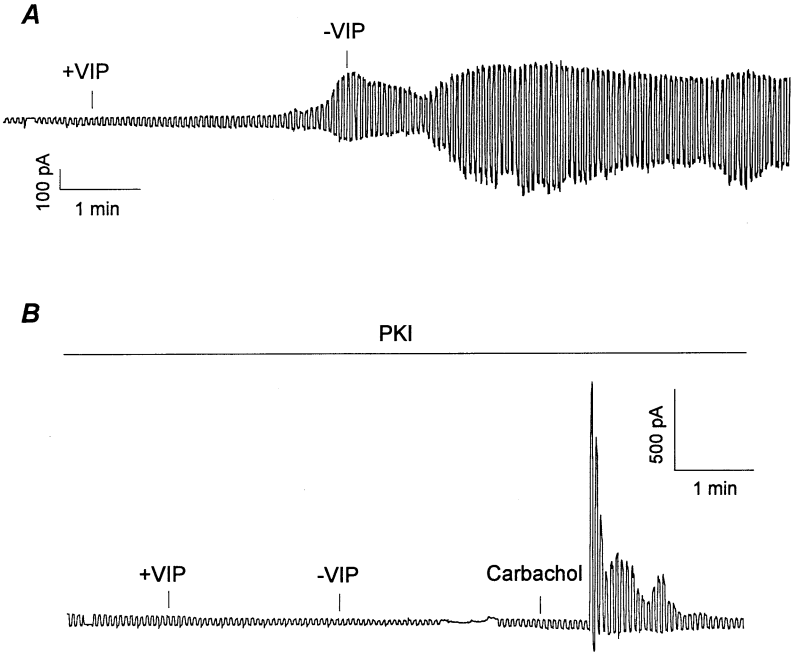

Fig. 3. Membrane current for 2 cells exposed to VIP. The plus (+) and minus (−) sign indicate beginning and end of 300 nM VIP exposure. (*A*) Membrane current for a cell at 30°C measured by the perforated patch-clamp technique. (*B*) Effect of the cAMP-dependent kinase inhibitor peptide (PKI) on membrane current. The whole-cell patch pipette contained standard pipette solution plus 100 nM PKI. The cell was exposed to 300 nM VIP followed by 100 μM carbachol. VIP exposure occurred 5 min after breaking the patch to allow PKI to diffuse into the cell.

VIP Activates K^+ and Inward Currents in Perforated Patches

To confirm these conclusions, we repeated these experiments with nystatin perforated-patch configuration because cell dialysis in the whole-cell configuration could result in the loss of vital intracellular constituents [15]. During perforated patching, 4 of 13 cells (31%) responded to VIP. For these patches, the mean K^+ current was 105 ± 52 pA and the mean inward current was 30 ± 12 pA. The outward currents measured by this technique were not statistically different from those activated during standard whole-cell voltage clamp. In contrast, the inward currents were significantly smaller. These results establish that the standard whole-cell configuration can be used to measure K^+ currents without concern for the loss of intracellular signaling components during whole-cell dialysis.

VIP Response Is Temperature-dependent

The responses to VIP seen in Fig. 2 were transient, usually lasting only about 30 sec to 1 min despite the continued presence of VIP. However, VIP-activated Cl^- secretion across T84 monolayers is sustained [8, 36]. The duration of our currents may be short because our measurements are made at room temperature (22–25°C), while the monolayer measurements were made at 37°C. To determine whether VIP responses are temperature-dependent, we repeated our measurements at 28–37°C using the perforated-patch configuration. Cells were voltage-clamped between E_{K^+} and E_{Cl^-} and exposed to VIP (300 nM). Figure 3*A* shows a representative current response to VIP (300 nM) at 30°C. As can be seen, VIP elevated both K^+ and inward currents, a response that was sustained longer than room-temperature responses. In fact, the membrane currents remained elevated 5 min after switching back to normal bathing solution. Similar responses were observed in 18 other cells tested at temperatures between 28–37°C; approximately 70% of the cells responded to VIP (*see* average values for measurements made at 37°C below). These results demonstrate the advantage of making measurements at higher temperatures. We performed most experiments at room temperature because our intracellular Ca^{2+} indicator, fura-2, rapidly leaks out of T84 cells at higher temperatures (unpublished observation), which would prevent correlation of the results of current measurements and $[Ca^{2+}]_i$ measurements (*see* below). Temperature-dependent leakage of fura-2 has been observed in other tissues and has been attributed to a yet to be identified anion transporter [40].

The Effects of VIP on Membrane Currents Are Mediated by cAMP-dependent Protein Kinase (PKA)

We hypothesize that the elevated membrane currents observed in the presence of VIP arise from the phosphorylation of the underlying channels by PKA. To test this hypothesis, we added the 20-amino-acid peptide cAMP-dependent protein kinase inhibitor, PKI (100 nM, K_i = 2.3 nM) [10], to whole-cell patch pipettes. To allow PKI to diffuse into the cells, we waited about 5 min after breaking the patches before

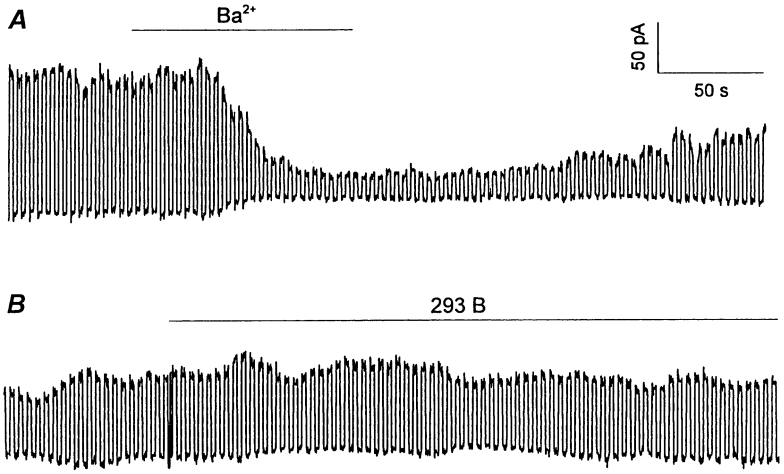

Fig. 4. Membrane current for cells exposed to 300 nM VIP at 37°C. Current was measured by the whole-cell patch-clamp technique. The cells were pre-exposed to VIP, which remained in the bathing solutions for the duration of each record. (*A*) The bath was switched to one containing 5 mM Ba^{2+} ($BaCl_2$), as indicated by the bar. (*B*) The bath was switched to one containing 100 μM 293B as indicated by the bar.

stimulating with VIP. Figure 3*B* shows that PKI abolished both K^+ and inward current responses to VIP. Subsequent exposure to carbachol (100 μM) elicited a robust K^+ current but little inward current. In the whole-cell configuration, we previously observed that carbachol activates an oscillatory increases in K^+ conductance [16] corresponding to oscillations of $[Ca^{2+}]_i$ [14]. When this experiment was carried out in 4 cells, VIP did not elicit any response. Following the application of VIP, carbachol exposure stimulated K^+ currents but no inward currents in 3 of these cells. Based upon these results, we conclude that VIP stimulates elevated K^+ and inward currents through PKA-dependent phosphorylation. Furthermore, we also demonstrate that K^+ conductance regulated by $[Ca^{2+}]_i$ is not dependent upon PKA-dependent phosphorylation.

Ba^{2+} Blocks VIP-activated K^+ Current

While the results of our whole-cell voltage-clamp measurements demonstrate that VIP activates a membrane K^+ current, these results do not identify the K^+ channels that underlie this current. To characterize these channels, we stimulated membrane currents with VIP in voltage-clamped cells using the whole-cell technique. Following activation of K^+ current by VIP, the cells were exposed to Ba^{2+}, an ion that has been shown to inhibit VIP-activated K^+ efflux (as $^{86}Rb^+$) across the basolateral membrane of T84 monolayers [19]. These measurements were made at 37°C so that VIP exposure would cause sustained responses (Fig. 3*A*). Figure 4*A* shows the response of one cell stimulated by VIP (300 nM) and exposed to Ba^{2+} (5 mM). As illustrated, Ba^{2+} inhibited the K^+ current. This effect is reversible. In 6 of 8 cells that responded to VIP, the VIP-activated K^+ current (85 ± 18 pA) was inhibited 100% by Ba^{2+}; in the remaining 2 cells Ba^{2+} had no effect. To further characterize this conductance, we repeated the experiment using the chromanol, 293B. Figure 4*B* demonstrates that 293B (100 μM) did not inhibit the K^+ current activated by VIP. No effect of 293B was seen in the 5 cells tested. When currents were measured using the perforated-patch technique, Ba^{2+} inhibited the VIP-activated K^+ current (56 ± 14 pA) by 100% in 7 of 9 cells that responded to VIP; in the remaining 2 cells Ba^{2+} had no effect. The chromanol failed to inhibit the VIP-activated K^+ current (68 ± 17 pA) in all 8 cells that were tested; while subsequent exposure of 5 of these cells to Ba^{2+} blocked the current by an average of 95%. These results suggest that VIP activates a unique K^+ conductance in isolated T84 cells.

Caffeine Activates K^+ and Inward Currents via a Ca^{2+}-independent Mechanism

Our results suggest that VIP activates K^+ and inward currents by an elevation of intracellular cAMP, presumably as a result of activation of adenylate cyclase [18, 36]. We attempted to confirm the involvement of cAMP in membrane channel activation by measuring ion currents while raising the intracellular cAMP concentration by an alternative mechanism. For this purpose, we chose to use a phosphodiesterase inhibitor rather than an agonist that activates PKA because PKA is known to activate a cAMP-specific phosphodiesterase [21]. We used caffeine, which can raise intracellular cAMP concentration by inhibiting phosphodiesterase [5]. While caffeine is known to raise $[Ca^{2+}]_i$ in many cell types [42, 53], it dose not raise $[Ca^{2+}]_i$ in T84 cells (*see* below). A preferred phosphodiesterase inhibitor, 3-isobutyl-l-methylxanthine (IBMX), was not used because it raises $[Ca^{2+}]_i$ in these cells (unpublished observation). To study the currents activated by caffeine, we used the perforated-patch configuration. Cells were voltage-clamped between E_{K^+} and E_{Cl^-} and exposed to caffeine (5 mM). Figure 5 illustrates a typical response in which

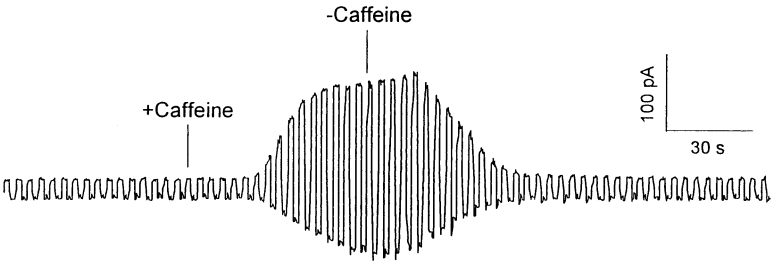

Fig. 5. Membrane currents activated by 5 mM caffeine. Current was measured by the perforated patch-clamp technique.

caffeine elevated both K^+ and inward currents. These currents return to their baseline values when caffeine is removed. Eight of 12 cells responded to caffeine (67%). The mean peak K^+ current was 184 ± 56 pA and the mean peak inward current was 84 ± 13 pA. Seven cells produced K^+ and inward currents, while one cell only had inward current. There was no difference between the peak caffeine-activated K^+ current measured by the perforated-patch configuration and the K^+ current activated by VIP in the same configuration. However, peak inward caffeine-activated current was different from peak inward VIP-activated current. Unlike the transient responses seen with VIP, the currents remained elevated as long as caffeine was present.

VIP and Caffeine Do not Raise $[Ca^{2+}]_i$

We demonstrated previously that carbachol activates K^+ current in perforated patches on T84 cells by raising $[Ca^{2+}]_i$ [14, 15]. To test whether current responses to VIP are mediated by intracellular Ca^{2+}, we measured the fura-2AM fluorescence ratio as a measure of $[Ca^{2+}]_i$. Figure 6*A* illustrates the result of an experiment in which VIP (300 nM) did not change $[Ca^{2+}]_i$ during a 4-min exposure. However, carbachol (100 μM) resulted in an elevation and oscillation of $[Ca^{2+}]_i$. This experiment was done on 57 cells from 19 coverslips. VIP did not change $[Ca^{2+}]_i$ in any of the 57 cells, however, in all cells carbachol caused $[Ca^{2+}]_i$ to rise. In some cases, carbachol caused $[Ca^{2+}]_i$ to oscillate, while in others the rise was sustained. In all but 3 cells, $[Ca^{2+}]_i$ returned to baseline values after removing carbachol. These results demonstrate that Ca^{2+} is not an intermediary of the action of VIP.

Besides having the ability to inhibit phosphodiesterase, caffeine has been observed to raise $[Ca^{2+}]_i$ in some cell types, such as hepatocytes [43] as well as skeletal [53] and cardiac [42] muscle cells. Therefore, we tested for involvement of Ca^{2+} in the current responses to caffeine by measuring $[Ca^{2+}]_i$ in a series of cells during exposure to caffeine. Figure 6*B* shows that caffeine (5 mM) had no effect on the fura-2 fluorescence ratio in 1 cell, while subsequent exposure to carbachol elicited the anticipated elevation and oscillation of $[Ca^{2+}]_i$. We monitored $[Ca^{2+}]_i$ changes to caffeine in 11 cells. In the cells tested, there was no change in $[Ca^{2+}]_i$ associated with caffeine applications, however, all responded to a previous or subsequent exposure to carbachol.

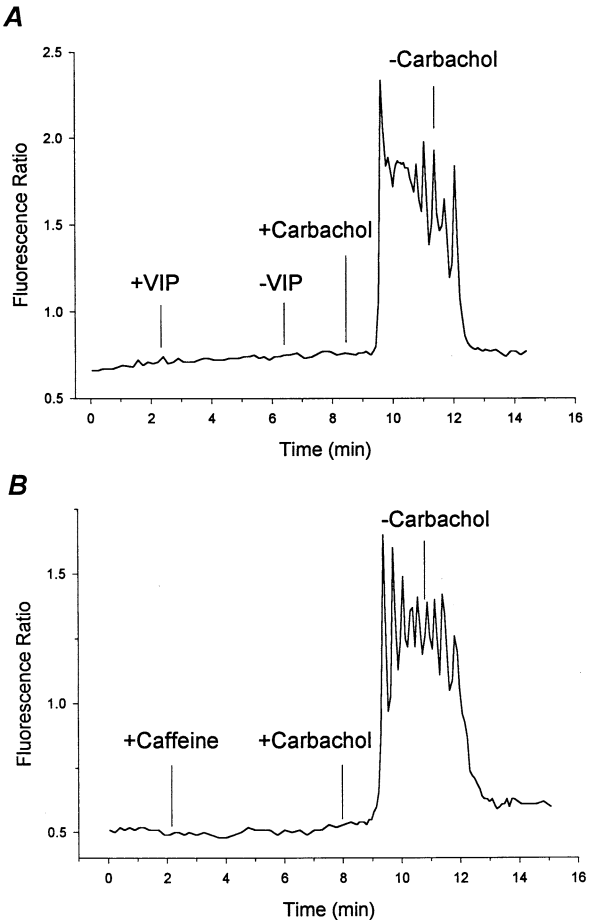

Fig. 6. Fura-2 fluorescence ratio recorded at 340 nm and 380 nm for 2 cells. (*A*) Fura-2 fluorescence ratio recorded for a cell exposed to 300 nM VIP followed by 100 μM carbachol. (*B*) Fura-2 fluorescence ratio for a cell exposed to 5 mM caffeine followed by 100 μM carbachol.

VIP Activates K^+ Channels

In order to demonstrate channels that underlie the K^+ current activated by VIP (Fig. 2), we used the cell-attached patch-clamp technique to study these channels. For these studies, patch pipettes contained

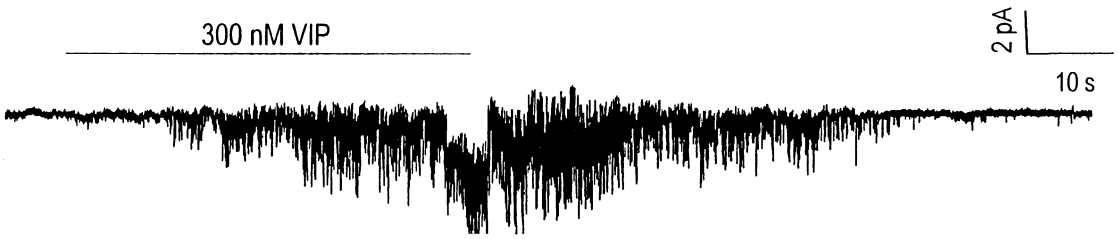

Fig. 7. Current activated by VIP in a cell-attached patch. The patch was voltage-clamped at 0 mV. The cell was exposed to 300 nM VIP during the period marked by the bar.

145 mM KCl and the bath contained 145 mM NaCl. Membrane patches were voltage-clamped to 0 mV so that the cell's membrane potential was the only electrical driving force across the cell membrane. Figure 7 illustrates that VIP (300 nM) activated channels in a cell-attached patch, and the activation was reversible. Prior to the addition of VIP, there was little spontaneous channel activity. These channel openings were likely to be the result of K^+ leaving the pipette and entering the cell through K^+ channels in the cell membrane. Figure 8*A* shows 2-sec traces of VIP-activated channel activity at various applied pipette potentials (V_{pip}). As V_{pip} was made more positive than 0 mV, the current flowing through these channels increased. However, as V_{pip} was made more negative than 0 mV, the current flow decreased until, at -40 mV, current was not detectable. At -60 mV the single-channel current was very small, but appeared to flow in the opposite direction. At -120 mV single-channel events were clearly detectable and reversed from the direction seen at positive potentials. Figure 8*B* summarizes this VIP-activated single-channel activity in a current-voltage (I/V) relationship. In this figure, the mean current amplitudes for each applied potential were determined for traces 5 sec in length by the techniques described in the Methods section. The x-axis represents $-V_{pip}$, so that currents were referenced to the cell interior. The I/V plot demonstrated that the single-channel currents are inward-rectifying. Similar results were observed in 2 other cells. Thus, the channels activated by VIP are likely to be inward-rectifying K^+ channels.

Discussion

Basolateral Membrane K^+ Conductance Contributes to VIP-activated I_{sc}

As the principal regulator of intestinal fluid secretion [9, 59], VIP stimulates the secretion of body water and electrolytes through a cAMP-dependent pathway [4, 9]; whereas ACH activates intestinal secretion through a Ca^{2+}-mediated pathway [12, 16, 19, 29]. In both pathways, the central event driving fluid secretion is Cl^- efflux from the apical membrane of secretory crypt cells [4, 26]. The secondary active process of Cl^- secretion generates an osmotic as well as an electrochemical gradient across the intestinal epithelium that favors the passive movement of body water and cations into the intestinal lumen. In order to sustain the electrical driving force for Cl^- secretion, both signaling pathways must synchronize apical membrane Cl^- efflux with basolateral membrane K^+ efflux. The Ca^{2+}-mediated basolateral K^+ conductance, along with the underlying K^+ channels, are well characterized, while the cAMP-dependent basolateral K^+ conductance is not as well understood. In the work reported here, we attempted to characterize the VlP-activated, cAMP-dependent K^+ conductance as well as the signaling pathway that regulates the underlying K^+ channels in the intestinal secretory cell line, T84.

As seen previously [8, 36], we demonstrate that serosal application of VIP generated an I_{sc} in monolayers of T84 cells (Fig. 1). Through isotopic flux measurements, this I_{sc} has been shown to correspond to the rate of Cl^- secretion [8, 36]. Since Cl^- secretion depends upon a favorable electrical gradient established by basolateral K^+ efflux, it follows that inhibitors of the underlying K^+ channels of this cAMP-dependent conductance will reduce the I_{sc} stimulated by VIP, thereby providing an inhibitor profile of the underlying K^+ channels. We show that serosal application of Ba^{2+} returned the VIP-stimulated I_{sc} to basal levels (Fig. 1). These results correspond to other studies demonstrating that the cAMP-dependent K^+ conductance is sensitive to external Ba^{2+}. For instance, serosal application of Ba^{2+} inhibited VIP-stimulated $^{86}Rb^+$ efflux across the basolateral membrane of T84 monolayers [36], the VIP-stimulated I_{sc} across monolayers of the HT29-C1.16E human colonic Cl^-- and mucin-secreting cell line [39], and the forskolin-stimulated I_{sc} across human colon [37]. As external application of Ba^{2+} inhibits a variety of K^+ channels [36], we also applied a more specific K^+ channel inhibitor, 293B. Figure 1*B* demonstrates that the serosal addition of 293B inhibited about 75% of the VIP-stimulated I_{sc}, and subsequent serosal addition of Ba^{2+} reduced the

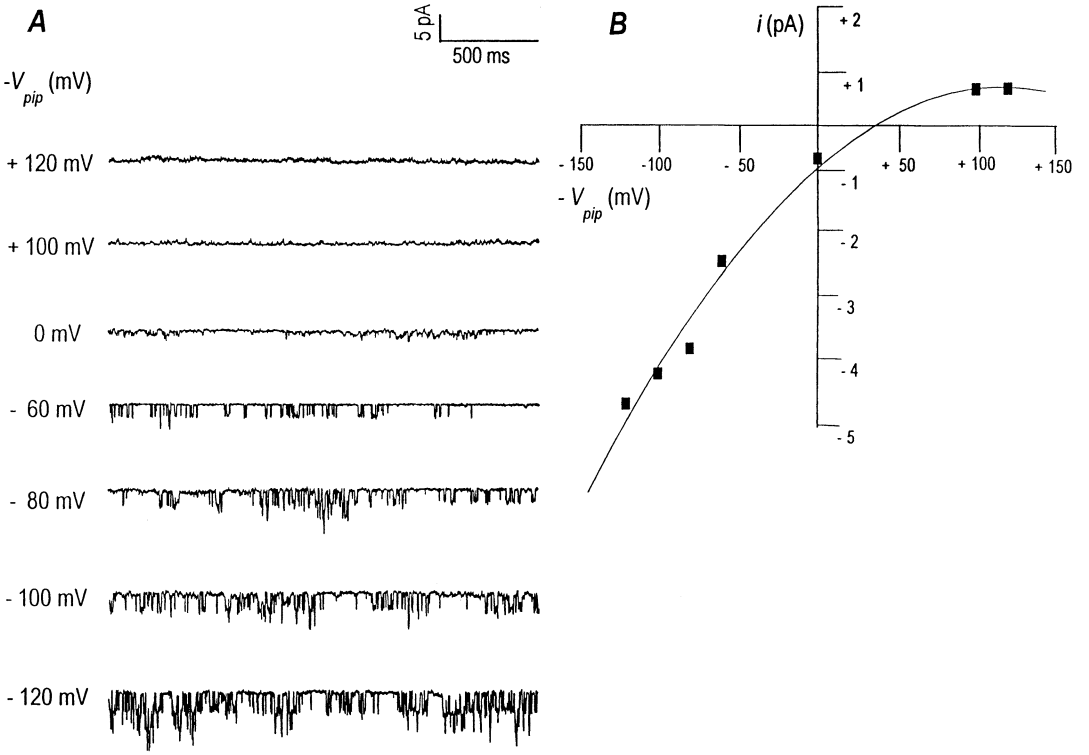

Fig. 8. During an application of VIP, the patch was voltage-clamped between ±120 mV in 20-mV steps of 1 min duration. Two-second traces of VIP-activated channel activity are shown at various applied pipette potentials (V_{pip}). (*B*) Current-voltage relationship (I/V) for the VIP-activated single channel currents of (*A*). The x-axis represents $-V_{pip}$ so that currents are referenced to the cell interior.

VIP-stimulated I_{sc} to near basal levels. These observations suggest that a large portion of the VIP-stimulated current in T84 monolayers is sensitive to the inhibitor, 293B. In the rabbit colonic epithelium [33] and the base cells of the rat crypt [60], 293B has also been shown to be an inhibitor of a K^+ conductance regulated through a cAMP-dependent pathway.

Serosal applications of CTX, a classic inhibitor of the Ca^{2+}-mediated K^+ conductance [24], did not reduce the VIP-stimulated I_{sc} (Fig. 1*A*). Thus, our results correspond to other studies demonstrating that the cAMP-dependent K^+ conductance is insensitive to CTX. For example, serosal application of CTX to T84 monolayers [13] and murine colonic epithelium [34] did not reduce the I_{sc} stimulated by forskolin. In contrast to the inhibitor profile of the cAMP-dependent conductance, the Ca^{2+}-activated I_{sc} has been shown to be insensitive to serosal applications of Ba^{2+} [36]. Illustrating the selectivity of this inhibitor for the cAMP-dependent K^+ conductance, 293B did not significantly reduce the I_{sc} stimulated by the Ca^{2+}-ionophore ionomycin in rabbit colonic epithelium [33]. Additionally, applications of 1-EBIO, an agonist of the underlying channels of the Ca^{2+}-mediated K^+ conductance, stimulated an I_{sc} that was insensitive to 293B and sensitive to CTX [13].

Mechanism Responsible for the VIP-activated K^+ Conductance

To characterize the K^+ conductance activated by VIP as well as the signaling pathway regulating this conductance, we used the whole-cell voltage-clamp technique to measure membrane currents in isolated T84 cells. Figure 2 demonstrates that VIP activated both K^+ and inward currents in isolated cells. In contrast to the prolonged VIP-stimulated I_{sc} observed in our Ussing chamber measurements (Fig. 1*A* and 1*B*) as well as in previous studies [8, 36], the whole-cell current responses to VIP in isolated T84 cells lasted from 30 to 90 sec at room temperature (Fig. 2). Since the monolayer measurements were done at 37°C, we measured currents in cells at 28–37°C to determine if temperature altered the magnitude and the duration of the VIP-stimulated conductances. In the perforated-patch configuration, VIP stimulated an enhanced response at 30°C (Fig. 3*A*). The underlying reason for this difference could be that activation of membrane channels by VIP involves a cascade of enzyme-catalyzed reactions that are both time- and temperature-dependent. For example, VIP binding to the basolateral membrane of rat and rabbit enterocytes is greatly enhanced by temperature elevation from room temperature to 37°C [18]. Additionally, it

has been shown in both rat and mouse macrophages that the amount of cAMP generated by VIP-stimulated adenylate cyclase increases with temperature and can require 30 min to reach a steady-state value [45].

Since Ca^{2+}-activated K^+ channels are found in many cell types, including T84 cells [12, 47, 55], the VIP-stimulated K^+ conductance may have resulted from a rise in $[Ca^{2+}]_i$. Receptors related to VIP receptors, such as the glucagon receptor [57] and the type I PACAP receptors [31] have been shown to increase $[Ca^{2+}]_i$ through an IP_3-mediated pathway along with intracellular cAMP through an increase in adenylate cyclase activity. VIP-stimulation has elevated $[Ca^{2+}]_i$ in some cell types and cell lines [30, 54]. This mechanism has been proposed to explain the epinephrine-stimulated basolateral membrane K^+ conductance in the canine tracheal epithelium [61]. Epinephrine, which stimulates Cl^- secretion in canine tracheal epithelium, increases intracellular cAMP as well as causes the release of Ca^{2+} from intracellular stores. Although it has been established that VIP stimulates adenylate cyclase and the consequent elevation of intracellular cAMP in the human colonic crypts [20] and T84 cells [36], it was not previously determined whether VIP stimulation triggers an elevation of $[Ca^{2+}]_i$ in T84 cells. Our measurements of $[Ca^{2+}]_i$ in T84 cells using fura-2 (Fig. 6*A*) demonstrate that VIP (300 nM) did not alter $[Ca^{2+}]_i$. These results are consistent with the observation that forskolin does not alter $[Ca^{2+}]_i$ in human colonic crypts [37] or the HT29-C1. 16E human colonic cell line [39].

Based upon the observation that VIP did not elevate $[Ca^{2+}]_i$ at room temperature, we proposed that VIP activates the channels underlying the K^+ conductance through a cAMP-dependent pathway. To test this hypothesis, caffeine, a phosphodiesterase inhibitor that generates a consequential elevation of intracellular cAMP [5], was applied to single T84 cells in the perforated-patch configuration. Figure 5 demonstrates that caffeine activated a K^+ current similar to the K^+ current activated by VIP. Caffeine, known to cause the release of Ca^{2+} from intracellular stores [42, 53], could have activated a Ca^{2+}-mediated K^+ conductance. However, in T84 cells, caffeine did not elevate $[Ca^{2+}]_i$ (Fig. 6*B*). These results support the hypothesis that a K^+ conductance regulated through a cAMP-dependent signaling pathway exists in T84 cells.

In single T84 cells dialyzed with the cAMP-dependent protein kinase inhibitor, PKI, application of VIP failed to generate an elevation of K^+ conductance (Fig. 3*B*). Intracellular cAMP could also activate membrane K^+ current by direct nucleotide binding to the underlying K^+ channels; however, the ability of PKI to inhibit VIP-stimulated K^+ conductances suggests that VIP may regulate the underlying K^+ channels through PKA-catalyzed phosphorylation. Subsequent application of the cholinergic agonist carbachol activated a K^+ current despite the inhibition of PKA-catalyzed phosphorylation. Since the K^+ current stimulated by carbachol was mediated by an elevation of $[Ca^{2+}]_i$ [14, 16], this result suggested that blocking PKA-catalyzed phosphorylation does not inhibit the ability of the K^+ channels to respond to Ca^{2+}. Therefore, there could be two separate populations of K^+ channels, respectively, regulated by either Ca^{2+} or cAMP-dependent phosphorylation. Combined with previous studies, the inhibitor profile of T84 monolayers supports this assumption because the VIP-stimulated I_{sc} was sensitive to 293B and insensitive to CTX, the inhibitor of the Ca^{2+}-mediated K^+ conductance [24]. Alternatively, both the Ca^{2+}-mediated and cAMP-dependent pathways may control the same population of K^+ channels as observed in pancreatic duct cells [25].

In order to determine whether the inhibitor profile of single T84 cells corresponds to that of T84 monolayers, the channel blockers Ba^{2+} and 293B were applied to isolated T84 cells stimulated by VIP in the perforated-patch and whole-cell configuration. As shown in Fig. 4, Ba^{2+} reversibly inhibits the K^+ current activated by VIP, while 293B does not inhibit the VIP-activated current. The inhibition by Ba^{2+} is consistent with the observation mentioned above of reduced VIP-activated $^{86}Rb^+$ efflux across the T84 monolayers exposed to serosal application of Ba^{2+} [36] and forskolin-stimulated I_{sc} across human colon [37]. These results conflict with the observation that 293B inhibits the VlP-stimulated I_{sc} across T84 monolayers (Fig. 1) and the forskolin-activated Cl^- secretory current [13]. One explanation for the apparent conflict is that in monolayers 293B may inhibit the VIP-stimulated I_{sc} by blocking CFTR, a possibility suggested by the recent observation that 293B can block CFTR Cl^- current *Xenopus* oocytes that express CFTR [1]. However, it seems unlikely that inhibition of I_{sc} by 293B in T84 monolayers was caused by block of CFTR since 293B was applied in the serosal bath, while CFTR is located in the apical membrane, and the response to 293B is very rapid (Fig. 1). Another explanation for the conflicting effects of 293B could be that differences exist between isolated cells and monolayers in expression or conformation of membrane K^+ channels. In polarized monolayers, trafficking, sorting and attachment of K^+-channel proteins at the basolateral membrane may alter the proteins in ways that lead to alterations in blocker effectiveness. While this phenomenon has not previously been seen, *activation* of K^+ channels can be dramatically affected by intracellular proteins involved in polarization, like A-kinase anchoring proteins (AKAPs) [44].

Identity of the K^+ Channel Activated by VIP

Figure 7 demonstrates that VIP activates ion channel currents in the cell-attached patch configuration. We

cannot definitively characterize these channels because we do not accurately know the intracellular concentrations or the membrane potential across the patch membrane. Initially, these experiments were performed with a bath containing K-gluconate so as to negate membrane potential as a driving force and minimize the occurrence of Cl^- channels in the traces. T84 cells are known to express CFTR [11], a Cl^- channel which may be responsible for the inward currents observed in the whole-cell and perforated-patch configuration (Figs. 2 and 3). However, we observed that high concentrations of K-gluconate stimulated spontaneous channel activity (unpublished observation). Therefore, we used standard NaCl ringers in the bath and a pipette solution high in KCl. Since CFTR exhibits a linear current-voltage profile [6], we could distinguish the outwardly activated channel currents from the linear channel currents. As the channel currents that we observed were inwardly rectifying, we propose that the channel currents observed in the cell-attached patches stimulated by VIP were K^+ channels and not Cl^- channels.

Ranging from 16 to 37 pS, an intermediate-conductance K^+ channel has been characterized in both colonic crypts and T84 cells [12, 38, 47, 48, 55]. This channel has been proposed to be the channel responsible for the Ca^{2+}-mediated K^+-conductance. In the cell-attached and excised inside-out patch configuration, this channel exhibits an inwardly-rectifying current-voltage profile. Ca^{2+}-mediated agonists, such as ACH and carbachol, along with Ca^{2+} ionophores, activate these channels in the cell-attached patch configuration [12, 56]. Based upon the sensitivity to intracellular Ca^+ and the inhibitor CTX, as well as on the current-voltage profile, the intermediate-conductance K^+ channel responsible for the Ca^{2+}-mediated K^+ conductance is likely to be the cloned channel hIK1 that shares these characteristics [28].

In the cell-attached patch configuration, agonists that increase intracellular cAMP stimulate channels similar to the Ca^{2+}-activated K^+ channels [38, 47, 48]. In the presence of elevated bath [Ca^{2+}], the activity of these K^+ channels is augmented by the application of ATP alone or of ATP in combination with the catalytic subunit of protein kinase A to the cytoplasmic face of an inside-out excised patch [55]. Despite these observations, it is improbable that this channel is responsible for the VIP-activated K^+ conductance in T84 monolayers (Fig. 1*A* and 1*B*). In contrast to the VIP-activated I_{sc}, the Ca^{2+}-activated I_{sc} is insensitive to 293B and sensitive to CTX [13]. It is more likely that the channel that underlies the VIP-activated K^+ conductance in T84 monolayers is related to the channel responsible for the ≈3-pS single-K^+-channel currents activated in the cell-attached patch configuration at the base of rat colonic crypt by forskolin [60]. These channel currents are sensitive to external Ba^{2+} and the more selective inhibitor of cAMP-mediated K^+ current, 293B. The channel responsible for these currents is likely to be the cloned K^+ channel subunit, K_vLQT1 [2, 49]. Support for this proposal comes from the observations that T84 cells [51], and rat and mouse colonic crypts [32, 51], express K_vLQT1. In addition, a recent study reports that cloned rat K_vLQTl expressed in oocytes is activated by cAMP [32]. Finally, 293B inhibits K^+ current in oocytes injected with K_vLQT1 mRNA [7]. Yet to be determined are the mechanisms by which VIP and other cAMP-mobilizing agonists regulate this K^+ channel.

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