Distinct K⁺ conductive pathways are required for Cl⁻ and K⁺ secretion across distal colonic epithelium

Susan Troutman Halm, Tianjiang Liao, and Dan R. Halm

Department of Neuroscience, Cell Biology, and Physiology, Wright State University Boonshoft School of Medicine, Dayton, Ohio

Submitted 31 October 2005; accepted in final form 1 April 2006

Distinct K⁺ conductive pathways are required for Cl⁻ and K⁺ secretion across distal colonic epithelium. Am J Physiol Cell Physiol 291: C636–C648, 2006. First published April 26, 2006; doi:10.1152/ajpcell.00557.2005.—Secretion of Cl⁻ through the colonic epithelium operates through a cellular mechanism requiring K⁺ conductive channels other than KCa3.1. The secretory response to various secretagogue substances (11, 14, 26, 37, 49) is the complete bumetanide sensitivity, suggesting an absolute requirement for Na⁺-K⁺-2Cl⁻ cotransporters (26, 55). In addition, apical and basolateral membrane K⁺ channels allow exit of K⁺ from the cell. The rates of K⁺ secretion can vary relative to that for Cl⁻ secretion, colonic secretory cells may control K⁺ secretion, in part, by modulating apical and basolateral K⁺ channel activity to alter the amount of K⁺ exiting in the lumen.

Active secretion of ions across colonic epithelia serves to produce a driving force for fluid secretion and to modify the composition of that secreted fluid (25, 26). Excessive rates of secretion occur in pathophysiological states such as secretory diarrhea and ulcerative colitis (11, 49). As in other fluid-secreting epithelia, electrogenic Cl⁻ secretion is commonly via prostanoid pathways (28, 49, 55). The highest rates of secretion are seen with the combined activation of prostanoïd and cholinergic pathways, suggesting a synergistic interaction (8, 18, 76). This apparent regulatory synergism may result from the actions of cytosolic cAMP and Ca²⁺ to stimulate the K⁺ channels necessary for secretion (23, 47).

Several types of K⁺ channels have been detected in colonic epithelial cells (23, 40, 60, 71, 72). Two of these types have been proposed to support cAMP- and Ca²⁺-activated secretion, Kv7.1 (KvLQT1, KCNQ1) and KCa3.1 (IK1, SK4, KCNn4), respectively (23). Although other ion transport components of the secretory mechanism also are likely regulated, activation of both these K⁺ channel types would serve to permit the large rate of secretion observed during synergistic stimulation. A broader corollary of this idea would be that the secretory cells use a distinct set of K⁺ channel types in response to each secretagogue. The number of available K⁺ channel types is large (24, 39, 77), but likely only some of these would best serve the regulatory constraints needed for each activation mode. With the use of the specific inhibitor HMR-1556, Kv7.1 was demonstrated to not play a crucial role in modulatory, flushing, or synergistic secretion (41). In particular, synergistic secretory activation was essentially independent of Kv7.1 activation. The study reported here uses the selective inhibitor TRAM-34 to examine the involvement of KCa3.1 in these three secretory modes. The lower inhibitory specificity at high con-
centrations of TRAM-34 also allowed an examination of involvement by other K\(^+\) channel types.

**METHODS**

Guinea pigs (Hartley, male, 500–700 g body wt) and rats (Sprague-Dawley, male or female, 150–250 g body wt) received standard chow and water ad libitum. Guinea pigs and rats were killed by decapitation in accordance with protocols approved by the Wright State University Institutional Laboratory Animal Care and Use Committee. The colon was removed, cut open along the mesenteric line, and flushed with ice-cold Ringer solution to remove fecal pellets. The mucosa was separated from underlying submucosa and muscle layers using a glass slide to gently scrape along the length of the colonic segment. The plane of dissection occurred at the base of the crypts such that only components of the mucosa immediately adherent to the epithelium remained. These isolated colonic mucosal sheets were used for measurement of transepithelial current and conductance (41). Colonic segments used were from the distal portion, in guinea pigs the \(20\) cm-long segment ending roughly \(5\) cm from the border of the parietal cavity and in rats the \(6\) cm-long segment ending roughly \(1\) cm from the peritoneal border.

**Tissue fixation and immunolocalization.** Colonic tissues were fixed after isolation, as described previously (41). Briefly, fixation was accomplished by pinning isolated mucosal sheets in a Sylgard-coated dish for immersion in fixation solutions (30–40 min, room temperature). These mucosal tissues were prepared for immunofluorescence by dehydration, sectioning, and mounting on gelatin-coated slides. Sections were permeabilized, blocked, and then incubated for 48 h (4°C) with primary antibody. A donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), conjugated to fluorescein isothiocyanate, was used to detect immunoreactivity (15 ng/\(\mu\)l, 2 h, room temperature). Sections were washed and mounted in Vectashield (Vector Laboratories, Burlingame, CA), and fluorescence was visualized with an Olympus BX60 epifluorescence microscope.

Dr. John B. Furness (University of Melbourne, Parkville, Australia) provided antisera IK38/6 for the \(K_{cnn4}\) K\(^+\) channel (residues 2–16 of rat \(Kcn4\)), used at a 1:2,000 dilution for 48 h at 4°C (20). A peptide was generated with the identical sequence employed to produce antisera IK38/6 (GGELVVTGLGALKRR; Genemed Synthesis, South San Francisco, CA), dissolved in water (0.6 mM), and used in controls of nonspecific interactions of antisera IK38/6. Anti-\(K_{cnn4}\), (residues 350–363 of rat \(Kcn4\)) from Alomone Laboratories (Jerusalem, Israel) was used (1:200, 1:100, and 1:50 dilution) but did not produce any distinct labeling in guinea pig distal colonic mucosa. Detection of the \(K_{cnn4}\) protein also was accomplished using immunoblotting (antisera IK38/6 at a 1:5,000 dilution), as described previously (20, 41).

**Transepithelial current measurement.** Isolated mucosal sheets were used for measurement of transepithelial current and conductance (28, 41). Four mucosal sheets from each animal were mounted in Ussing chambers (0.64 cm\(^2\) aperture), supported on the serosal face by Nuclepore filters \((\sim 10\) \(\mu\)m thick, 5 \(\mu\)m pore diameter; Whatman, Clifton, NJ). Bathing solutions (10 ml) were circulated by gas lift through water-jacketed reservoirs (37°C). Standard Ringer solution contained (in mM): 145 Na\(^+\), 5.0 K\(^+\), 2.0 Ca\(^2+\), 1.2 Mg\(^2+\), 125 Cl\(^-\), 25 HCO\(_3\)\(^-\), 4.0 H\(_2\)PO\(_4\)\(^-\), and 10 D-glucose. Solutions were continually gassed with 95% O\(_2\) and 5% CO\(_2\), which maintained solution pH at 7.4. Chambers were connected to automatic voltage clamps (Physiologic Instruments, San Diego, CA) that permitted compensation for solution resistance and continuous measurement of short-circuit current \((I_{sc})\). Transepithelial electrical potential difference (PD) was measured by paired calomel electrodes, current was passed across the tissue through two Ag-AgCl electrodes, and electrical connections to the chambers were made by Ringer-agar bridges. \(I_{sc}\) was referred to as positive for cation flow across the epithelium from the mucosal-to-serosal side (Cl\(^-\) secretion would produce a positive \(I_{sc}\) and K\(^+\) secretion would produce a negative \(I_{sc}\)). Transepithelial conductance \((G_{sc})\) was calculated from currents produced by bipolar square voltage pulses imposed across the mucosa (\(\pm 5\) mV, 3-s duration, 1-min intervals).

A quiescent basal condition was induced by suppressing the neural and paracrine activators persisting in the isolated colonic mucosa (28, 41). The mucosal preparation removes influences from nerves in the underlying muscle layers such that only mucosal nerve endings remain which have only minimal contribution to the stimulation by secretagogues (6, 19, 28, 55). Indomethacin (2 \(\mu\)M) and the cyclooxygenase-2 inhibitor NS-398 (2 \(\mu\)M) were used to inhibit production of prostanooids within the isolated mucosa. Other compounds released from mucosal cells in the bathing solutions as a result of mucosal isolation were reduced in concentration (\(\sim 8,000\)-fold) by replacing the solutions three times, after mounting the mucosa in the chamber (28).

PGE\(_2\), indomethacin, and NS-398 were obtained from Cayman Chemical (Ann Arbor, MI), epinephrine from Elkins-Sinn (Cherry Hill, NJ), 1-EBIO from Tocris Bioscience (Ellisville, MO), and clotrimazole from Wako Chemicals (Richmond, VA). K\(^+\) channel inhibitors TRAM-34 {1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole} was provided by Dr. Heike Wulff (University of California, Davis, CA), and KV7 (K\(_{VLQT}\)) inhibitor HMR-1556 {[3-hydroxy-2,2-dimethyl-6-(4,4,4-trifluorobutoxy)chroman-4-yl]-N-methyl-ethanesulfonamide} was provided by Dr. Uwe Gerlach (Aventis Pharma, Frankfurt am Main, Germany). All other chemicals, including clotrimazole and clofolium tosylate (4-chloro-N,N-diethyl-4-heptylbenzenesulfonamid). were obtained from Sigma Chemical (St. Louis, MO). Drugs were added in small volumes from concentrated stock solutions. PGE\(_2\) was prepared in an ethanol stock solution that added 0.03% ethanol at 3 \(\mu\)M of PGE\(_2\). TRAM-34 and clotrimazole were prepared in a DMSO stock solution (final added DMSO concentration of 0.2% at 100 \(\mu\)M of inhibitor). Stock solutions of HMR-1556 (10 mM) were made with DMSO. Additions of 1% ethanol or DMSO alone did not alter transepithelial measures of K\(^+\) or Cl\(^-\) secretion (28).

Inhibitor-sensitive components of \(I_{sc}\) and \(G_{sc}\) were calculated using the paired responses of adjacent mucosal tissues. A nonlinear least-squares procedure was used to fit Henri-Michaelis-Menten binding curves to the responses of \(I_{sc}\) and \(G_{sc}\) to inhibitors (28, 41). Strip-chart recordings of \(I_{sc}\) were digitized at 10-s intervals to examine secretory onset. Results are reported as means and SE. Statistical comparisons were made using a two-tailed Student’s t-test for paired responses, with significant difference accepted at \(P < 0.05\).

**RESULTS**

The actions of the K\(^+\) channel inhibitors TRAM-34 and clofolium on responses to physiological secretagogues were examined after first producing a quiescent basal condition. By limiting variability because of stimulatory status (28), this consistent basal state allowed clearer comparisons of results from adjacent tissue pairs. Distinct secretory states were produced (after attaining a basal condition) by adding specific physiologically secretagogues of the modulatory and flushing types (27, 28, 41, 55). Addition of the modulatory secretagogue epinephrine (5 \(\mu\)M) stimulates electrogenic K\(^+\) secretion without sustained Cl\(^-\) secretion. Addition of PGE\(_2\) (3 \(\mu\)M) stimulates sustained Cl\(^-\) secretion together with K\(^+\) secretion, a flushing type response. The cholinergic agonist carbachol (CCh; 10 \(\mu\)M) added cumulatively with PGE\(_2\) (3 \(\mu\)M) produces a synergistic flushing secretion (8, 18, 76). However, CCh addition from the basal state stimulates a negative \(I_{sc}\) consistent with modulatory type K\(^+\) secretion (41). Thus the three secretory modes examined for sensitivity to K\(^+\) channel
inhibitors were 1) modulatory-type K⁺ secretion, 2) flushing-type Cl⁻ and K⁺ secretion, and 3) synergistic-type Cl⁻ secretion.

Sensitivity of secretion to TRAM-34. The Kc₃.1 K⁺ channel (IK1, SK4, Kcnn4) is inhibited by TRAM-34, a derivative of clotrimazole (75). TRAM-34 inhibits Kc₃.1 in human, mouse, and rat cells with an IC₅₀ of 20 nM, but lacks the inhibitory action on P-450 enzymes of the parent compound clotrimazole (1, 3, 75). Both TRAM-34 and clotrimazole also inhibit a range of K⁺ channel types with IC₅₀ values of ~5 μM and higher so that specificity for Kc₃.1 occurs only at a lower concentration. A concentration of 0.5 μM was chosen to provide 95% inhibition of Kc₃.1 while only inhibiting other K⁺ channels by 10% or less. Similarly, concentrations of clotrimazole from 0.2 to 0.5 μM have been used to demonstrate specificity for Kc₃.1 in cellular systems (36, 69). Because TRAM-34 is lipid soluble, inhibitory actions could occur at either basolateral or apical membranes regardless of the side of addition. Other lipophilic molecules (HMR-1556 and PGE₂) as well have been delivered with high potency to colonic mucosa (28, 41).

TRAM-34 added to the serosal solution at 0.5 μM (Fig. 1) did not alter the response of guinea pig distal colonic mucosa to the modulatory secretagogue epinephrine or the flushing secretagogue PGE₂, which supports a lack of involvement by Kc₃.1 in these secretory responses. Mucosal addition produced similar results (data not shown). A similar lack of inhibition by 0.5 μM TRAM-34 was observed for secretory responses of the modulatory and flushing types in rat distal colonic mucosa (data not shown).

An ability to compensate for inhibition of Kc₃.1 by activation of Kv7.1 (KᵥLQT1, Kcnq1) has been suggested as a means for the secretory cells to maintain adequate K⁺ conductance (15, 42, 73). Sequential addition of TRAM-34 (0.5 μM) and HMR-1556 (10 μM) did not alter modulatory or flushing responses in guinea pig distal colon (data not shown). Similarly, in rat distal colon, the limited inhibitory action of HMR-1556 (41) was not augmented by TRAM-34 addition (data not shown). Apparently, activation by physiological secretagogues did not include a mechanism for compensation of Kc₃.1 or Kᵥ7.1 inhibition.

Inhibition by TRAM-34 at high concentration. The lower specificity of TRAM-34 at high concentrations leads to inhibition of many K⁺ channels (75) such that 100 μM TRAM-34 would inhibit one group of channels by >90% (Kᵥ1.1, Kᵥ1.2, Kᵥ1.3, Kᵥ1.4, Kᵥ1.5/Kcnal−5, Kᵥ4.2/Kcn2) and another group by ~80% (Kᵥ1.1/Kcnma1, Kᵥ2.2, Kᵥ2.3/Kcn2−3, Kᵥ3.1/Kcnf1). TRAM-34 added at 100 μM produced a small reduction (<4%) of modulatory K⁺ secretion, suggesting only a minor inhibition of apical membrane K⁺ conductance (Fig. 2); TRAM-34 at 100 μM did not influence this modulatory response in rat distal colonic mucosa (data not shown). The apparent Cl⁻ secretory rate during the flushing response was reduced substantially (~30%) by high-concentration TRAM-34 (Fig. 2A), but the initial positive change in Iₛ (data not shown) also suggested a modest reduction of K⁺ secretion through inhibition of apical membrane K⁺ conductance followed by a larger influence on basolateral membrane K⁺ channels. Addition of TRAM-34 to the mucosal bath did not increase the apparent inhibition of the K⁺ secretory responses (data not shown). A similar inhibition of this flushing response by 100 μM TRAM-34 was observed in rat distal colonic mucosa (data not shown).

A large synergistic stimulation of secretion occurs in the simultaneous presence of the secretagogues PGE₂ and CCh (41, 76). Addition of TRAM-34 at 0.5 μM produced a small reduction (~9%, P < 0.05) in the Iₛ stimulated during synergistic secretion (10 μM CCh added in the presence of 3 μM PGE₂; ΔIₛ = −28.4 ± 7.0 μA/cm², ΔGₛ = −0.84 ± 0.67 mS/cm², n = 5), suggesting only a limited involvement of Kc₃.1 in this secretory mode. However, TRAM-34 at high concentration markedly inhibited the synergistic response. Addition of CCh to ongoing flushing secretion (Fig. 3) stimulated a synergistic response largely resistant to TRAM-34 (100 μM) over the first ~10 min followed by a progressive increase in

---

**Fig. 1.** TRAM-34 sensitivity of modulatory and flushing secretion. Guinea pig colonic mucosae were stimulated cumulatively by epinephrine (5 μM) and PGE₂ (3 μM) from the standard basal condition. The basal condition was produced by 3 successive bath replacements with indomethacin (2 μM) and NS-398 (2 μM) in both bathing solutions and amiloride (100 μM) in the mucosal bathing solution. Short-circuit current (Iₛ) and transepithelial conductance (B, Gₛ) are shown. Gₛ shown (ΔGₛ) had the prestimulation value subtracted (Δ: 10.3 mS/cm²; ○: 10.2 mS/cm²). TRAM-34 (0.5 μM) was added to the serosal bath (*) for an adjacent pair of mucosae either during stimulation by epinephrine (○) or PGE₂ (●). Differences within the pair for Iₛ and Gₛ (C and D) revealed the TRAM-34-sensitive components (shaded region) of epinephrine and PGE₂ responses. Abrupt changes with TRAM-34 were not apparent, and the paired TRAM-34 responses during epinephrine (ΔLₛ = −0.3 ± 1.3 μA/cm², ΔGₛ = −0.02 ± 0.21 mS/cm², n = 6) or PGE₂ (ΔLₛ = −2.0 ± 2.1 μA/cm², ΔGₛ = −0.19 ± 0.38 mS/cm², n = 9) were not significantly different from 0 (P < 0.05).
TRAM-34-sensitive $I_{sc}$ over the next 30 min. Interestingly, the order of secretagogue activation influenced the time course of $I_{sc}$ increase (Figs. 3 and 4), unlike an earlier report (76). Stimulation by CCh before PGE$_2$ addition dramatically reduced the TRAM-34-resistant $I_{sc}$ component of the synergistic response (Fig. 4A). The time course of the $G_I$ response (Fig. 4, B and D) suggested the presence of an intricate compensatory reaction by the secretory cells. In addition, the modulatory response to CCh (Fig. 4, A and B) was unaltered by TRAM-34 at high concentration (100 µM), supporting a lack of involvement by KCa3.1 or any of several other K$^+$ channel types. The IC$_{50}$ for TRAM-34 inhibition of the synergistic response was 4.0 µM (Fig. 5), such that the minor inhibition seen at 0.5 µM (~9%) likely was because of low-affinity inhibition of K$^+$ channels other than KCa3.1. Clotrimazole produced similar results at both low and high concentrations (data not shown).

**Secretory augmentation by a K$^+$ channel opener.** Because KCa3.1 has been shown to be present in colonic epithelia (12, 20, 36, 73) and the K$^+$ channel opener 1-EBIO activates KCa3.1 (16), 1-EBIO was used to examine further the action of TRAM-34 in the colonic mucosa. Addition of 1-EBIO in the basal condition had minimal influence on $I_{sc}$ but increased $G_I$ (Fig. 6). Subsequent addition of secretagogues led to higher $I_{sc}$ and greater $G_I$ increases than seen with the control modulatory and flushing responses. Although the EC$_{50}$ was ~500 µM for 1-EBIO augmentation of the PGE$_2$ response, a concentration of 300 µM was used to limit the possible influence of cAMP generation (15). Roughly, 1-EBIO appeared to convert the modulatory response of epinephrine into a flushing response consistent with sustained Cl$^-$ secretion and converted the flushing response of PGE$_2$ into a synergistic-level response. The addition of CCh...
to these stimulated mucosae did not produce further increases in the electrogenic response, although changes in the time course of $I_{sc}$ and $G_{t}$ suggested an alteration in the details of the secretory state that was attained.

Testing the 1-EBIO augmented state with TRAM-34 at low concentration (0.5 μM) indicated a lack of dependence on KCa3.1 activity (Fig. 7). A high concentration of TRAM-34 (100 μM), however, inhibited a small portion of the augmented epinephrine response and a large portion of the augmented PGE2 response (Fig. 8). Apparently, the ability of 1-EBIO to open KCa3.1 K$^+$ channels was not directly responsible for the greatly increased Cl$^-$ secretion observed with physiological secretagogues.

Inhibition of secretion by clofilium. The TRAM-34 sensitivity of secretory activity (Fig. 5) indicated a lack of involvement by KCa3.1 but supported the action of additional K$^+$ channel types. Other inhibitors were used to further define the K$^+$ channels involved in secretion. Addition of clofilium (100 μM) revealed the TRAM-34-sensitive components (shaded region) of CCh and PGE2 responses. The paired TRAM-34 responses during CCh ($\Delta I_{sc} = -2.2 \pm 6.7 \, \mu A/cm^2, \Delta G_{t} = -0.72 \pm 0.30 \, mS/cm^2, n = 7$; $P < 0.05$) were not significantly different from 0. Abrupt changes with TRAM-34 were apparent during CCh and PGE2, and the paired TRAM-34 $I_{sc}$ response ($\Delta I_{sc} = -271.2 \pm 21.4 \, \mu A/cm^2, \Delta G_{t} = -0.89 \pm 0.77 \, mS/cm^2, n = 8$) was significantly different from 0 ($P < 0.05$).

---

Fig. 4. Sensitivity of cholinergic stimulated secretion to TRAM-34 at high concentration. Guinea pig colonic mucosae were stimulated cumulatively by CCh (10 μM) and PGE2 (3 μM) from the standard basal condition as in Fig. 1. $I_{sc}$ (A) and $G_{t}$ (B) are shown. $G_{t}$ shown (6Gt) had the prestimulation value subtracted ($\sim 9.6 \, mS/cm^2$; •, 13.6 mS/cm$^2$). TRAM-34 (100 μM) was added to the serosal bath (*) for an adjacent pair of mucosae either during stimulation by CCh (•) or PGE2 (○). Differences within the pair for $I_{sc}$ and $G_{t}$ revealed the TRAM-34-sensitive components (shaded region) of CCh and PGE2 responses. The paired TRAM-34 responses during CCh ($\Delta I_{sc} = -2.2 \pm 6.7 \, \mu A/cm^2, \Delta G_{t} = -0.72 \pm 0.30 \, mS/cm^2, n = 7$; $P < 0.05$) were not significantly different from 0. Abrupt changes with TRAM-34 were apparent during CCh + PGE2, and the paired TRAM-34 $I_{sc}$ response ($\Delta I_{sc} = -271.2 \pm 21.4 \, \mu A/cm^2, \Delta G_{t} = -0.89 \pm 0.77 \, mS/cm^2, n = 8$) was significantly different from 0 ($P < 0.05$).

---

Fig. 5. Concentration-dependent inhibition of synergistic secretion by TRAM-34. Guinea pig colonic mucosae were stimulated to produce a synergistic secretory response with PGE2 (3 μM) and CCh (10 μM), followed (after ~40 min) by cumulative additions of TRAM-34 to the stimulated state. The responses to TRAM-34 were compared with adjacent time controls. The TRAM-34-resistant proportion of $I_{sc}$ and $G_{t}$ is shown at 5 concentrations; the resistant proportion was calculated as $(I_{sc} - I_{sc}^{P})/(I_{sc}^{T} - I_{sc}^{P})$. The IC$\text{50}$ was 4.0 ± 1.0 μM for $I_{sc}$ and 2.9 ± 0.6 μM for $G_{t}$ from a fit to a single component response (solid line). The dashed line indicates the inhibition expected (IC$\text{50}$ = 20 nM) if the response depended only on KCa3.1 (75). The dotted curve shows a fit assuming two components, one with IC$\text{50}$ = 20 nM and the second freely adjustable; <5% of the response could be ascribed to the high affinity consistent with KCa3.1. Across the top are plotted TRAM-34 IC$\text{50}$ values (○) for various K$^+$ channels (24, 75); the left group is from KCa3.1 and the right two groups represent Kv1.2, Kv1.3, Kv4.2, Kv1.5, Kv1.4, Kv1.1 and KCa2.2, KCa2.3, KCa1.1, and Kv3.1, respectively.

---

Fig. 6. Augmentation of modulatory and flushing secretagogues by 1-EBIO. Guinea pig colonic mucosae were stimulated cumulatively by epinephrine (5 μM); PGE2 (3 μM), and CCh (10 μM; standard basal condition as in Fig. 1). $I_{sc}$ (A) and $G_{t}$ (B) are shown. $G_{t}$ shown (6Gt) had the prestimulation value subtracted ($\sim 5.3 \, mS/cm^2$; •, 6.5 mS/cm$^2$). 1-EBIO (300 μM) was added to the mucosal and serosal bath (*) for one mucosa of an adjacent pair before secretory stimulation.

---

AJP-Cell Physiol • VOL 291 • OCTOBER 2006 • www.ajpcell.org
μM), a quaternary ammonium inhibitor of K⁺ channels (13), produced a small and slowly developing inhibition of the epinephrine-stimulated modulatory response (Fig. 9) that varied among animals and was not statistically significant. Stimulation by low-concentration PGE₂ (10 nM) also produced a modulatory response that, however, exhibited a statistically significant inhibition of $I_{sc}$ by clofilium ($\Delta I_{sc} = 22.5 \pm 11.7 \, \mu A/cm^2$, $\Delta G_t = -2.05 \pm 0.56 \, mS/cm^2$, $n = 4$). Clofilium inhibition of the PGE₂ flushing response was statistically significant with a rapid component and a slowly developing decrease in $I_{sc}$ and $G_t$ (Fig. 9). E-4031 (10 μM), an inhibitor of Kv11 (erg, Kcnh2) and Kv10 (eag, Kcnh1; see Refs. 13 and 21), did not alter the modulatory, flushing, or synergistic secretory responses when added to the mucosal or serosal bathing solution (data not shown).

Cholinergic responses, both modulatory and synergistic, were inhibited rapidly by 100 μM clofilium added to the serosal solution (data not shown). The relatively high $IC_{50}$ of 26 ± 5 μM ($n = 3$) and inhibitory half-time of <1 min suggested that this clofilium inhibition of cholinergic responses likely was influenced by antagonism at the muscarinic receptors responsible for the secretory response. Also, clofilium added at 300 μM to either the mucosal or serosal bathing solution led to a slow progressive increase in $I_t$ over a period of 30–90 min, independent of inhibitory actions on secretory responses; small progressive increases in $G_t$ occasionally were seen at 100 μM clofilium.

**Synergistic secretory response.** The time course of synergistic stimulation in the presence of a high concentration TRAM-34 (Figs. 3 and 4) suggested that a similar steady-state condition could be attained through two distinct activation pathways. Comparison of the stimulation by PGE₂ + CCh and CCh + PGE₂ at higher time resolution (Fig. 10) illustrated these two routes for secretory onset. Cholinergic stimulation from ongoing flushing secretion (PGE₂ + CCh) produced a large transient phase followed by a sustained phase (Fig. 10A), whereas the reversed order of stimulation (CCh + PGE₂) produced primarily just the sustained phase (Fig. 10C). This diminution of the transient component coincided with the loss of the TRAM-34-resistant component (Fig. 10C), indicating that CCh inhibited the synergistic response by inhibiting K⁺ channels with little sensitivity to TRAM-34. Cholinergic stimulation (PGE₂ + CCh) also activated a TRAM-34-sensitive $I_{sc}$ component with a relatively slow onset (Fig. 10A), but cholin-
Fig. 9. Clofilium sensitivity of modulatory and flushing secretion. Guinea pig colonic mucosae were stimulated as in Fig. 1. 
$I_{sc}$ (A) and $G_t$ (B) are shown. $G_t$ shown ($G_t$) had the prestimulation value subtracted ($G_t$ = 4.1 mS/cm$^2$, $G_t$ = 8.0 mS/cm$^2$). Clofilium (100 μM) was added to the serosal bath (•) for an adjacent pair of mucosae either during stimulation by epinephrine (○) or PGE$_2$ (●). Differences within the pair for $I_{sc}$ and $G_t$ (C and D) revealed the clofilium-sensitive components (shaded region) of epinephrine and PGE$_2$ responses. The paired clofilium responses during epinephrine ($I_{sc}$ = 20.7 ± 10.8 μA/cm$^2$, $G_t$ = −2.81 ± 1.78 mS/cm$^2$, n = 6) were not significantly different from 0, and those during PGE$_2$ ($I_{sc}$ = −28.5 ± 5.7 μA/cm$^2$, $G_t$ = −4.25 ± 0.83 mS/cm$^2$, n = 5) were significantly different from 0 (P < 0.05).

Fig. 10. Sensitivity of synergistic activation to TRAM-34 at high concentration. Guinea pig colonic mucosae were stimulated cumulatively (as in Fig. 3) by epinephrine (5 μM) and PGE$_2$ (3 μM) followed by CCh (10 μM) at time 0 (PGE$_2$ + CCh, A and B). Average CCh-stimulated $I_{sc}$ (A) and $G_t$ (B) are shown (n = 7) for control stimulation (●), with TRAM-34 (100 μM; ○), and for the paired difference values between these conditions (◆). TRAM-34 was present in treated mucosa 25 min before secretory activation. The half-times for $I_{sc}$ activation were 17.7 ± 1.4 s for control, 35.8 ± 2.5 s for the TRAM-34-resistant component, and 15.4 ± 2.3 s for the TRAM-34-sensitive component. The half-time for activation of the TRAM-34-resistant component was significantly longer than control (paired difference of 18.1 ± 2.7 s, P < 0.05). The TRAM-34-sensitive component also had a slow secondary activation with a half-time of 12.2 ± 1.1 min. The half-times for $I_{sc}$ inactivation were 11.9 ± 1.1 min for the TRAM-34-resistant component and 2.2 ± 0.6 min for the TRAM-34-sensitive component. Paired mucosae (n = 6) also were stimulated (as in Fig. 4) by CCh (10 μM) followed with PGE$_2$ (3 μM) at time 0 (CCh + PGE$_2$, C and D), showing control (●), TRAM-34 (100 μM; ○), conditions and the difference values between these conditions (◆). The half-times for $I_{sc}$ activation were 39.5 ± 1.4 s for control, 45.4 ± 15.7 s for the TRAM-34-resistant component, and 39.0 ± 5.8 s for the TRAM-34-sensitive component. The half-time for $I_{sc}$ inactivation was 13.3 ± 1.0 min for the TRAM-34-resistant component. The TRAM-34-sensitive $I_{sc}$ and $G_t$ (A–C) were significantly different from 0 during the entire activation time course. The TRAM-34-sensitive $G_t$ (CCh + PGE$_2$, D) was significantly different from 0 for the first 12 min and again after 26 min.
ergic induction of this $I_{sc}$ component apparently could occur without the need for channel activity, since with the CCh + PGE$_2$ activation order TRAM-34-sensitive $I_{sc}$ maintained a steady, high level beginning immediately after PGE$_2$ stimulation (Fig. 10C).

The large $I_{sc}$ of the synergistic response (Figs. 3 and 4) allowed another test of the sidedness of TRAM-34 action, raising the possibility of action at apical and basolateral membrane K$^+$ channels. The time course of TRAM-34 inhibition with serosal addition was relatively slow (Fig. 11), consistent with inhibition occurring at a site on the intracellular side of the channel (74). Mucosal addition also produced inhibition but with a further delay likely because of a time lag resulting from diffusion to the basolateral membrane site of action. Examination at higher time resolution (Fig. 11B) supported the possibility that the delay may have included inhibition of apical membrane K$^+$ conductance and basolateral membrane K$^+$ conductance. Specifically, $I_{sc}$ was briefly elevated above the expected level due likely to the more rapid loss of the K$^+$ secretory current component than the Cl$^-$ secretory component, similar to the result during PGE$_2$ stimulation (Fig. 2A). Because inhibition at both apical and basolateral sites appeared to progress with additions to either side, the degree of apical inhibition could not be quantified. Also, although the possible inhibition at apical K$^+$ channels complicates interpretation of TRAM-34 action (Fig. 10), inhibition of Cl$^-$ secretion appeared clearly to be the larger response (since a large negative $I_{sc}$ consistent with K$^+$ secretion occurred with steady-state inhibition; Fig. 11A), such that the dominant action was to inhibit basolateral membrane K$^+$ channels.

**Localization of the KCa3.1 K$^+$ channel.** Immunoreactivity for the K$^+$ channel protein KCa3.1 (IK1, SK4, Kcnn4) was detected in a location consistent with the plasma membrane of colonic epithelial cells (Fig. 12). As with rat colon (20), the guinea pig colon had prominent labeling in the lateral membrane of crypt and surface epithelial cells. The luminal margins of crypt epithelial cells also were labeled (Fig. 12C), supporting the presence of KCa3.1 in the apical membrane. The uniform lateral labeling in crypts (Fig. 12D) supported the possible presence of KCa3.1 in goblet cells and columnar cells.

The presence of the K$^+$ channel protein KCa3.1 in guinea pig distal colonic mucosa also was examined by immunoblot of the membrane fraction (Fig. 13). Immunoreactive bands consistent with the KCa3.1 protein were observed. The band at 48 kDa was similar to the anticipated size of 47.8 kDa, as was found in rat and mouse colonic smooth muscle containing the myenteric plexus (51, 64). The smaller 41-kDa band was similar in size to the product obtained from in vitro translation of the mouse Kcnn4 gene (67) and that found in rat ileal smooth muscle containing the myenteric plexus (20). Therefore, the 48-kDa band actually may represent a posttranslational modification of the KCa3.1 protein. The faint band at ~130 kDa (Fig. 13) was similar to that obtained from HEK-293 cells transfected with rat Kcnn4, which was interpreted as the assembled KCa3.1 tetramer (20). These results support further the presence of the KCa3.1 K$^+$ channel protein in the colonic mucosa.

**DISCUSSION**

The cellular model for electrogenic Cl$^-$ secretion in epithelia requires the operation of K$^+$ channels (23, 25). These channels not only permit conductive exit of K$^+$ from the cell, but in so doing also aid in assuring conductive exit of Cl$^-$ across the apical membrane. Both of these actions are central to the transepithelial flow of Cl$^-$ during stimulated secretion. Cl$^-$ enters the epithelial cells across the basolateral membrane via Na$^+$-K$^+$-2Cl$^-$ cotransporters. Intracellular K$^+$ concentration remains in a steady state, even while K$^+$ continues to enter across the basolateral membrane through the turnover of these Na$^+$-K$^+$-2Cl$^-$ cotransporters and Na$^+$-K$^+$ pumps because of the balance created by the conductive K$^+$ exit through channels. The high intracellular K$^+$ concentration maintained by the Na$^+$-K$^+$ pumps and Na$^+$-K$^+$-2Cl$^-$ cotransporters assures diffusive exit of K$^+$ that makes the membrane electrical PD negative on the inside compared with the outside of the cell. Also, it is this electrical PD that must be large enough to drive Cl$^-$ exit across the apical membrane against the prevailing Cl$^-$ concentration gradient for transepithelial Cl$^-$ secretion to occur.

In colonic epithelia, K$^+$ channels are present in the apical and basolateral membrane so that the conductive K$^+$ exit that

![Fig. 11. Mucosal vs. serosal TRAM-34 sensitivity during synergistic activation. Guinea pig colonic mucosae were stimulated (as in Figs. 3 and 4) to produce synergistic activation. Average $I_{sc}$ (A) are shown ($n = 6$) for serosal (gray line) or mucosal (black line) addition of TRAM-34 (100 µM) after 35 min of synergistic activation. Results were similar with either order of secretagogue addition so both were included ($n = 3$ for PGE$_2$ + CCh; $n = 3$ for CCh + PGE$_2$). Half-times for $I_{sc}$ inhibition were 12.6 ± 1.9 min for serosal addition and 21.4 ± 2.5 min for mucosal addition (the paired difference of 8.8 ± 1.4 min was significantly different from 0, $P < 0.05$). The early time course is shown (B) to illustrate the positive-going change in $I_{sc}$ during mucosal addition ($\circ$) compared with serosal addition (gray circles). The dashed lines are extrapolations of the $I_{sc}$ before TRAM-34 addition. The SE in $B$ were calculated after normalization to the maximal response for a better indication of time course variability.](http://ajpcell.physiology.org/)

AJP-Cell Physiol • VOL 291 • OCTOBER 2006 • www.ajpcell.org
acts to maintain Cl\(^-\) secretion also can result in transepithelial K\(^+\) secretion (26, 36, 49, 71). An active absorptive process for K\(^+\) also is present in colonic epithelia such that net K\(^+\) transport is determined by a balance between secretory and absorptive activities (26, 49, 55). K\(^+\) absorption is driven by an apical membrane H\(^+\)-K\(^+\) pump that operates by electroneutral exchange such that the transepithelial process also is electroneutral. Thus the active electrogenic K\(^+\) secretory rate can be measured separately from the active electroneutral K\(^+\) absorptive rate by using the \(I_{sc}\).

**Role of KCa3.1 in colonic ion secretion.** The presence of KCa3.1 (IK1, SK4, Kcnn4) in the colonic epithelium has been demonstrated by several experimental means. Most specifically, the mRNA for KCa3.1 has been observed by Northern blot analysis and RT-PCR in colonic epithelial cells from human, mouse, and rat (5, 12, 34, 35, 36, 67, 73). Immunoreactivity for KCa3.1 is present in the colonic mucosa of human (12) and rat (20, 36) and also was detected in guinea pig colonic mucosa (Figs. 12 and 13). The KCa3.1 immunoreactivity was present in the basolateral membrane of colonic epithelial cells at both surface and crypt locations (Refs. 20 and 36 and Fig. 12). Apical membrane KCa3.1 immunoreactivity was detected in crypt cells (Refs. 20 and 36 and Fig. 12C), whereas surface epithelial cell apical membranes of rat are immunoreactive (20) but those of guinea pig were negative (Fig. 12, A and B). Ca\(^{2+}\)-activated K\(^+\) channel activity consistent with KCa3.1 also has been observed in the basolateral membrane of colonic crypt cells from human and rat (5, 60, 72).

Involvement of KCa3.1 in colonic ion secretion has been substantiated by stimulation of Ca\(^{2+}\)-dependent K\(^+\) channel activity with secretagogues, but primarily by the sensitivity of secretion and KCa3.1 to inhibition by clotrimazole (23, 36, 50, 72). Use of clotrimazole for identifying KCa3.1 involvement is confounded by its equal potency at inhibiting P-450 enzymes and by inhibiting a range of K\(^+\) channels at concentrations higher than \(~1\) \(\mu\)M (65, 75). In colonic epithelia, clotrimazole has been shown to be effective at inhibiting Cl\(^-\) secretion only at high concentrations, which could include inhibitory action at several K\(^+\) channel types. The IC\(_{50}\) is \(~5\) \(\mu\)M for clotrimazole inhibition of Cl\(^-\) secretory \(I_{sc}\) stimulated by CCh or vasoactive...
intestinal polypeptide (VIP) in the colonic tumor cell line T84 (57), suggesting dependence on K^+ channels other than KCa3.1. Similarly, forskolin-stimulated Cl^- secretory I_sc in T84 cells has an IC_{50} of 5.2 μM for clotrimazole (17). Interestingly, the concentration-response curve for VIP-stimulated secretory I_sc has a shape at low clotrimazole concentrations, suggesting a possible KCa3.1-dependent component of up to 20% of the total (57). Clotrimazole at 30 μM inhibits Cl^- secretory I_sc in human, rabbit, and rat distal colon (9, 45, 58, 73), which leaves unanswered the identity of the K^+ channels involved. A study of rat proximal colon indicates that clotrimazole at 0.5 μM inhibits K^+ secretion, apparently acting on apical membrane KCa3.1 K^+ channels, since this concentration provides specificity among K^+ channel types (36); however, the size of this K^+ secretion is small, only ~20% of the epinephrine-stimulated K^+ secretory I_sc in rat distal colon (41). Furthermore, cholinergic-stimulated K^+ secretory I_sc in human colon is insensitive to clotrimazole at 30 μM (46), similar to the result in guinea pig distal colon (Fig. 4A). Together with the results obtained using TRAM-34 in guinea pig (Fig. 1) and rat colon, the previous studies using clotrimazole suggest that the involvement of KCa3.1 in secretory responses may be limited to a minor role.

The ability of the K^+ channel opener 1-EBIO to activate KCa3.1 and colonic Cl^- secretory I_sc suggests involvement of KCa3.1 in the cellular mechanism for ion secretion (15, 16, 35, 62, 68). Interestingly, 1-EBIO did not stimulate secretory I_sc from the basal state in guinea pig colon (Fig. 6), unlike murine colon and T84 cells (15, 16), which may have occurred because of the reduction of endogenous secretagogue substances in these guinea pig colon experiments. Although the EC_{50} for 1-EBIO activation of KCa3.1 is 60–80 μM (35, 62, 68), the 1-EBIO EC_{50} for stimulating secretion was approximately eightfold higher at 500–600 μM in murine colonic epithelia, T84 cells, and guinea pig distal colonic mucosa, supporting the possibility of additional actions for 1-EBIO in epithelial cells (15, 16). Clotrimazole inhibited the 1-EBIO-activated Cl^- secretory I_sc in T84 cells with an IC_{50} of 0.27 μM, suggestive of inhibition at KCa3.1 (17), whereas TRAM-34 was ineffective at 0.5 μM in guinea pig distal colonic mucosa (Fig. 7). The ability of TRAM-34 to inhibit 1-EBIO-augmented secretory I_sc in guinea pig colon only at high concentration (Fig. 8) was consistent with inhibiting K^+ channels other than KCa3.1 (75).

A role for KCa3.1 K^+ channels in the function of colonic epithelia appears certain given the strong support for their presence in these cells (5, 12, 20, 34, 35, 36, 73). Contrary to the previous hypothesis (23), however, KCa3.1 would not appear to be one of the K^+ channels involved in ion secretory responses, based on the inhibitory characteristics of TRAM-34 (Fig. 5). Of course, if KCa3.1 takes on a TRAM-34-insensitive character in colonic epithelia, then the inhibition observed at high concentration could include KCa3.1 activity. However, TRAM-34 inhibition of KCa3.1 activity does occur at concentrations <0.5 μM in several cell types, indicating that KCa3.1 in native cells from complex tissues retains sensitivity and that TRAM-34 can be delivered effectively to these cells during physiological measurements. In particular, cellular responses apparently dependent on KCa3.1, such as cytokine production in T lymphocytes and bactericidal peptide secretion in paneth cells, are sensitive to TRAM-34 at these low concentrations (1, 10, 75). Emerging evidence supports a role for KCa3.1 in epithelial cell volume control, including human intestinal cells and colonic crypt cells (59, 69). Mice lacking KCa3.1 (Kcnm4 null) have erythrocytes and T lymphocytes with severely impaired cell volume regulation but parotid salivary glands with normal rates of activated fluid secretion (4), which further supports the likelihood that KCa3.1 is not involved obligatorily in secretory responses.

Synergistic stimulation of colonic ion secretion. Secretion of Cl^- and K^+ across colonic epithelia is stimulated by a variety of secretagogues (11, 14, 26, 49, 72). The observation that a combination of secretagogues can produce more secretory I_sc than expected from simple addition of the individual responses suggests a synergy within the intracellular signaling pathways (18, 70, 76). Because combinations of cAMP- and Ca^{2+}-mobilizing agents reproduce the synergistic stimulation of secretory I_sc, these two intracellular messengers are thought to produce the signaling interactions leading to synergistic stim-
ulation (8, 48, 63, 70, 76). This concept of regulatory interaction fits within the more general synergistic (acting together) regulation observed for cAMP- and Ca$^{2+}$-dependent cellular responses (54). The pattern in the colonic mucosa is one of redundant control in which both signals lead to the same ultimate response, but with elements of hierarchical control that has one signal potentiating the response to the other as well as antagonistic control between the signals. A commonly studied secretagogue pair is PGE$_2$ and CCh, thought to represent cAMP and Ca$^{2+}$ signaling, respectively. From a physiological perspective, CCh represents neural input and PGE$_2$ represents the paracrine/autocrine regulation of immunomodulation (14, 37, 49).

The guinea pig distal colonic mucosa exhibits a large synergistic secretory $I_{sc}$ in response to stimulation by PGE$_2$ and CCh (41, 56, 76) as well as by PGE$_2$ and the tachykinin substance P (33). The robustness of the guinea pig secretory response allows for a fuller appreciation of all the facets of this synergistic control. For the colonic tumor cell line T84, synergism is most pronounced when PGE$_2$ and CCh are added together or when CCh follows PGE$_2$ addition (18, 63, 66). This dependence on the order of addition results from the additional antagonistic cholinergic control that inhibits the secretory $I_{sc}$ over a time course of 5–30 min (2, 38). In the guinea pig distal colonic mucosa, this cholinergic inhibition was apparent as a large transient component of the secretory $I_{sc}$ (Fig. 10A) that was nearly eliminated by pretreatment with CCh for 30 min (Fig. 10C). Importantly, in guinea pig colon, the synergistic secretory $I_{sc}$ response had a large component that was resistant to cholinergic inhibition, whereas T84 cells have mostly just the transient component.

Sensitivity of the synergistic response to a high concentration of TRAM-34 distinguished these same two components, with the transient component resistant to TRAM-34 inhibition and the steady-state component sensitive to TRAM-34 (Fig. 10, A and C). The TRAM-34-sensitive portion of the response included a brief transient phase during PGE$_2$ + CCh addition (Fig. 10A). The slow time course of activation ($\tau_{1/2} = 12.1$ min) for the second TRAM-34-sensitive phase mirrored the time course of inhibition ($\tau_{1/2} = 11.9$ min) for the resistant portion, suggesting that the cholinergic inhibition of TRAM-34-resistant K$^+$ conductance was linked to this activation of a TRAM-34-sensitive K$^+$ conductance. The nature of this activation was such that pretreatment with CCh resulted in a nearly immediate stimulation of the TRAM-34-sensitive portion upon PGE$_2$ addition (Fig. 10C). The first and second phases may still have been present for CCh + PGE$_2$ addition but simply merged because of overlap of activation and inactivation times. The candidate K$^+$ channel types for this portion of the secretory $I_{sc}$ would be any with significant sensitivity to TRAM-34 at 100 $\mu$M (75), except for Kv7.1 (41) and KvCa3.1 (Fig. 5); the two TRAM-34-sensitive phases also could be different K$^+$ channel types from within this class. The major distinction between the synergistic response in T84 cells and guinea pig colonic epithelial cells would be that T84 cells are much less capable of activating these TRAM-34-sensitive K$^+$ channels to support secretion. The K$^+$ channel types making up the K$^+$ conductance during the transient component of synergistic stimulation found in both T84 cells and guinea pig colon cells would be any of the TRAM-34-resistant K$^+$ channels. Overall, several separable groups of K$^+$ channels appear to be activated to produce the distinctly different secretagogue-stimulated rates of ion secretion observed.

**ACKNOWLEDGMENTS**

We are grateful to Dr. Robert Yffle for use of equipment in the Center for Brain Research.

**GRANTS**

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-65845 and the Wright State University Research Challenge program.

**REFERENCES**


SECRETAGOGUE-STIMULATED K+ CONDUCTIVE PATHWAYS


