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

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Halm, Susan Troutman, Tianjiang Liao, and Dan R. Halm. 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^- and K^+ in the colonic epithelium operates through a cellular mechanism requiring K^+ channels in the basolateral and apical membranes. Transepithelial current [short-circuit current (I_{sc})] and conductance (G_t) were measured for isolated distal colonic mucosa during secretory activation by epinephrine (Epi) or PGE_2 and synergistically by PGE_2 and carbachol (PGE_2 + CCh). TRAM-34 at 0.5 μM, an inhibitor of $K_{Ca}3.1$ (IK, *Kcnn4*) K^+ channels (H. Wulff, M. J. Miller, W. Hänsel, S. Grissmer, M. D. Cahalan, and K. G. Chandy. *Proc Natl Acad Sci USA* 97: 8151–8156, 2000), did not alter secretory I_{sc} or G_t in guinea pig or rat colon. The presence of $K_{Ca}3.1$ in the mucosa was confirmed by immunoblot and immunofluorescence detection. At 100 μM, TRAM-34 inhibited I_{sc} and G_t activated by Epi (~4%), PGE_2 (~30%) and PGE_2 + CCh (~60%). The IC_{50} of 4.0 μM implicated involvement of K^+ channels other than $K_{Ca}3.1$. The secretory responses augmented by the K^+ channel opener 1-EBIO were inhibited only at a high concentration of TRAM-34, suggesting further that $K_{Ca}3.1$ was not involved. Sensitivity of the synergistic response (PGE_2 + CCh) to a high concentration TRAM-34 supported a requirement for multiple K^+ conductive pathways in secretion. Clofilium (100 μM), a quaternary ammonium, inhibited Cl^- secretory I_{sc} and G_t activated by PGE_2 (~20%) but not K^+ secretion activated by Epi. Thus Cl^- secretion activated by physiological secretagogues occurred without apparent activity of $K_{Ca}3.1$ channels but was dependent on other types of K^+ channels sensitive to high concentrations of TRAM-34 and/or clofilium.

epinephrine; prostaglandin E_2; cholinergic; *Kcnn4*; TRAM-34; clofilium

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 a major mechanism for producing fluid flow (25). Stimulating Cl^- secretion by this mechanism requires that K^+ entering via the Na^+-K^+ pumps and Na^+-K^+-$2Cl^-$ cotransporters must exit via basolateral membrane K^+ channels (23, 26, 49, 71, 72). In mammalian colon, K^+ secretion is stimulated together with Cl^- secretion, contributing to the relatively high luminal K^+ concentration (26, 31, 55). The cellular mechanism for this K^+ secretion is electrogenic and similar to the mechanism for Cl^- secretion, and localization studies support the presence of both K^+ and Cl^- secretory capacities in columnar cells of colonic crypts (29–31). A key feature of this electrogenic K^+ secretion 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. Because the rate 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.

Secretion of Cl^- and K^+ in colonic epithelia is stimulated by various secretagogue substances (11, 14, 26, 37, 49). These responses can be grouped into the following three major modes based on Cl^- and K^+ secretory rate: modulatory, flushing, and synergistic (41). The modulatory mode consists of sustained electrogenic K^+ secretion without sustained Cl^- secretion, activated via adrenergic, prostanoid, and cholinergic pathways (7, 28, 44, 55). In contrast, the more familiar flushing mode exhibits both sustained Cl^- and K^+ secretion stimulated most commonly via prostanoid pathways (28, 49, 55). The highest rates of secretion are seen with the combined activation of prostanoid and cholinergic pathways, suggesting a synergistic interaction (8, 18, 76). This apparent regulatory synergism may result from the actions of cytosolic cAMP and Ca^{2+} 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 Ca^{2+}-activated secretion, $K_V7.1$ (K_VLQT1, *Kcnq1*) and $K_{Ca}3.1$ (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, $K_V7.1$ was demonstrated to not play a crucial role in modulatory, flushing, or synergistic secretion (41). In particular, synergistic secretory activation was essentially independent of $K_V7.1$ activation. The study reported here uses the selective inhibitor TRAM-34 to examine the involvement of $K_{Ca}3.1$ in these three secretory modes. The lower inhibitory specificity at high con-

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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 peritoneal 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/μl, 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 antiserum IK38/6 for the $K_{Ca}3.1$ K^+ channel (residues 2–16 of rat *Kcnn4*), 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 antiserum IK38/6 (GGELVTGLGALRRRK; Genemed Synthesis, South San Francisco, CA), dissolved in water (0.6 mM), and used in controls of nonspecific interactions of antiserum IK38/6. Anti-$K_{Ca}3.1$ (residues 350–363 of rat *Kcnn4*) 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 $K_{Ca}3.1$ protein also was accomplished using immunoblotting (antiserum 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 (~10 μm thick, 5 μm 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 $H_2PO_4^-$, 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_t) was calculated from currents produced by bipolar square voltage pulses imposed across the mucosa (±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 μM) and the cyclooxygenase-2 inhibitor NS-398 (2 μM) were used to inhibit production of prostanoids within the isolated mucosa. Other compounds released from mucosal cells in the bathing solutions as a result of mucosal isolation were reduced in concentration (~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 E-4031 from Wako Chemical (Richmond, VA). K^+ channel inhibitor TRAM-34 {1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole} was provided by Dr. Heike Wulff (University of California, Davis, CA), and K_V7 (K_VLQT) inhibitor HMR-1556 {(3R,4S)-(+)-*N*-[3-hydroxy-2,2-dimethyl-6-(4,4,4-trifluorobutoxy)chroman-4-yl]-*N*-methylethanesulfonamide} was provided by Dr. Uwe Gerlach (Aventis Pharma, Frankfurt am Main, Germany). All other chemicals, including clotrimazole and clofilium tosylate (4-chloro-*N*,*N*-diethyl-*N*-heptylbenzenebutanaminium tosylate), 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 μM of PGE_2. TRAM-34 and clotrimazole were prepared in a DMSO stock solution (final added DMSO concentration of 0.2% at 100 μM 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_t 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_t 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 clofilium 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 physiological secretagogues of the modulatory and flushing types (27, 28, 41, 55). Addition of the modulatory secretagogue epinephrine (5 μM) stimulates electrogenic K^+ secretion without sustained Cl^- secretion. Addition of PGE_2 (3 μM) stimulates sustained Cl^- secretion together with K^+ secretion, a flushing type response. The cholinergic agonist carbachol (CCh; 10 μM) added cumulatively with PGE_2 (3 μM) 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 $K_{Ca}3.1$ K^+ channel (IK1, SK4, *Kcnn4*) is inhibited by TRAM-34, a derivative of clotrimazole (75). TRAM-34 inhibits $K_{Ca}3.1$ in human, mouse, and rat cells with an IC_{50} 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 IC_{50} values of ~5 μM and higher so that specificity for $K_{Ca}3.1$ occurs only at a lower concentration. A concentration of 0.5 μM was chosen to provide 95% inhibition of $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 PGE_2) 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 PGE_2, which supports a lack of involvement by $K_{Ca}3.1$ 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 $K_{Ca}3.1$ by activation of $K_V7.1$ (K_VLQT1, *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 $K_{Ca}3.1$ or $K_V7.1$ 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% ($K_V1.1$, $K_V1.2$, $K_V1.3$, $K_V1.4$, $K_V1.5$/*Kcna1–5*, $K_V4.2$/*Kcnd2*) and another group by ~80% ($K_{Ca}1.1$/*Kcnma1*, $K_{Ca}2.2$, $K_{Ca}2.3$/*Kcnn2–3*, $K_V3.1$/*Kcnc1*). 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. 2*A*), but the initial positive change in I_{sc} 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 PGE_2 and CCh (41, 76). Addition of TRAM-34 at 0.5 μM produced a small reduction (~9%, $P < 0.05$) in the I_{sc} stimulated during synergistic secretion (10 μM CCh added in the presence of 3 μM PGE_2; $\Delta I_{sc} = -28.4 \pm 7.0$ μA/cm^2, $\Delta G_t = -0.84 \pm 0.67$ mS/cm^2, $n = 5$), suggesting only a limited involvement of $K_{Ca}3.1$ 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

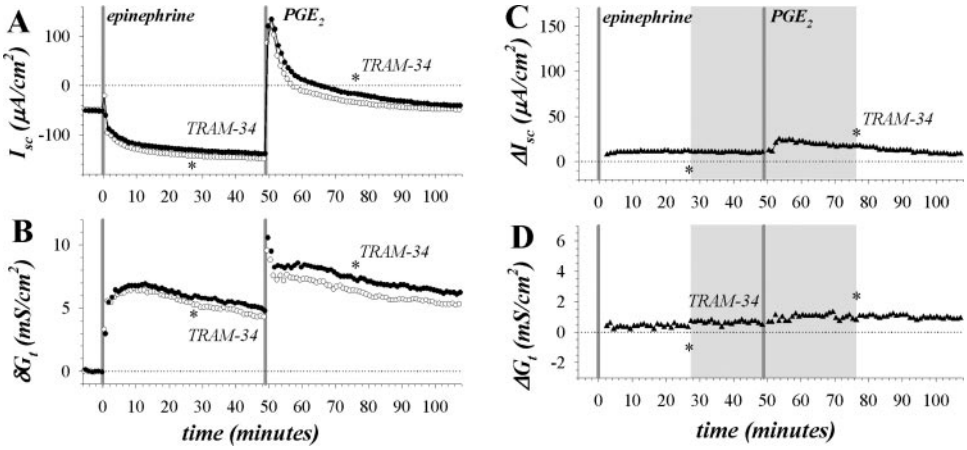

Fig. 1. TRAM-34 sensitivity of modulatory and flushing secretion. Guinea pig colonic mucosae were stimulated cumulatively by epinephrine (5 μM) and PGE_2 (3 μM) from the standard basal condition. The basal condition was produced by 3 successive bath replacements with indomethacin (2 μM) and NS-398 (2 μM) in both bathing solutions and amiloride (100 μM) in the mucosal bathing solution. Short-circuit current (*A*, I_{sc}) and transepithelial conductance (*B*, G_t) are shown. G_t shown (δG_t) had the prestimulation value subtracted (○, 10.3 mS/cm^2; ●, 10.2 mS/cm^2). TRAM-34 (0.5 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 TRAM-34-sensitive components (shaded region) of epinephrine and PGE_2 responses. Abrupt changes with TRAM-34 were not apparent, and the paired TRAM-34 responses during epinephrine ($\Delta I_{sc} = -0.3 \pm 1.3$ μA/cm^2, $\Delta G_t = -0.02 \pm 0.21$ mS/cm^2, $n = 6$) or PGE_2 ($\Delta I_{sc} = -2.0 \pm 2.1$ μA/cm^2, $\Delta G_t = -0.19 \pm 0.38$ mS/cm^2, $n = 9$) were not significantly different from 0 ($P < 0.05$).

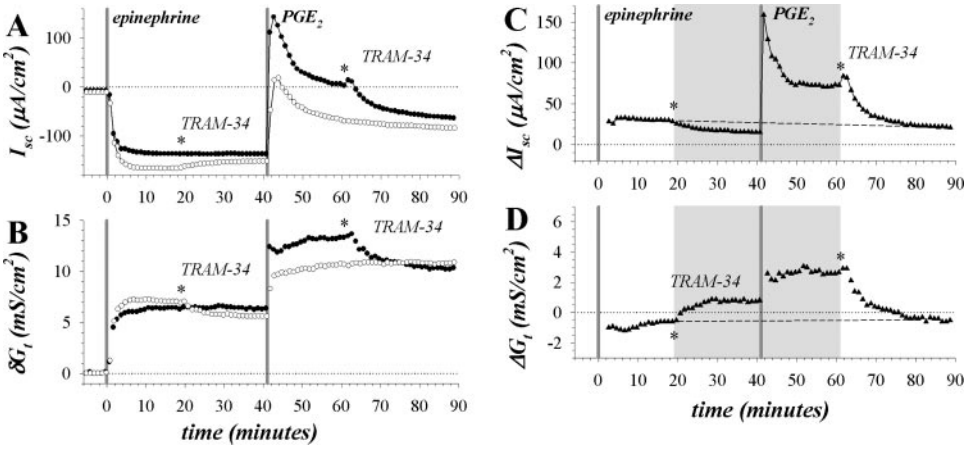

Fig. 2. Sensitivity of modulatory and flushing secretion to TRAM-34 at high concentration. Guinea pig colonic mucosae were stimulated as in Fig. 1. I_{sc} (*A*) and G_t (*B*) are shown. G_t shown (δG_t) had the prestimulation value subtracted (○, 8.4 mS/cm^2; ●, 5.9 mS/cm^2). TRAM-34 (100 μM) was added 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 TRAM-34-sensitive components (shaded region) of epinephrine and PGE_2 responses. The dashed line connects periods of identical treatment conditions for the tissue pair. Abrupt changes with TRAM-34 were apparent, and the paired TRAM-34 I_{sc} response during epinephrine (ΔI_{sc} = 4.7 ± 1.5 μA/cm^2, ΔG_t = −0.23 ± 0.20 mS/cm^2, n = 12) and paired I_{sc} and G_t responses during PGE_2 (ΔI_{sc} = −28.9 ± 5.0 μA/cm^2, ΔG_t = −0.93 ± 0.37 mS/cm^2, n = 9) were 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. 4*A*). The time course of the G_t 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$. Clotrimazole produced similar results at both low and high concentrations (data not shown).

Secretory augmentation by a K^+ channel opener. Because $K_{Ca}3.1$ has been shown to be present in colonic epithelia (12, 20, 36, 73) and the K^+ channel opener 1-EBIO activates $K_{Ca}3.1$ (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_t (Fig. 6). Subsequent addition of secretagogues led to higher I_{sc} and greater G_t 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

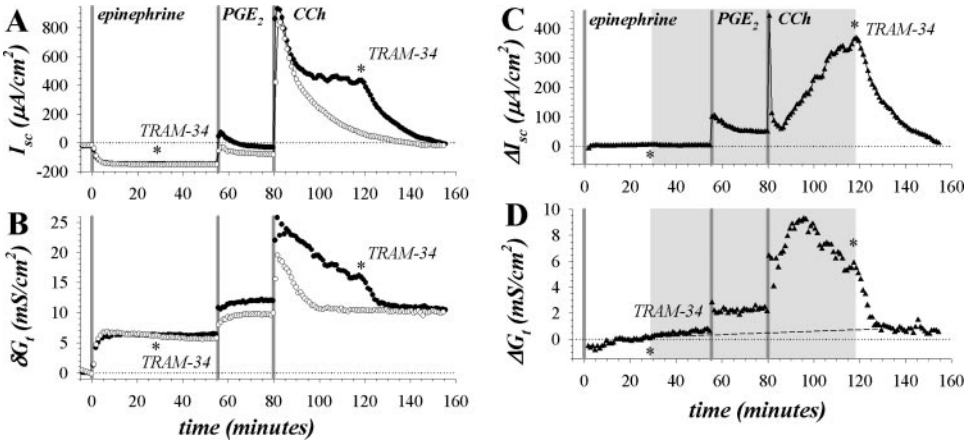

Fig. 3. Sensitivity of synergistically stimulated secretion to TRAM-34 at high concentration. Guinea pig colonic mucosae were stimulated cumulatively by epinephrine (5 μM) and PGE_2 (3 μM) followed by CCh (10 μM) from the standard basal condition as in Fig. 1. I_{sc} (A_c) and G_t (*B*) are shown. G_t shown (δG_t) had the prestimulation value subtracted (○, 8.6 mS/cm^2; ●, 9.2 mS/cm^2). TRAM-34 (100 μM) was added to the serosal bath (*) for an adjacent pair of mucosae either during stimulation by epinephrine (○) or CCh (●). Differences within the pair for I_{sc} and G_t (*C* and *D*) revealed the TRAM-34-sensitive components (shaded region) of epinephrine, PGE_2, and carbachol (CCh) responses. Abrupt changes with TRAM-34 were apparent, and the paired TRAM-34 responses during PGE_2 + CCh (ΔI_{sc} = −174.6 ± 26.1 μA/cm^2, ΔG_t = −2.70 ± 0.59 mS/cm^2, n = 13) were significantly different from 0 ($P < 0.05$).

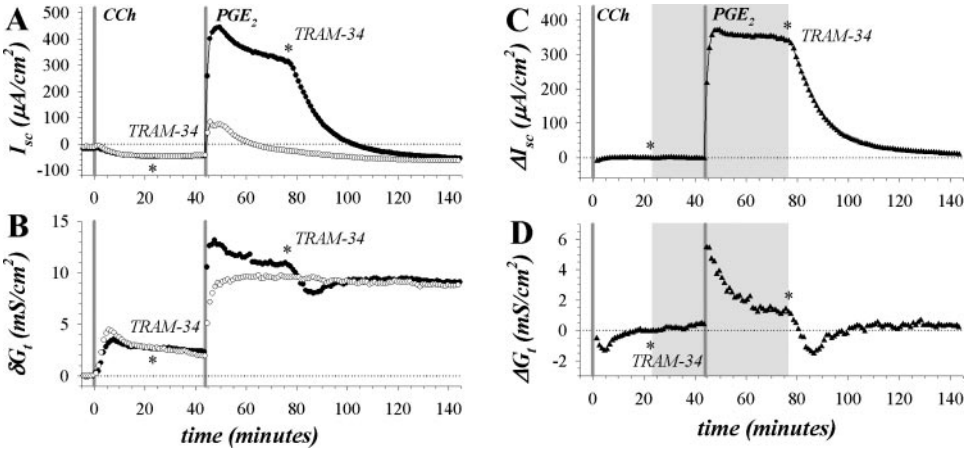

Fig. 4. Sensitivity of cholinergic stimulated secretion to TRAM-34 at high concentration. Guinea pig colonic mucosae were stimulated cumulatively by CCh (10 μM) and PGE_2 (3 μM) from the standard basal condition as in Fig. 1. I_{sc} (*A*) and G_t (*B*) are shown. G_t shown (δG_t) had the prestimulation value subtracted (○, 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 PGE_2 (●). Differences within the pair for I_{sc} and G_t (*C* and *D*) revealed the TRAM-34-sensitive components (shaded region) of CCh and PGE_2 responses. The paired TRAM-34 responses during CCh ($\Delta I_{sc} = -2.2 \pm 6.7$ μA/cm^2, $\Delta G_t = -0.72 \pm 0.30$ mS/cm^2, $n = 7$) were not significantly different from 0 ($P < 0.05$). Abrupt changes with TRAM-34 were apparent during CCh + PGE_2, and the paired TRAM-34 I_{sc} response ($\Delta I_{sc} = -271.2 \pm 21.4$ μA/cm^2, $\Delta G_t = -0.89 \pm 0.77$ mS/cm^2, $n = 8$) was significantly different from 0 ($P < 0.05$).

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.

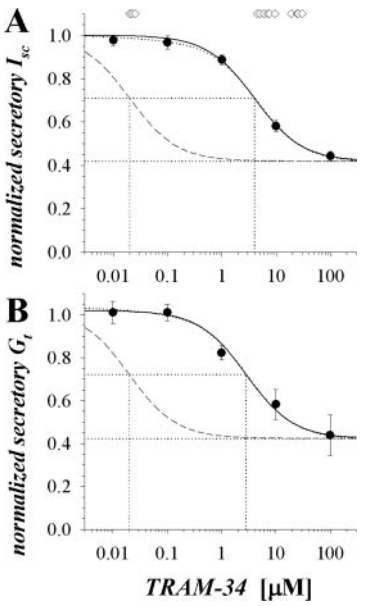

Fig. 5. Concentration-dependent inhibition of synergistic secretion by TRAM-34. Guinea pig colonic mucosae were stimulated to produce a synergistic secretory response with PGE_2 (3 μM) and CCh (10 μM), followed (after ~40 min) by cumulative additions of TRAM-34 to the stimulated state. The responses 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 $(I_{TRAM\text{-}34} - I_{Epi})/(I_{CCh} - I_{Epi})$. The IC_{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 ($IC_{50} \equiv 20$ nM) if the response depended only on $K_{Ca}3.1$ (75). The dotted curve shows a fit assuming two components, one with $IC_{50} \equiv 20$ nM and the second freely adjustable; <3% of the response could be ascribed to the high affinity consistent with $K_{Ca}3.1$. Across the top are plotted TRAM-34 IC_{50} values (◇) for various K^+ channels (24, 75); the left group is from $K_{Ca}3.1$ and the right two groups represent $K_V1.2$, $K_V1.3$, $K_V4.2$, $K_V1.5$, $K_V1.4$, $K_V1.1$ and $K_{Ca}2.2$, $K_{Ca}2.3$, $K_{Ca}1.1$, and $K_V3.1$, respectively.

Testing the 1-EBIO augmented state with TRAM-34 at low concentration (0.5 μM) indicated a lack of dependence on $K_{Ca}3.1$ 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 PGE_2 response (Fig. 8). Apparently, the ability of 1-EBIO to open $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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

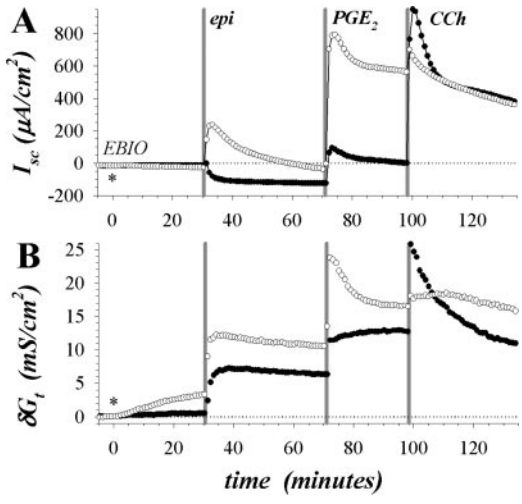

Fig. 6. Augmentation of modulatory and flushing secretagogues by 1-EBIO. Guinea pig colonic mucosae were stimulated cumulatively by epinephrine (5 μM), PGE_2 (3 μM), and CCh (10 μM; standard basal condition as in Fig. 1). I_{sc} (*A*) and G_t (*B*) are shown. G_t shown (δG_t) had the prestimulation value subtracted (○, 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.

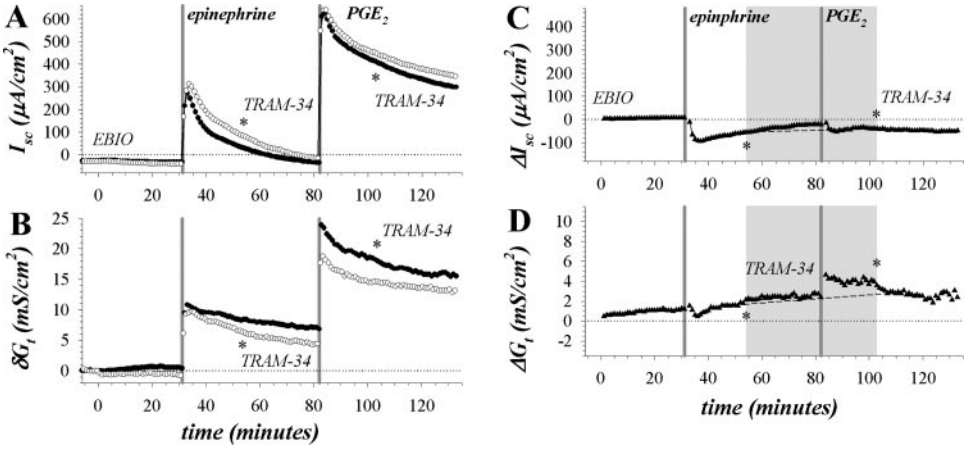

Fig. 7. TRAM-34 sensitivity of 1-EBIO augmented secretion. Guinea pig colonic mucosae were stimulated cumulatively by epinephrine (5 μM) and PGE_2 (3 μM) from the standard basal condition as in Fig. 1. I_{sc} (*A*) and G_t (*B*) are shown. G_t shown (δG_t) had the prestimulation value subtracted (○, 28.2 mS/cm^2; ●, 22.8 mS/cm^2). 1-EBIO (300 μM) was added to the serosal bath (*) for both mucosae of an adjacent pair 30 min before secretory stimulation. TRAM-34 (0.5 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 TRAM-34-sensitive components (shaded region) of epinephrine and PGE_2 responses. The dashed line connects periods of identical treatment conditions for the tissue pair. Abrupt changes with TRAM-34 were not apparent.

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 PGE_2 (10 nM) also produced a modulatory response that, however, exhibited a statistically significant inhibition of G_t by clofilium (ΔI_{sc} = 22.5 ± 11.7 μA/cm^2, ΔG_t = −2.05 ± 0.56 mS/cm^2, n = 4). Clofilium inhibition of the PGE_2 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 K_V11 (erg, *Kcnh2*) and K_V10 (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 G_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 PGE_2 + CCh and CCh + PGE_2 at higher time resolution (Fig. 10) illustrated these two routes for secretory onset. Cholinergic stimulation from ongoing flushing secretion (PGE_2 + CCh) produced a large transient phase followed by a sustained phase (Fig. 10*A*), whereas the reversed order of stimulation (CCh + PGE_2) produced primarily just the sustained phase (Fig. 10*C*). This diminution of the transient component coincided with the loss of the TRAM-34-resistant component (Fig. 10*C*), indicating that CCh inhibited the synergistic response by inhibiting K^+ channels with little sensitivity to TRAM-34. Cholinergic stimulation (PGE_2 + CCh) also activated a TRAM-34-sensitive I_{sc} component with a relatively slow onset (Fig. 10*A*), but cholin-

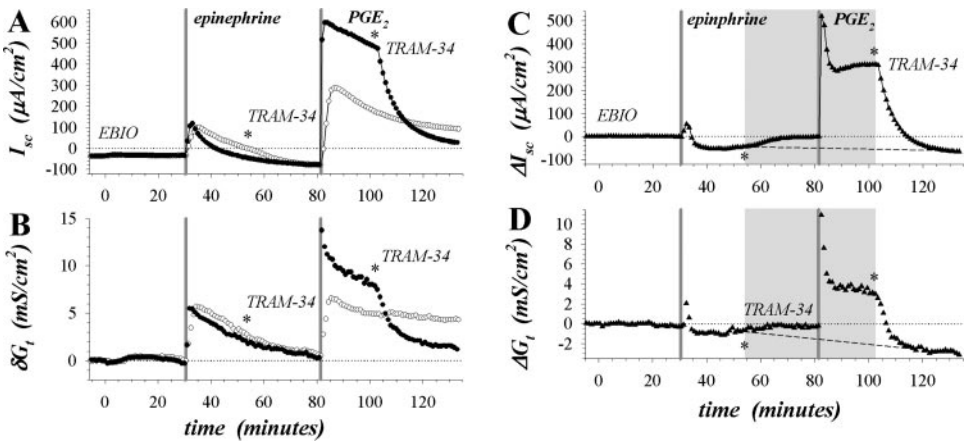

Fig. 8. Sensitivity of 1-EBIO augmented secretion to TRAM-34 at high concentration. Guinea pig colonic mucosae were stimulated as in Fig. 7. I_{sc} (A_c) and G_t (*B*) are shown. G_t shown (δG_t) had the prestimulation value subtracted (○, 15.8 mS/cm^2; ●, 17.0 mS/cm^2). 1-EBIO (300 μM) was added to the serosal bath (*) for both mucosae of an adjacent pair 30 min before secretory stimulation. TRAM-34 (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 TRAM-34-sensitive components (shaded region) of epinephrine, PGE_2, and CCh responses. The dashed line connects periods of identical treatment conditions for the tissue pair. Abrupt changes with TRAM-34 were apparent.

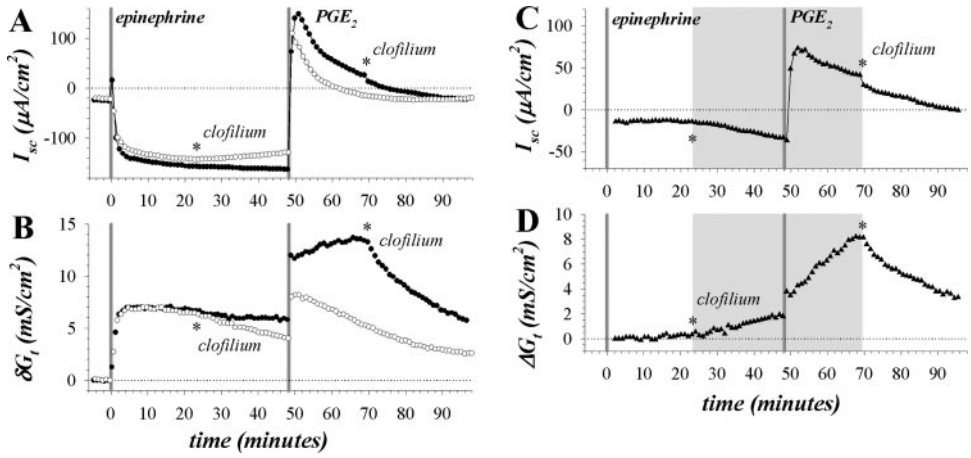

Fig. 9. Clofilium sensitivity of modulatory and flushing secretion. Guinea pig colonic mucosae were stimulated as in Fig. 1. I_{sc} (*A*) and G_t (*B*) are shown. G_t shown (δG_t) had the prestimulation value subtracted (○, 4.1 mS/cm^2; ●, 8.0 mS/cm^2). Clofilium (100 μM) was added to the serosal bath (*) 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 ($\Delta I_{sc} = 20.7 \pm 10.8$ μA/cm^2, $\Delta G_t = -2.81 \pm 1.78$ mS/cm^2, $n = 6$) were not significantly different from 0, and those during PGE_2 ($\Delta I_{sc} = -28.5 \pm 5.7$ μA/cm^2, $\Delta G_t = -4.25 \pm 0.83$ mS/cm^2, $n = 5$) were significantly different from 0 ($P < 0.05$).

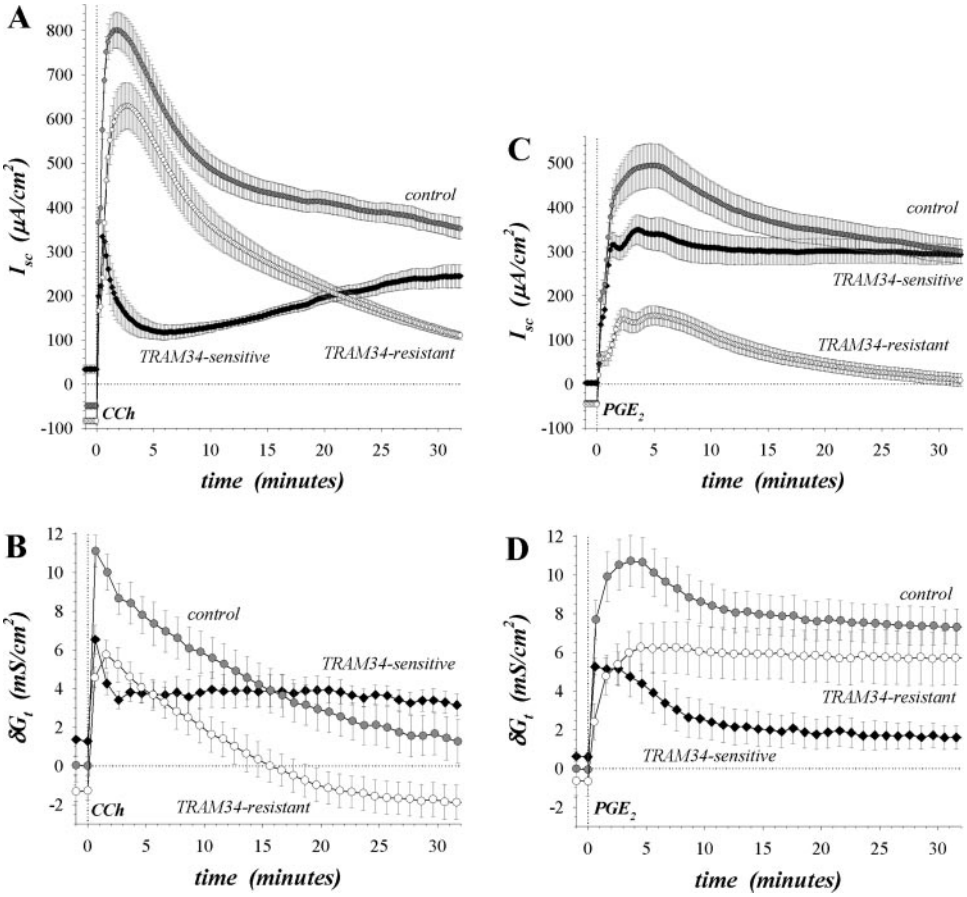

Fig. 10. Sensitivity of synergistic activation to TRAM-34 at high concentration. Guinea pig colonic mucosae were stimulated cumulatively (as in Fig. 3) by epinephrine (5 μM) and PGE_2 (3 μM) followed by CCh (10 μM) at *time 0* (PGE_2 + CCh, *A* and *B*). Average CCh-stimulated I_{sc} (*A*) and G_t (*B*) 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. 10*C*).

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. 11*B*) 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. 2*A*). 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. 11*A*), such that the dominant action was to inhibit basolateral membrane K^+ channels.

Localization of the $K_{Ca}3.1$ K^+ channel. Immunoreactivity for the K^+ channel protein $K_{Ca}3.1$ (IK1, SK4, *Kcnn4*) was detected in a location consistent with the plasma membrane of colonic epithelial cells (Fig. 12), similar to previous reports (12, 20, 36). 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. 12*C*), supporting the presence of $K_{Ca}3.1$ in the apical membrane. The uniform lateral labeling in crypts (Fig. 12*D*) supported the possible presence of $K_{Ca}3.1$ in goblet cells and columnar cells.

The presence of the K^+ channel protein $K_{Ca}3.1$ in guinea pig distal colonic mucosa also was examined by immunoblot of the membrane fraction (Fig. 13). Immunoreactive bands consistent with the $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$ tetramer (20). These results support further the presence of the $K_{Ca}3.1$ 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

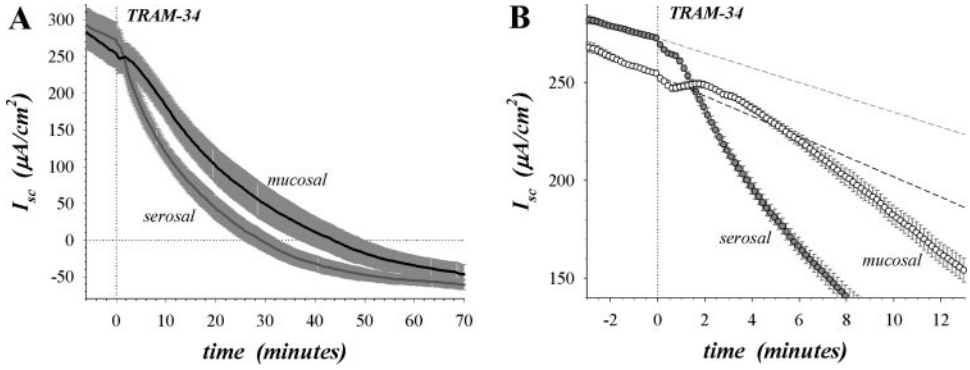

Fig. 11. Mucosal vs. serosal TRAM-34 sensitivity during synergistic activation. Guinea pig colonic mucosae were stimulated (as in Figs. 3 and 4) to produce synergistic activation. Average I_{sc} (*A*) are shown ($n = 6$) for serosal (gray line) or mucosal (black line) addition of TRAM-34 (100 μM) after 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 (○) 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.

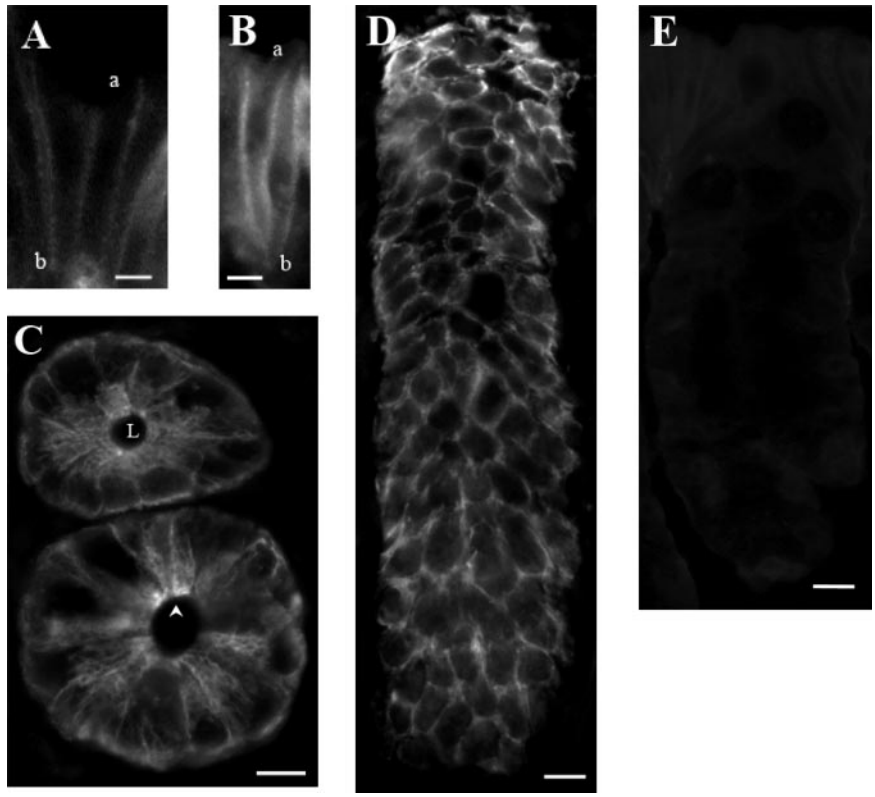

Fig. 12. Localization of $K_{Ca}3.1$ immunoreactivity in guinea pig distal colonic mucosa. $K_{Ca}3.1$ (IK1, SK4, *Kcnn4*) was detected by immunofluorescence (anti-$K_{Ca}3.1$ antiserum IK38/6; see Ref. 20) in guinea pig distal colonic mucosa. *A* and *B*: surface epithelial cells had dark nuclei and prominent immunoreactive labeling of lateral membranes. Labeling for apical (a) and basal (b) membranes was not apparent. *C*: cross sections of crypts are shown [lumen (L) at the center] showing distinct lateral and basal membrane labeling. The luminal margin labeled brightly in some cells (arrowhead), and the apical pole had a filamentous labeling. *D*: longitudinal profile of a crypt sectioned at roughly the midcell height (for those cells along the longitudinal crypt axis) showed a ring of labeling surrounding each cell. The continuous array of labeling suggested that all crypt cells exhibited immunoreactivity, both columnar and goblet cells. Use of the secondary antibody alone eliminated all membrane labeling of epithelial cells (data not shown), indicating that the IK38/6 antiserum was necessary for the observed labeling. *E*: preabsorption of the IK38/6 antiserum with the antigenic peptide (48 h at 4°C, 100:1 volume ratio of antigenic peptide to antiserum) eliminated all labeling of epithelial cells, similar to secondary antibody alone. Scale bars, 5 μm for *A* and *B*, 10 μm for *C–E*.

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}.

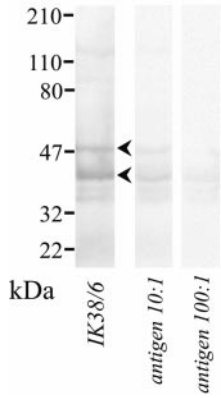

Fig. 13. K^+ channel immunoblot. The membrane fraction from guinea pig colonic mucosa was immunoblotted with antiserum IK38/6 against the $K_{Ca}3.1$ (*Kcnn4*) K^+ channel protein (20, 64). Immunoreactive bands occurred at 48 and 41 kDa (arrowheads); a fainter band also was apparent (~130 kDa). Preabsorption of the IK38/6 antiserum with the antigenic peptide (72 h at 4°C; volume ratio of antigenic peptide to antiserum 10:1, 100:1) progressively reduced the intensities of the immunoreactive bands.

Role of $K_{Ca}3.1$ in colonic ion secretion. The presence of $K_{Ca}3.1$ (IK1, SK4, *Kcnn4*) in the colonic epithelium has been demonstrated by several experimental means. Most specifically, the mRNA for $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$ immunoreactivity was detected in crypt cells (Refs. 20 and 36 and Fig. 12*C*), 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 $K_{Ca}3.1$ also has been observed in the basolateral membrane of colonic crypt cells from human and rat (5, 60, 72).

Involvement of $K_{Ca}3.1$ 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 $K_{Ca}3.1$ to inhibition by clotrimazole (23, 36, 50, 72). Use of clotrimazole for identifying $K_{Ca}3.1$ 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 μM (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 μM 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 $K_{Ca}3.1$. 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 $K_{Ca}3.1$-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 $K_{Ca}3.1$ 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. 4*A*). 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 $K_{Ca}3.1$ in colonic secretion may be limited to a minor role.

The ability of the K^+ channel opener 1-EBIO to activate $K_{Ca}3.1$ and colonic Cl^- secretory I_{sc} suggests involvement of $K_{Ca}3.1$ 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 $K_{Ca}3.1$ 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 $K_{Ca}3.1$ (*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 $K_{Ca}3.1$ (75).

A role for $K_{Ca}3.1$ 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, $K_{Ca}3.1$ 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 $K_{Ca}3.1$ takes on a TRAM-34-insensitive character in colonic epithelia, then the inhibition observed at high concentration could include $K_{Ca}3.1$ activity. However, TRAM-34 inhibition of $K_{Ca}3.1$ activity does occur at concentrations <0.5 μM in several cell types, indicating that $K_{Ca}3.1$ 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 $K_{Ca}3.1$, 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 $K_{Ca}3.1$ in epithelial cell volume control, including human intestinal cells and colonic crypt cells (59, 69). Mice lacking $K_{Ca}3.1$ (*Kcnn4* 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 $K_{Ca}3.1$ is not involved obligatorily in secretory responses.

Involvement of other K^+ channels in colonic ion secretion. In summary, modulatory K^+ secretion stimulated by either epinephrine or CCh as well as flushing secretion stimulated by PGE_2 and synergistic stimulation by the combination of PGE_2 and CCh was not sensitive to inhibition by TRAM-34 at a concentration specific for $K_{Ca}3.1$ (Figs. 1 and 5). Because $K_{Ca}3.1$ was likely not required during these secretory modes, other K^+ channel types must be involved in the responses. The inwardly rectified K^+ channel observed in the basolateral membrane of guinea pig distal colonic crypts (with a single-channel conductance similar to $K_{Ca}3.1$) was stimulated by either forskolin or CCh but was insensitive to changes in Ca^{2+} activity on the cytoplasmic side contrary to the expectation for $K_{Ca}3.1$ (40); the molecular identity of this channel is presently unknown. The group of channels involved was defined further by sensitivity to the inhibitors clofilium and E-4031. Quaternary ammonium compounds have been extensively studied as inhibitors of K^+ channels with tetraethylammonium$^+$ (TEA^+) as the archetypal example (*32*). Clofilium, an aromatic quaternary ammonium, inhibits K^+ channels with higher affinity than TEA^+ and has been suggested to have some specificity among the types of K^+ channels, particularly $K_V1.5$ (*Kcna5*), $K_V7.1$ (K_VLQT1, *Kcnq1*), $K_V10.1$ (eag, *Kcnh1*), $K_V11.1$ (erg, *Kcnh2*), and $K_{2P}5.1$ (TASK-2, *Kcnk5*; see Refs. 13, 21, 22, 43, 52, 53, 61). The partial inhibition of flushing secretion by clofilium (Fig. 9) was unlikely the result of involvement by $K_V7.1$, $K_V10.1$, or $K_V11.1$, since flushing secretion in guinea pig mucosa is insensitive to the K_V7 inhibitor HMR-1556 (41) and was not inhibited by the K_V11/K_V10 inhibitor E-4031. Given the present extent of information on clofilium sensitivity, $K_V1.5$ and $K_{2P}5.1$ would remain as possible candidates for involvement in secretion. Because a high concentration TRAM-34 inhibited flushing secretion by only ~30% (Fig. 2*A*), two classes of K^+ channels at the least must be required to produce flushing secretion. The class of K^+ channels sensitive to TRAM-34 at high concentration (75) includes $K_V1.1$, $K_V1.2$, $K_V1.3$, $K_V1.4$, $K_V1.5$ (*Kcna1–5*), $K_V3.1$ (*Kcnc1*), $K_V4.2$ (*Kcnd2*), $K_{Ca}1.1$ (*Kcnma1*), $K_{Ca}2.2$, $K_{Ca}2.3$ (*Kcnn2–3*), and likely others that are presently untested. The larger TRAM-34-resistant component of secretory I_{sc} could include any of the other K^+ channel types, except $K_V7.1$ and $K_{Ca}3.1$. Clearly, the K^+ channel types needed for secretory responses in the colonic mucosa still remain ill defined.

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 synarchic (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. 10*A*) that was nearly eliminated by pretreatment with CCh for 30 min (Fig. 10*C*). 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. 10*A*). 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-sensistive 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. 10*C*). 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 μM (75), except for $K_V7.1$ (41) and $K_{Ca}3.1$ (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.

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