β-Adrenergic activation of electrogenic K\(^+\) and Cl\(^-\) secretion in guinea pig distal colonic epithelium proceeds via separate cAMP signaling pathways

Susan T. Halm, Jin Zhang, and Dan R. Halm

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

Submitted 1 February 2010; accepted in final form 19 April 2010

Halm ST, Zhang J, Halm DR. β-Adrenergic activation of electrogenic K\(^+\) and Cl\(^-\) secretion in guinea pig distal colonic epithelium proceeds via separate cAMP signaling pathways. Am J Physiol Gastrointest Liver Physiol 299: G81–G95, 2010. First published April 22, 2010; doi:10.1152/ajpgi.00035.2010.—Adrenergic stimulation of isolated guinea pig distal colonic mucosa produced transient Cl\(^-\) and sustained K\(^+\) secretion. Transient short-circuit current (\(I_{sc}\)) depended on β2-adrenergic receptors (β2-AdrR), and sustained \(I_{sc}\) relies on a β1-AdrR/β2-AdrR complex. Epinephrine (epi) increased cAMP content with a biphasic time course similar to changes in epi-activated \(I_{sc}\) (\(\text{epi}\)/\(I_{sc}\)). Inhibition of transmembrane adenylyl cyclases (tmACs) reduced peak \(\text{epi}\)/\(I_{sc}\) and cAMP to near zero without decreasing sustained \(\text{epi}\)/\(I_{sc}\), consistent with cAMP from tmAC signaling for only Cl\(^-\) secretion. Inhibition of soluble adenylyl cyclase (sAC) reduced sustained \(\text{epi}\)/\(I_{sc}\) and cAMP to near zero without decreasing peak \(\text{epi}\)/\(I_{sc}\) or cAMP, consistent with cAMP from sAC signaling for K\(^+\) secretion. Sensitivity to phosphodiesterase (PDE) inhibitors and peptide YY (PYY) stimulation further supported separate signaling for the two components. PDE3 or PDE4 inhibitors enhanced peak \(\text{epi}\)/\(I_{sc}\) but not sustained \(\text{epi}\)/\(I_{sc}\), consistent with these PDEs as part of the β2-AdrR signaling domain. PYY suppressed peak \(\text{epi}\)/\(I_{sc}\) in a pertussis toxin (PTx)-sensitive manner, supporting Gs\(_{i}\)-independent inhibition of tmACs producing cAMP for Cl\(^-\) secretion. Since PYY or PTx did not alter sustained \(\text{epi}\)/\(I_{sc}\), signaling for K\(^+\) secretion occurred via a Gs\(_{i}\)-independent mechanism. Presence of multiple sAC variants in colonic epithelial cells was supported by domain-specific antibodies. Responses to specific activators and inhibitors suggested that protein kinase A was not involved in activating peak or sustained components of \(\text{epi}\)/\(I_{sc}\), but the cAMP-dependent guanine nucleotide exchange factor, Epac, may contribute. Thus β-adrenergic activation of electrogenic Cl\(^-\) and K\(^+\) secretion, respectively, required tmAC- and sAC-dependent signaling pathways.

Sympathetic stimulation suppresses digestion via norepinephrine (norepi) released from nerves and blood-borne epinephrine (epi). This slowing of digestion occurs by a combination of reduced intestinal motility and ion secretory activity as well as vasoconstriction that limits blood flow to the gut. Together, this inhibition of Cl\(^-\) secretion that stops fluid flow into the lumen, coupled with reduced blood flow and motility, helps conserve whole body water resources (15, 24). Also, direct sympathetic innervation of the mucosa in the distal colon stimulates a modulatory mode of secretion consisting of sustained electrogenic K\(^+\) secretion (73, 74). The generally lower rates of this secretory mode contrast dramatically with the flushing and synergistic modes stimulated by enteric ganglia that lead to rapid fluid accumulation in the lumen. Thus a sympathetic event overrides any enteric Cl\(^-\) secretory tone and replaces it with electrogenic K\(^+\) secretion.

In the distal colon, the sympathetic conversion from electrogenic Cl\(^-\) secretion to electrogenic K\(^+\) secretion involves several features of enteric signaling. The first feature that limits Cl\(^-\) secretion during a sympathetic event is the long-appreciated inhibition of enteric secretomotor nerves (15, 24). The second feature is the transient nature of Cl\(^-\) secretory activation via β-adrenergic receptors (β-AdrR). This secretory response peaks at −1 min and largely subsides within 5 min (73, 74). Thirdly, adrenergic nerves release neuropeptide Y (NPY) together with norepi, which in guinea pig distal colon directly inhibits Cl\(^-\) secretion via Y2-neuropeptide receptors (Y2-NpR) (15, 74). And fourth, β-adrenergic activation of enteroreendocrine L-cells releases peptide YY (PYY), which also inhibits Cl\(^-\) secretion via Y2-NpR (52, 74). As is apparent from the combined action of these agonists, the sympathetic response at the distal colonic mucosa is not simply to end secretory activity, but instead it produces almost solely electrogenic K\(^+\) secretion.

Clinical manifestations of colonic K\(^+\) secretion are apparent in patients with cystic fibrosis and those with acute colonic pseudo-obstruction (aCPO). The lack of CFTR Cl\(^-\) channels in the apical membrane for cystic fibrosis patients makes normally Cl\(^-\) secretory stimuli produce primarily electrogenic K\(^+\) secretion in the rectal colon (27, 49). The absence of Cl\(^-\) secretion from these responses illustrates the underlying capacity to produce modulatory secretion. In those aCPO patients having high fecal K\(^+\) concentrations (9, 13, 41, 67), hypokalemia likely results from inappropriately prolonged modulatory secretion. The accompanying colonic distension caused by the dominance of sympathetic over parasympathetic influences on gut motility provides a large reservoir for K\(^+\) accumulation from the continual K\(^+\) secretion, likely activated via a β1-AdrR/β2-AdrR complex (74). Inappropriate switching between the Cl\(^-\) secretion of the flushing mode and the K\(^+\) secretion of the modulatory mode may underlie many types of secretory dysfunction.

The cellular mechanism producing electrogenic K\(^+\) secretion closely resembles the standard model of Cl\(^-\) secretion with the addition of apical membrane K\(^+\) channels and basolateral membrane Cl\(^-\) channels (30, 31, 46, 57). This similarity makes electrogenic K\(^+\) secretion and Cl\(^-\) secretion compatible enough in transport mechanism to occur in the same epithelial cell type. As with any cellular process, a key requirement for physiologically appropriate responses is to have intracellular signaling pathways that can coordinate the activities of all the required elements. β-Adrenergic activation of secretion during
a sympathetic event likely occurs as a sequence of activations. Initially, opening of apical membrane Cl⁻ channels and K⁺ channels allows secretion to begin. Cl⁻ secretory rate exceeds K⁺ secretion as seen by the upswing of the transient component. Maintenance of secretion requires entry of Cl⁻ and K⁺ through the basolateral membrane to match the apical exit, which also supports the electrochemical driving forces needed for apical flow to be out of the cell. Basolateral membrane localized Na⁺⁺-K⁺⁺-2Cl⁻ cotransporters serve to bring Cl⁻ and K⁺ into the cell, thereby increasing intracellular concentration; Na⁺/K⁺ pumps also bring K⁺ into the cell and extrude the Na⁺ entering via Na⁺⁺-K⁺⁺-2Cl⁻ cotransporters. Basolateral K⁺ channels contribute to cellular K⁺ balance and creation of a membrane electrical potential difference that drives Cl⁻ exit via apical channels. The unique feature of β-adrenergic activation is the transition from a transient phase of flushing secretion to a sustained phase when only electrogenic K⁺ secretion is apparent (57, 74). As apical Cl⁻ channels close and produce the fall of the transient component, basolateral membrane Cl⁻ channels would open. Cellular balance is maintained as earlier with the distinction that conductive Cl⁻ exit occurs only at the basolateral membrane. Left largely unexplained are the signaling events that underlie this secretory activation by β-AdRs.

β-Adrenergic receptors were among the first G protein-coupled receptors (GPCR) to be linked to cAMP production as the means to actuate cellular responses (12). A hallmark of elective Cl⁻ secretion is the involvement of cAMP in the activation of apical membrane Cl⁻ conductance (5). Upon binding of epi (or norepi), β-AdRs activate Gαs by the exchange of GDP for GTP, which then enhances adenylyl cyclase production of cAMP (43, 63). Phosphodiesterases (PDEs) cleave cAMP into AMP, thereby limiting cAMP buildup and aiding in response termination after cAMP production ends (6). Both β₁-AdR and β₂-AdR are required to produce the sustained K⁺ secretion of the modulatory response (73, 74). Since the adenyl cyclase activator forskolin also stimulates a sustained K⁺ secretion (46), the β₁-AdR/β₂-AdR response likely occurs via cAMP production. Of the 10 mammalian class III adenylyl cyclases, nine have transmembrane domains (tmAC1–9) and one is soluble in the cytoplasm (sAC) (43, 63). Several of the tmACs have been found in colonic epithelial cells (23), but those responsible for specific GPCR signaling are not known. Interestingly, Y2-NpR activation by PYY or NPY inhibits the transient Cl⁻ secretory component of β-adrenergic activation but not the sustained K⁺ secretory component (74). Since a dominant mode of signaling by Y2-NpR occurs via Goi inhibition of tmACs (50), distinct ACs must contribute to activation of each secretory component. Similarly, the PDEs associated with each secretory component of β-adrenergic activation may be distinct. Although cAMP appears as a likely signaling molecule, the means by which cAMP promotes modulatory secretion is comparatively undefined.

The present study examined the involvement of cAMP in the β-adrenergic activation of electrogenic Cl⁻ and K⁺ secretion. As in previous studies from this laboratory (74), secretory responses were studied after suppressing endogenous agonists in the ex vivo mucosa. These procedures allowed a clearer distinction of the features controlling each component of the response. The experiments presented here examined the type of β-AdR required for activation of transient Cl⁻ secretion, the adenylyl cyclase involvement in each secretory component, the changes in cAMP content during β-adrenergic activation, as well as functional evidence for the cAMP-dependent actuators of Cl⁻ and K⁺ secretion.

METHODS

Male guinea pigs (500–800 g body wt, Hartley strain, Hilltop Lab Animals, Scottsdale, PA) received standard chow and water ad libitum and were housed on site at least 2 wk before experiments. Guinea pigs were euthanized with an animal decapitator (Harvard Apparatus, Holliston, MA) in accordance with a protocol approved by the Wright State University Laboratory Animal Care and Use Committee. Colonic mucosa was isolated as described previously (73). These isolated colonic mucosal sheets were used for protein detection by immunoblot, biochemical assays, and measurement of transepithelial electrical parameters.

Detection of proteins. Proteins were isolated from colonic mucosa as described previously (47, 73). Briefly, after disruption by sonication in a buffered solution containing protease inhibitors, samples were centrifuged to obtain a membrane sample. Following SDS-PAGE and transfer to polyvinylidene difluoride membranes, incubation with specific primary antibody and then with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) allowed detection of specific proteins. Antibodies for sAC were generously provided by L. R. Levin and J. Buck at the Weill Medical College of Cornell University, New York, NY (monoclonal mouse-anti-sAC, R21, residues 203–216 in exon 5 of human adcy10; 1:1,000) and by P. Huang at the Hong Kong University of Science and Technology, Kowloon, Hong Kong [polyclonal rabbit-anti-sAC, sAC(N-term), residues 1–17 of human adcy10; 1:250].

Assay for cAMP and activated Rap1. Isolated mucosal sheets were used for assays of cAMP content and Rap1 activation. These sheets were glued apical surface up to stainless steel rings (1.2 mm thick, 2.4-cm OD, 0.64-cm² opening) with cyanoacrylate glue. The annuli holding the mucosae were positioned vertically by using dental wax within beakers in a temperature-controlled water bath (38°C), and the bathing solution was gassed continuously to provide aeration and mixing. A basal condition was produced, as done for transepithelial electrical measurements. Mucosae were treated with secretagogues and inhibitors, punched from the rings on an ice-cold glass plate, placed in a microcentrifuge tube, and then quick frozen in a dry-ice/isopropanol bath (−78°C). Three mucosae were assigned to each experimental condition and chosen to minimize any variation along the distal colon segment used (~40 cm). Samples were stored at −80°C until use with cAMP EIA kit (Cayman Chemical, Ann Arbor, MI) or Active Rap1 Pull-Down and Detection kit (Pierce/ThermoFisher Scientific, Waltham, MA). Assays were conducted according to manufacturer’s instructions on a pooled sample from the three mucosae for each experimental condition. Acetylation was used in the cAMP assay to increase sensitivity. All cAMP contents were converted to apparent intracellular concentration by using the mucosal surface area (1.5 cm²), estimates for cell height (20 μm), and the ratio of cell number in crypt and surface zones (1:1). Immunoblot membranes were probed for Rap1, stripped, and reprobed with Rap2 antibody from Santa Cruz Biotechnology, Santa Cruz, CA (polyclonal goat-anti-Rap2, V-19, COOH-terminus of human rap2; 0.4 ng/μl), since RapGDS-RBD can be used as an activation probe for Rap2 as well as Rap1 (20).

Transmepithelial current measurement. Isolated mucosal sheets were used for measurement of transepithelial current and conductance as described previously (47). Mucosal sheets were mounted in Ussing chambers (0.64 cm² 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...
β-ADRENERGIC CAMP SIGNALING FOR K\(^{+}\) AND CL\(^{-}\) SECRETION

through water-jacketed reservoirs (38°C). Standard Ringer’s 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\(_3\)PO\(_4\)/H\(_2\)PO\(_4\), 10 d-glucose. Solutions were continually gassed with 95% O\(_2\) and 5% CO\(_2\), which maintained solution pH at 7.4. Low Cl\(^{-}\) solutions were made by substituting NaCl and KCl with gluconate salts and increasing Ca\(^{2+}\) to 8 mM to compensate for buffering by gluconate. Automatic voltage clamps (Physiologic Instruments, San Diego, CA) permitted measurement of short-circuit current (I\(_{sc}\)) and calculation of transepithelial conductance (G\(_{t}\)) from current responses to voltage pulses imposed across the mucosa (±5 mV, 3-s duration, 60-s intervals). I\(_{sc}\) was referred to as positive for cation flow across the epithelium from mucosal to serosal side.

Mucosal responses to physiological secretagogues and to inhibitors were examined after producing a quiescent basal condition. This basal state was produced by suppressing the neural and paracrine activators persisting in the isolated colon mucosa (74). The mucosal preparation removes influences from nerves in the underlying muscle layers. Compounds released into the bathing solutions were reduced in concentration (~8,000-fold) by replacing the solutions three times after mounting the mucosa. Prostanoid production within the isolated mucosa was suppressed by the cyclooxygenase-1 inhibitor SC560 (1 mM) and the cyclooxygenase-2 inhibitor CAY10404 (1 μM) added to both bathing solutions. The action of PYY and NPY released from mucosal cells was inhibited by adding the Y2-NpR antagonist BIIE0246 (1 mM) to the serosal bath. Amiloride (10 μM) was added to the mucosal bath to inhibit electrogenic Na\(^{+}\) absorption. Sequential addition of secretagogues (epi, prostaglandin-E2, carbachol) was used to examine the range of secretory response from modulatory to synergistic. Prior addition of epi does not alter subsequent PGE\(_2\) responses (32, 33, 47), and combined addition of PGE\(_2\) and carbachol (C) produces the synergistic mode of secretion (74).

Bicarbonate (bicarb)-free solutions were made by replacing HCO\(_3\)^{-} with either HEPES or Bis-Tris propane (BTP). HEPES Ringer’s solution contained (in mM) 142 Na\(^{+}\), 5.0 K\(^{+}\), 2.0 Ca\(^{2+}\), 1.2 Mg\(^{2+}\), 143 Cl\(^{-}\), 4.0 H\(_3\)PO\(_4\)/H\(_2\)PO\(_4\), 10 HEPES, 10 d-glucose. Bis-Tris propane Ringer’s solution contained (in mM) 141 Na\(^{+}\), 5.0 K\(^{+}\), 2.0 Ca\(^{2+}\), 1.2 Mg\(^{2+}\), 151 Cl\(^{-}\), 4.0 H\(_3\)PO\(_4\)/H\(_2\)PO\(_4\), 5 Bis-Tris propane, 10 d-glucose. The solutions were titrated to a pH of 7.4 with NaOH or HCl and continuously gassed with atmospheric or 100% O\(_2\). The influence of bicarb was examined by replacing the bathing solutions with a bicarb-free Ringer, after an initial equilibration of the mucosae in standard Ringer bubbled with 95% O\(_2\)-5% CO\(_2\) for 30 min. Within 5 min of the transition to bicarb-free Ringer, G\(_{t}\) began increasing and reached a peak approximately threefold higher than control over 15–30 s, with a small second peak apparent at 60–90 s, followed by the sustained negative I\(_{sc}\) (Fig. 1A). Activation of the transient component was minimal at 0.1 μM epi even though sustained secretion was near maximal. The concentration dependence for peak ΔI\(_{sc}\) activation (ΔI\(_{sc}\) difference between stimulated and basal I\(_{sc}\)) indicated a ~700-fold lower sensitivity to epi (Fig. 1B) compared with the previously determined sensitivity of the sustained component (74). Preference for epi over norepi in activating peak ΔI\(_{sc}\) was consistent with β\(_2\)-AdrR involvement, but with an overall lower sensitivity (Fig. 1C). β-Adrenergic activation of the sustained component requires both β\(_1\)- and β\(_2\)-adrenergic receptor types as indicated by synergistic antagonism with CGP20712A (β\(_{1}\)-AdrR) and ICI-118551 (β\(_{2}\)-AdrR) (73, 74). In contrast, ICI-118551 alone reduced peak epi-activated ΔI\(_{sc}\) (Fig. 1D), but CGP20712A (1.0 μM) did not reduce this peak ΔI\(_{sc}\) associated with Cl\(^{-}\) secretion (data not shown).

The low K\(_{d}\) for ICI-118551 antagonism (Fig. 1E) was consistent with a response acting solely via β\(_{2}\)-AdrR (3).

Dependence of ion secretion on soluble adenylyl cyclase.

Involvement of cAMP produced by β-adrnergic activation of cAC was examined by using KH7, an inhibitor selective for sAC in preference over tmAC (34). Addition of KH7 (30 μM) during the basal condition significantly reduced I\(_{sc}\) toward zero by 25.5 ± 2.8 μA/cm\(^2\) (n = 4, P < 0.05), consistent with inhibiting basal K\(^{+}\) secretion. Epi activation produced the early peak I\(_{sc}\), with KH7 increasing the peak by 36.4 ± 10.4 μA/cm\(^2\) (n = 4, P < 0.05) compared with the control peak (Fig. 2A, Table 1). KH7 also made the sustained plateau of ΔI\(_{sc}\) (at 20–30 min) more positive by 68.0 ± 5.5 μA/cm\(^2\) (Fig. 2A, Table 1) and decreased G\(_{t}\) by 0.71 ± 0.24 mS/cm\(^2\) (n = 4, P < 0.05), consistent with inhibiting K\(^{+}\) secretion. Low Cl\(^{-}\) bathing solutions attenuated the transient ΔI\(_{sc}\) in control and KH7-treated mucosae (data not shown), consistent with Cl\(^{-}\) secretion as the source of the ΔI\(_{sc}\). Also, low Cl\(^{-}\} bathing solutions eliminated the sustained ΔI\(_{sc}\) as well as the inhibitory action of KH7 (data not shown), consistent with the Cl\(^{-}\} dependence of K\(^{+}\} secretion (31). Given the uniform positive shift of the I\(_{sc}\) during epi activation, the primary action of KH7 was to inhibit K\(^{+}\} secretion 68 ± 6%, leaving the transient Cl\(^{-}\} secretory component unaltered.

Addition of PGE\(_{2}\) (3 mM) to epi-activated mucosae stimulated a positive change in I\(_{sc}\) consistent with Cl\(^{-}\} secretion (Fig. 2B, Table 1). The time course of PGE\(_{2}\) (3 mM) activation included an early transient at 15–30 s followed by a second peak at 60–90 s and then a decline to a sustained plateau, a
Timing similar to β2-AdrR-dependent activation (Fig. 1D). KH7 (30 μM) significantly increased the ISc at these three points in the PGE2 response compared with control, with the first peak 143.6 ± 15.2 μA/cm² higher, the second peak 137.8 ± 22.1 μA/cm² higher, and the plateau (20–30 min) 97.5 ± 19.9 μA/cm² higher (n = 4, P < 0.05). The difference in G0 between the KH7 and control conditions during plateau PGE2 activation was not significant (+0.57 ± 0.55 mS/cm², n = 4, P < 0.05). Similar to epi activation, the primary action of KH7 during PGE2 activation likely was to inhibit ~100 μA/cm² of K⁺ secretion. Cholinergic activation by CCh (10 μM) in the presence of epi and PGE2 exhibited a large positive ISc consistent with Cl⁻ secretion composed of two early peaks followed by a plateau. KH7 treatment did not alter ISc significantly at any of these times (data not shown, n = 4, P < 0.05).

**Dependence of ion secretion on transmembrane adenylyl cyclase.** Involvement of cAMP produced by β-adrenergic activation of tmAC was examined by using 2′,5′-dideoxyadenosine (ddAdo), an inhibitor selective for tmAC in preference to sAC (39, 43). Addition of ddAdo (200 μM) during the basal condition significantly decreased ISc by ~23.2 ± 5.5 μA/cm² and increased G0 by 0.44 ± 0.14 mS/cm² (n = 4, P < 0.05), consistent with suppressing basal K⁺ secretion (Fig. 3, A and B). ddAdo inhibited the epi-activated peak ISc in a concentration-dependent manner as expected for an action on Cl⁻ secretion (Fig. 3B, Table 2). The sustained epi response had a larger negative ISc in the presence of ddAdo together with a decrease in G0 by 1.19 ± 0.15 mS/cm² (n = 4, P < 0.05), consistent with suppressing the tail of this Cl⁻ secretory transient. Combining ddAdo (200 μM) with KH7 (30 μM) inhibited both the transient and sustained components (Fig. 3A), supporting the concept that cAMP from these two sources sufficed to complete β-adrenergic activation of secretion.

ddAdo reduced PGE2-activated ISc during the peaks and at the plateau level (Fig. 3B), ddAdo (200 μM) also significantly reduced the PGE2-activated plateau G0 by 0.94 ± 0.30 mS/cm² (n = 4, P < 0.05) as expected for suppression of the secretory

| Table 1. Dependence of secretagog responses on sAC: inhibition by KH7 |
|------------------|-------------------|
|                  | 1st Peak          | 2nd Peak          | Sustained         |
| Epi              | +19.2 ± 27.1       |                   |                   |
| Epi + KH7        | +82.6 ± 34.4*     |                   |                   |
| PGE2             |                   | +220.3 ± 7.0      |                   |
| PGE2 + KH7       | +363.9 ± 13.2*    | +307.0 ± 27.2*    | +115.2 ± 15.9*    |

Short-circuit current (ISC) values (μA/cm²) are means ± SE. n = 4, stimulated as in Fig. 2. sAC, soluble adenylyl cyclase; epi, epinephrine. *Responses with KH7 (30 μM) significantly different from control (P < 0.05).

Fig. 2. Inhibition of soluble adenylyl cyclase decreased sustained secretion. Isolated mucosae were stimulated sequentially by epi (5 μM) and PGE2 (3 μM), from the standard basal condition. A: ISc was measured in adjacent mucosae during activation by epi with 1 mucosa pretreated for 30 min with KH7 (○, 30 μM). B: ISc was measured in adjacent mucosae (as in A), ~30 min after epi stimulation, during activation by PGE2 with 1 mucosa pretreated with KH7 (○, 30 μM). The responses were representative of 4 similar experiments (see Table 1).
β-ADRENERGIC CAMP SIGNALING FOR K+ AND CL− SECRETION

Table 2. Dependence of secretagog responses on tmAC: fit parameters for concentration dependence of inhibition by ddAdo

<table>
<thead>
<tr>
<th></th>
<th>IC50</th>
<th>Imin</th>
<th>Imax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>36 ± 6</td>
<td>32 ± 8*</td>
<td>−59 ± 11*</td>
</tr>
<tr>
<td>Epi sustained</td>
<td>38 ± 4</td>
<td>21 ± 5*</td>
<td>−127 ± 11*</td>
</tr>
<tr>
<td>ΔEpi peak</td>
<td>70 ± 7</td>
<td>166 ± 34*</td>
<td>+3 ± 8</td>
</tr>
<tr>
<td>ΔPGE2 1st peak</td>
<td>39 ± 8</td>
<td>233 ± 59*</td>
<td>+268 ± 25*</td>
</tr>
<tr>
<td>ΔPGE2 2nd peak</td>
<td>58 ± 7</td>
<td>155 ± 40*</td>
<td>+208 ± 19*</td>
</tr>
<tr>
<td>ΔPGE2 sustained</td>
<td>48 ± 8</td>
<td>54 ± 11*</td>
<td>+103 ± 11*</td>
</tr>
<tr>
<td>CCh 1st peak</td>
<td>37 ± 6</td>
<td>527 ± 146*</td>
<td>+604 ± 52*</td>
</tr>
<tr>
<td>CCh 2nd peak</td>
<td>227 ± 42</td>
<td>405 ± 87*</td>
<td>+627 ± 35*</td>
</tr>
<tr>
<td>CCh sustained</td>
<td>220 ± 16</td>
<td>225 ± 22*</td>
<td>+95 ± 12*</td>
</tr>
</tbody>
</table>

The fit parameters (IC50, μM; Imin, μA/cm²) are shown for concentration dependence of ddAdo (means ± SE, n = 4), from experiments in Fig 3. The Imin components of the response sensitive to (I(min)) and resistant to (I(resis)) inhibition by 2′,5′-dideoxyadenosine (ddAdo) are shown. I(resis) = I(min) + I(resis).

*Significantly different from zero (P < 0.05).

response. The concentration dependence for ddAdo was fit best with a two-site cooperative model that suggested an interaction between catalytic domains of two tmAC proteins. The EC50 values clustered between 36 and 77 μM (Table 2), consistent with a common mechanism for ddAdo action during secretagogue activation. Although ddAdo inhibited 98 ± 6% of the transient β-adrenergic response, for the PGE2 response only 46 ± 5% of the first peak, 43 ± 9% of the second peak, and 34 ± 8% of the plateau were inhibited (Fig. 3B, Table 2), suggesting the presence of signaling agents in addition to cAMP during PGE2 activation. ddAdo inhibited cholinergic activation primarily during the first transient (47 ± 4%) that peaked at 10–15 s (Fig. 3C, Table 2). The second peak and plateau of the CCh response were sensitive only at the highest ddAdo concentration suggesting the possibility of a different mechanism for this inhibition.

PYY inhibition of transient β-adrenergic response. PYY acts through Y2-NpR to inhibit Cl− secretion in the distal colon (17, 74). Since the predominant signaling mode for this receptor occurs via activation of Goα(S), the resulting inhibition of tmAC would be responsible for inhibiting the CI− secretory component of β-adrenergic activation. Pretreatment with pertussis toxin (PTx) should inactivate Goα(S) and block the action of PYY, which was observed as a larger peak ΔIsc during PGE2 activation by 10.2 ± 0.32.247 on November 1, 2017 http://ajpgi.physiology.org/ Downloaded from http://ajpgi.physiology.org/ by 10.220.224.7 on November 1, 2017

AJP-Gastrointest Liver Physiol • VOL 299 • JULY 2010 • www.ajpgi.org
Expression of soluble adenylyl cyclase in distal colonic mucosa. The presence of sAC in colonic epithelial cells was detected by immunoblot (Fig. 6). Several splice forms of sAC have been identified: a full-length form (sAC-fl, ~180 kDa) and a truncated form (sAC-trnc, ~50 kDa) containing the NH₂-terminal catalytic domains as well as a somatic form (sAC-som, ~50 kDa) that lacks the initial four exons (22, 25, 40, 70). The R21 antibody recognizes three of these splice forms (22), and the sAC(N-term) antibody (70) cannot detect sAC-som, which lacks the NH₂-terminus. The band at ~55 kDa detected by the R21 antibody was likely the sAC-som because the sAC(N-term) antibody did not detect a band at this size. The ~73-kDa band detected by both antibodies was larger than sAC-trnc and smaller than sAC-fl, similar to another possible splice form found in kidney and airway epithelial cells (25, 60). In the membrane fraction the sAC(N-term) antibody detected two bands of higher molecular weight (Fig. 6), the larger consistent with the presence of sAC-fl, but did not detect the ~73-kDa variant. The ~120-kDa band may represent a dimer between the ~73-kDa and ~55-kDa variants that has the R21 epitope obscured. Also, by using R21 with membranes, the ~55 kDa variant appeared more prominently than the ~73 kDa variant. These results support the presence of sAC in colonic epithelial cells.

**Bicarb dependence of secretory activation.** Involvement of sAC in β-adrenergic activation of K⁺ secretion would confer HCO₃⁻ dependence due to the HCO₃⁻ sensitivity of sAC. The influence of HCO₃⁻ was examined by replacing the bathing solutions with a bicarb-free Ringer (see METHODS). Epi addition in the bicarb-free condition activated transient and sustained components of Iₜₑ, as in control with the peak response significantly more positive by 43.0 ± 15.0 μA/cm² and the sustained level more positive by 53.5 ± 7.3 μA/cm² (n = 7, P < 0.05), consistent with inhibiting K⁺ secretion. Suppressing the Cl⁻

A portion of the secretory response stimulated by PGE₂ was dependent on tmAC (Fig. 3B), suggesting that PYY suppression of this Cl⁻ secretion might act through inhibition of tmAC. In contrast to the epi response, PTx failed to reverse the PYY inhibition of the PGE₂ secretory response (data not shown, n = 4, P < 0.05). This result supported the presence of signaling mechanisms for Y2-NpR in addition to activation of Go, (50). Similarly, PTx failed to reverse the PYY sensitivity of the first peak and plateau responses to CCh (data not shown, n = 4, P < 0.05). However, PTx decreased the second peak of the CCh response by 97.5 ± 24.7 μA/cm² even though the second peak was insensitive to PYY (n = 4, P > 0.05), suggesting that CaMP had a modest suppressing influence or that Gβγ released together with Go, contributed to Cl⁻ secretory stimulation.

**Influence of PDEs on β-adrenergic activation.** PDEs contribute to regulation of cAMP concentration by mediating an inactivating cleavage to AMP (6). The action of PDEs was tested by using the general inhibitor IBMX and two typespecific inhibitors, trequinsin (PDE3) and rolipram (PDE4). IBMX (100 μM) and rolipram (30 μM) added during the basal condition both led to significantly more negative Iₜₑ and increased Gₜₑ (∼141 ± 3.3 μA/cm², 2.31 ± 0.84 mS/cm², n = 6, P < 0.05), suggesting a minor stimulation of K⁺ secretion. Rolipram significantly enhanced the initial epi peak (Fig. 5) by 63.3 ± 1.3 μA/cm² (n = 3, P < 0.05), whereas neither IBMX nor trequinsin enhanced this peak response significantly. These PDE inhibitors enhanced the smaller second peak in Iₜₑ by 52.4 ± 9.8 μA/cm² (n = 9, P < 0.05). Also, elevation of the sustained plateau at 20–30 min to a more positive value by these PDE inhibitors (28.7 ± 3.5 μA/cm², n = 9, P < 0.05) suggested a prolongation of the Cl⁻ secretory transient. Similarly, the PDE inhibitors increased the plateau of the subsequent PGE₂ (3 μM) response (33.5 ± 8.3 μA/cm², n = 9, P < 0.05), but the peaks were unaltered.

Fig. 4. Pertussis toxin (PTx) reversed peptide YY (PYY) inhibition of β₂-adrenergic response. Isolated mucosae were stimulated by epi (5 μM) from the standard basal condition, and Iₜₑ was measured in adjacent mucosae during activation with 1 mucosa in the standard basal condition serving as an activation control (○) and the others pretreated with PYY (7 nM) or PYY and PTx (△). PTx (1 μg/ml) was added 150 min prior to epi activation and PYY 20 min prior to activation. The responses were representative of 4 similar experiments, with PTx in the presence of PYY significantly increasing peak Iₜₑ by 4.6 ± 1.4-fold compared with PYY treatment alone (P < 0.05).

Fig. 5. Inhibition of phosphodiesterases (PDEs) enhanced β₂-adrenergic response. Isolated mucosae were stimulated sequentially by epi (5 μM), PGE₂ (3 μM), and CCh (10 μM) from the standard basal condition. Iₜₑ was measured in adjacent mucosae during secretagogue activation, with 1 mucosa serving as an activation control (○) and the others pretreated with the phosphodiesterase inhibitors trequinsin (trq, 7 nM, PDE3) and rolipram (rlp, △, 30 μM, PDE4). The responses were representative of 3 similar experiments. Iₜₑ responses during activation by PGE₂ and CCh were similar to those in Figs. 2 and 3; only the PGE₂ plateau Iₜₑ was significantly different from control (higher) and the response to CCh was not significantly different from control (P < 0.05).
bicarb-free treatment inhibiting K<sup>-</sup> secretion (Fig. 2A), with the action of KH7 on sustained I<sub>sc</sub> activation (Fig. 2A). The prolongation of the initial cAMP transient supported the possible suppression of tmAC by cAMP from sAC. Inhibition of tmAC with ddAdo eliminated the early cAMP transient (Fig. 8B), consistent with the concurrent loss of the cAMP transient (Fig. 3A). With ddAdo present, cAMP increased at 5 min, consistent with the independence of sustained I<sub>sc</sub> from tmAC activity. However, at 10 min of epi activation and longer, ddAdo suppressed cAMP content, indicating a supportive influence of tmAC on sustained cAMP production. Combined inhibition of sAC and tmAC suppressed cAMP over the entire epi activation time course, supporting the influence of tmAC on the early cAMP peak and of sAC on the sustained level.

Bicarb-free solutions significantly suppressed basal cAMP, as well as blunting the epi- and forskolin-induced increases in cAMP (Fig. 8A). Even with this suppression, a significant cAMP rise occurred after 2 min of epi stimulation. The peak of the early transient occurred individually at a wider range of times than in control (0.5–2 min), reaching 1.58 ± 0.01 μM. Addition of KH7 and ddAdo eliminated the epi stimulation of cAMP in bicarb-free conditions consistent with the requirement for sAC and tmAC in the β-adrenergic response.

cAMP-dependent actuators of secretory signaling. cAMP directly activates protein kinase A (PKA) (64, 65) and the guanine nucleotide exchange factor Epac (10, 26, 35, 59), and either protein could be involved in transducing the cAMP signaling into changes of ion permeability at the apical and basolateral membranes. The cAMP analog 8Br-cAMP has higher lipophilicity and lower susceptibility to PDE degradation than cAMP (55), and when added to the bathing solution it stimulated a negative I<sub>sc</sub> consistent with K<sup+</sup> secretion (Fig. 9). At concentrations >100 μM, 8Br-cAMP produced a positive change in I<sub>sc</sub>, suggesting the stimulation of Cl<sup-</sup> secretion in addition to the K<sup+</sup> secretion stimulated at lower concentration. This biphasic concentration dependence exactly followed the pattern of stimulation produced by increasing concentrations of forskolin (46). The shape for this concentration dependence suggested further that actuators of K<sup+</sup> secretion were either more sensitive to 8Br-cAMP or simply more accessible to the permeating cAMP analog than the actuators of Cl<sup-</sup> secretion. Other analogs of cAMP (35, 55) with preference for PKA (8Pip-cAMP, 6Bnz-cAMP) or Epac (8CPT2M-cAMP, 8MPT2M-cAMP) also were tested for ability to stimulate secretory I<sub>sc</sub>, and three (8Pip-cAMP, 8CPT2M-cAMP, 6Bnz-cAMP) produced negative I<sub>sc</sub> at 100 μM (Fig. 9). All of these analogs have higher lipophilicity than 8Br-cAMP (55), suggesting that the lower sensitivity for stimulating I<sub>sc</sub> occurred because neither PKA nor Epac was involved.
Involvement of PKA in secretory activation was examined further by using several inhibitors with distinct characteristics. Rp-8Br-cAMPS is a competitive inhibitor of PKA, and is \( \sim 30\% \) more lipophilic than 8Br-cAMP (55). The PKI inactivating peptide for PKA is made lipophilic by myristoylation and is the most specific inhibitor for PKA (65). KT5720 is a PKA specific analog of the protein kinase inhibitor staurosporine (19). Pretreatment with these inhibitors did not alter basal \( I_{sc} \) nor either the transient or sustained components of adrenergic activation (Fig. 10). H89 inhibits PKA (19), but has the confounding action of antagonism at \( \beta \)-AdR (54). H89 addition decreased \( I_{sc} \) in the basal condition to more negative values and produced a \( \sim 10\% \) inhibition of the sustained response (Fig. 10). Since staurosporine inhibited epi-activated \( I_{sc} \) at 0.3 \( \mu \)M (data not shown), a protein kinase may be involved, but the identity was undetermined.

Epac activation of the small GTPases Rap1 or Rap2 (10, 59) could provide a signal for producing epi-stimulated ion secretion. Involvement of Epac/Rap in the \( \beta \)-adrenergic pathways was examined by testing for activation of these small G proteins Rap1 and Rap2 in colonic mucosa stimulated by epi. Epi failed to activate Rap1 during the transient and sustained

| Table 3. Dependence of secretagog responses on HCO\(_3\)/CO\(_2\) buffer: sensitivity to bicarb-free conditions |
|-----------------|-----------------|-----------------|
|                | 1st Peak        | 2nd Peak        | Sustained      |
| Epi: control   | +123.7 \( \pm \) 21.1 | −137.3 \( \pm \) 8.3* |
| Epi: bicarb-free | +166.6 \( \pm \) 29.2† | −83.9 \( \pm \) 3.0† |
| PGE\(_2\): control | +265.3 \( \pm \) 17.4 | +204.0 \( \pm \) 20.0 | +19.1 \( \pm \) 13.2 |
| PGE\(_2\): bicarb-free | +385.1 \( \pm \) 29.4† | +259.9 \( \pm \) 23.0† | +56.1 \( \pm \) 6.8† |
| CCh: control   | +747.0 \( \pm \) 27.7 | +927.1 \( \pm \) 29.6 | +338.6 \( \pm \) 28.7 |
| CCh: bicarb-free | +842.7 \( \pm \) 36.5† | +884.0 \( \pm \) 68.1 | +222.1 \( \pm \) 22.0† |

\( I_{sc} \) values (\( \mu \)A/cm\(^2\)) are means \( \pm \) SE, \( n = 7 \), stimulated as in Fig 7. BIIE0246 was present for all responses, except sustained epi in which peptide YY replaced BIIE0246 (*). Responses in bicarbonate (bicarb)-free conditions significantly different from control (†\( P < 0.05 \).)
Fig. 9. cAMP analogs mimicked sustained β-adrenergic response. Isolated mucosae in the standard basal condition were stimulated by addition of cAMP analogs. I<sub>s</sub> responses to cumulative addition of 8-Br-adenosine-3′,5′-cyclic-monophosphate (8Br-cAMP) were measured for 2 adjacent mucosae from each colon (●, n = 3). A fit of Henri-Michaelis-Menten kinetics with 2 independent binding sites (A and B) stimulating separate responses was made to the concentration dependence of I<sub>s</sub> (dashed line; A EC<sub>50</sub> = 0.35 ± 0.03 μM, A I<sub>max</sub> = −154 ± 19 μA/cm<sup>2</sup> and B EC<sub>50</sub> = 1.1 ± 0.4 nM, B I<sub>max</sub> = +365 ± 40 μA/cm<sup>2</sup>). Other cAMP analogs were assessed for concentration-dependent stimulation (●, 8-piperidino-cAMP (8Pip-cAMP); P, n = 2, EC<sub>50</sub> ∼ 100 μM; △, 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8CPT2M-cAMP); C, n = 3, EC<sub>50</sub> ∼ 270 μM; ○, N<sup>6</sup>-benzoyl-cAMP (6Bnz-cAMP); B, n = 2, EC<sub>50</sub> ∼ 350 μM; ▽, 8-Br-adenosine-3′,5′-cyclic-monophosphorothioate-Rp-isomer (8MPT2M-cAMP); M, n = 3; KT5720 (100 μM, range shown for n = 2); KT5724 (200 μM, range shown for n = 3; KT5724 (200 μM, range shown for n = 3). Lipophilicities for the analogs relative to cAMP were 1.8, 8Br-cAMP; 6.0, 6Bnz-cAMP; 19, 8Pip-cAMP; 50, 8MPT2M-cAMP; 70, 8CPT2M-cAMP (Ref. 55).

Fig. 10. Sensitivity of epi stimulation to PKA inhibition. The transient and sustained responses to epi in standard conditions were compared with the epi responses in the presence of the PKA inhibitors Rp-8Br-c-AMPS (100 μM, n = 4), mPKI (15 μM, n = 3), KT5720 (10 μM, n = 3), and H89 (10 μM, n = 4). Those conditions that were inhibited compared with control are shown as black bars for emphasis. Paired differences compared with control conditions provide the delta produced by these inhibitors (hatched bars); * P < 0.05, deltas significantly different from zero.

β-ADRENERGIC CAMP SIGNALING FOR K+ AND CL− SECRETION

G90

Fig. 8. Epinephrine stimulated a biphasic increase in cAMP. Isolated mucosae were stimulated by epi (5 μM) from the standard basal condition, and cAMP content was measured by enzyme immunoassay at 7 time points (see METHODS). Estimates of mucosal histological parameters allowed a conversion to apparent intracellular concentration. A: control (●, n = 4) and bicarb-free (○, n = 4) solutions were used to assess HCO<sub>3</sub>− dependence. Additional mucosae from the same colon were stimulated with forskolin (frsk; 10 μM) for 5 min as a reference level to compare with epi. The action of AC inhibitors with bicarb-free solutions was assessed by pretreatment for 30 min (◇, 25 μM KH<sub>7</sub> + 200 μM ddAdo, n = 4). Control basal cAMP content was 1.79 ± 0.03 μM (n = 17), and bicarb-free basal cAMP content was significantly suppressed (1.28 ± 0.03 μM, n = 4, P < 0.05). * P < 0.05, epi-activated cAMP significantly different from basal levels. Control cAMP at 1 min was significantly different from the level at 30 s (P < 0.05), and control cAMP at 2 min epi stimulation was significantly different from the level at 1 or 5 min (P < 0.05). § Significant differences (P < 0.05) between the cAMP contents in bicarb-free conditions with AC inhibitors and the basal bicarb-free cAMP level without inhibitors. B: action of AC inhibitors in standard solutions was assessed by pretreatment for 30 min (◇, 25 μM KH<sub>7</sub>; KH<sub>7</sub> + 200 μM ddAdo, n = 3; ○, 25 μM KH<sub>7</sub> + 200 μM ddAdo, n = 3). Basal cAMP with KH<sub>7</sub> was 2.04 ± 0.12 μM (n = 3), 1.59 ± 0.10 μM (n = 3) with ddAdo, and 1.43 ± 0.20 μM (n = 3) with KH<sub>7</sub> + ddAdo; the level with KH<sub>7</sub> + ddAdo was significantly different from control (P < 0.05). * P < 0.05, cAMP contents significantly different from basal levels. After epi stimulation ddAdo significantly decreased cAMP compared with control at all time points, and KH<sub>7</sub> significantly decreased cAMP compared with control at 10 min and longer (P < 0.05). cAMP with ddAdo at 5 min after epi was significantly different from 2 min after epi (P < 0.05).
distinct pathways (Fig. 12). A receptor complexes with the in the mucosa during any sympathetic response. The signals for secretion is modified further by the PYY and NPY coreleased producing cAMP destined to stimulate Cl
secretory response likely involves combined activation of \( \beta_1 \)-AdrR and \( \beta_2 \)-AdrR (73, 74) with sAC producing the cAMP that stimulated K\(^+\) secretion (Fig. 2).

**Sympathetic activation of modulatory secretion.** Sympathetic stimulation initiates ion secretion that includes a transient flushing mode together with the sustained modulatory mode (73, 74) by using functionally separate \( \beta \)-AdrRs. The transient Cl\(^-\) secretion required \( \beta_2 \)-AdrR as indicated by high sensitivity to the \( \beta_2 \) antagonist ICI-118551 (Fig. 1B), similar to that reported for \( \beta_2 \)-AdrR in other cell types (3) but \( \sim 30 \)-fold more sensitive than for the sustained K\(^+\) secretory component (74). Since the combination of ICI-118551 and the \( \beta_1 \) antagonist CGP20712A was more effective than either alone at antagonizing sustained K\(^+\) secretion (73), a functional \( \beta_1 \)-AdrR/\( \beta_2 \)-AdrR oligomer likely activates this response. The transient character of the \( \beta_2 \)-AdrR-initiated Cl\(^-\) secretion conforms to the desensitization commonly found for \( \beta_2 \)-AdrR events, whereas involvement of \( \beta_1 \)-AdrR in the sustained response conforms to the resistance to desensitization often found with \( \beta_1 \)-AdrR-mediated events (45, 58). A major consequence of the difference in agonist sensitivity for the two components (Fig. 1C) is that Cl\(^-\) secretion would be stimulated only at high sympathetic discharge. Since neural NPY and enterococcal NPY secretion release also would be most pronounced at these intense levels (24), the transient component often would be severely suppressed. In this way the epithelial response to sympathetic stimulation generally would be limited to the sustained K\(^+\) secretory component of \( \beta \)-adrenergic activation.

**Epi-stimulated cAMP generation.** As expected for a \( \beta \)-adrenergic response, cAMP increased but with a biphasic time course (Fig. 8) mirroring the two components of the secretory \( I_{sc} \) (Fig. 1). Lending further support to the pharmacological evidence for two receptor complexes, each component of the

\[ \beta_2 \text{-AdrR} \]

\[ \beta_1 \text{-AdrR} \]

\[ \text{Y2-NpR} \]

\[ \text{sAC} \]

\[ \text{PDEs} \]

\[ \text{Epac} \]

\[ \text{PKA} \]

\[ \text{cAMP} \]

\[ \text{Cl}^- \text{secretion} \]

\[ \text{K}^+ \text{secretion} \]

**DISCUSSION**

\( \beta \)-Adrenergic receptors transmit sympathetic signals to the ion secretory elements of the colonic epithelial cells such that epi or norepi stimulates a transient Cl\(^-\) secretion and a sustained K\(^+\) secretion (73, 74). This \( \beta \)-adrenergic activated secretion is modified further by the PYY and NPY coreleased in the mucosa during any sympathetic response. The signals for the two secretory components appear to originate from separate receptor complexes with the \( \beta \)-AdrRs in each group activating distinct pathways (Fig. 12). A \( \beta_2 \)-AdrR complex initiated the Cl\(^-\) secretory component on the basis of antagonist sensitivity (Fig. 1). This \( \beta_2 \)-AdrR complex likely included a tmAC-producing cAMP destined to stimulate Cl\(^-\) secretion (Fig. 3) together with Y2-NpRs that inhibit the tmAC (Fig. 4). Proximity of PDEs also would limit the extent of cAMP emanating from this complex (Fig. 5). In contrast, the sustained-phase K\(^+\) secretion would be stimulated only at high sympathetic discharge. Since neural NPY and enteroendocrine NPY secretion release also would be most pronounced at these intense levels (24), the transient component often would be severely suppressed. In this way the epithelial response to sympathetic stimulation generally would be limited to the sustained K\(^+\) secretory component of \( \beta \)-adrenergic activation.

\[ \beta_1 \text{-AdrR} \]

\[ \beta_2 \text{-AdrR} \]

\[ \text{Y2-NpR} \]

\[ \text{sAC} \]

\[ \text{PDEs} \]

\[ \text{Epac} \]

\[ \text{PKA} \]

\[ \text{cAMP} \]

\[ \text{Cl}^- \text{secretion} \]

\[ \text{K}^+ \text{secretion} \]
secretory response required different adenylyl cyclases. Loss of both the sustained \( \frac{dI_c}{dt} \) (Fig. 2) and the plateau in cAMP (Fig. 8) with KH7 supported a primary involvement of sAC in activating K\(^+\) secretion. Although sAC (adcy10) was designated as soluble (43), because of the lack of transmembrane domains typical of tmACs (adcy1-adcy9), sAC localizes in membrane fractions, presumably via adapter proteins (76). The membrane localization of sAC in colonic epithelial cells (Fig. 6) would permit a close association with the proposed \( \beta_1 \)-AdrR/\( \beta_2 \)-AdrR complex (Fig. 12). Of the several splice variants of sAC presently described (22, 25, 40, 70), two may occur in colonic epithelial cells as indicated by antibodies to distinct domains of sAC (Fig. 6). The larger variant (\( \approx 73 \) kDa) presumably has both catalytic domains but not the full COOH-terminus that regulates activity, and the smaller variant (\( \approx 55 \) kDa) likely represented sAC-som, which also lacks the NH\(_2\)-terminal catalytic domain. Since cAMP production requires both catalytic domains (43), oligomers of these sAC variants may be involved in \( \beta_1 \)-AdrR/\( \beta_2 \)-AdrR signaling as suggested by the larger-sized membrane forms observed (Fig. 6). Although receptor-initiated activation of sAC has been observed in several cell types, heterotrimeric G proteins do not appear to be involved and the receptor-dependent activation mechanisms remain to be determined (11, 29, 43, 56, 62).

Loss of the transient \( \frac{dI_c}{dt} \) with a tmAC inhibitor without diminishing the sustained response (Figs. 3 and 7B) supported selective stimulation of Cl\(^-\) secretion via \( \beta_2 \)-AdrR activation of tmACs. Of the nine tmACs (63), seven (tmAC2–7/9) have been detected in colonic epithelial cells (23). tmAC5 and 6 are the most likely candidates for mediating the transient response, because both are sensitive to inhibition by G\(_{i/o}\) (14, 63, 71), as was the transient response (Fig. 4). All tmACs are inhibited by ddAdo, but some are more sensitive, with tmAC5 having one of the lower \( K_d \) values (39, 42). The fit of ddAdo inhibition to a cooperative two-site model supported cAMP generation via a tmAC dimer in which the catalytic domains interact (Fig. 3), consistent with evidence for tmAC oligomer formation and heterodimerization of adenylyl cyclase catalytic domains (16, 43). The ability of ddAdo to suppress epi-induced increases in cAMP occurring after \( \sim 10 \) min (Fig. 8B) implied that tmAC activity influenced sAC or that the basal component of cAMP production was reduced.

Activity of both tmACs and sAC influenced basal cAMP levels (Fig. 8), producing a concentration of 1.8 \( \mu \)M, similar to basal concentrations in cardiac myocytes (1.2 \( \mu \)M) obtained from a FRET-based cAMP biosensor (38). These basal cAMP levels were high enough to activate PKA strongly and Epac moderately (55), supporting the concept that cytoplasmic compartmentation maintained basal cAMP near the receptor complexes at levels lower than the global cytoplasmic average (2, 37, 38). Subsequent receptor activation would increase cAMP in the region of the receptor complexes and result in cAMP flow into the rest of the cytoplasm such that global cAMP increased (Fig. 12). Thus activated cAMP levels in the region of receptor complexes would be higher than the global measurement, especially if high PDE activity limited cAMP emerging from these regions. If all of the cells in the colonic epithelium did not respond to \( \beta \)-adrenergic activation, then the global measure of the response would be attenuated further from that actually occurring near activated \( \beta \)-AdrRs. The transient and sustained cAMP responses (Fig. 8) were similar to those measured by biosensors in cells with \( \beta_2 \)-AdrRs or \( \beta_1 \)-AdrRs. \( \beta_2 \)-AdrR produces a transient cAMP response with a time for half-activation (\( \tau_{0.5} \)) of \( \sim 0.5 \) min and a \( \tau_{0.5} \) for decline of \( \sim 2 \) min (69), whereas a \( \beta_1 \)-AdrR response remains stable for over 10 min (38).

Increasing CO\(_2\) stimulates both tmAC and sAC, whereas HCO\(_3\) stimulates only sAC (43, 66). Preferential reduction of sustained \( \frac{dI_c}{dt} \) by removing HCO\(_3\)/CO\(_2\) while leaving peak \( \frac{dI_c}{dt} \) unaltered (Fig. 7) suggested that sAC sensitivity was higher than that for tmAC. Attenuation of the early cAMP peaks (Fig. 8A) suggested that either sufficient cAMP still arrived to produce near normal transient Cl\(^-\) secretion or that this cAMP was not crucial for signaling. In contrast, the partial reduction of sustained K\(^+\) secretion conformed to the observed reduction in sustained cAMP. With the HCO\(_3\) EC\(_{50}\) of 9–11 mM for sAC (25, 48), and assuming an intracellular pH of \( \sim 7.0\), the \( \sim 40\%\) reduction in sustained secretion (Fig. 7B) suggested a drop of cell HCO\(_3\) from \( \sim 10 \) to \( \sim 4 \) mM, consistent with metabolism maintaining cell CO\(_2\) at \( \sim 0.5 \) mM compared with the 1.2 mM level in the standard HCO\(_3\)/CO\(_2\)-buffered conditions. This drop in CO\(_2\) may have been insufficient to limit tmAC-dependent cAMP production destined for activating Cl\(^-\) secretory elements, because a portion of tmAC activity appears CO\(_2\) independent and the CO\(_2\) EC\(_{50}\) for tmAC (\( \geq 6 \) mM) is much higher than the nominal CO\(_2\) level in the cells (66). The partial inhibition of the forskolin response (Fig. 8A) provided support for the extent of CO\(_2\) dependence for tmAC in these cells.

The response of cAMP content to epi also could be interpreted to suggest that cAMP is not the primary signal for the observed secretory events. However, since the measured cAMP content tracks the total cellular cAMP, the actual concentrations at the point of production near the tmAC and sAC may have a wider range such that a direct quantitative comparison between \( I_c \) and cAMP content will require spatial information concerning production and response (37, 38).

The response to 8Br-cAMP (Fig. 9), which mimics the action of cAMP at many signaling proteins (55), supported the direct dependence of secretion on cAMP. The preferential stimulation of K\(^+\) secretion at low concentration and Cl\(^-\) secretion only at higher concentrations suggested that actuators of K\(^+\) secretion were more sensitive to cAMP, or that those for Cl\(^-\) secretion were relatively isolated from global changes in cAMP. The high levels of cAMP needed for Cl\(^-\) secretion could be achieved by close localization of the signaling elements as illustrated in the model (Fig. 12). Such receptor signaling complexes have been observed in other cell types (16, 21, 71) and may be a general means to coordinate simultaneous activation of distinct responses.

**Termination of cAMP signaling during secretory activation.** Cellular signaling via cAMP occurs as a balance between production by ACs (63) and degradation by PDEs (6). PDE4 and PDE3 likely occurred in the \( \beta_2 \)-AdrR complex, which signals for Cl\(^-\) secretion (Fig. 5). PDE inhibition enhanced the second peak of transient \( \frac{dI_c}{dt} \), which was \( \beta_2 \)-AdrR dependent (Fig. 1D). Activation of two peaks also occurred in the much smaller \( \beta \)-adrenergic response in T84 cells (72). The lack of any enhancement in sustained secretion suggested that an IBMX/rolipram-insensitive PDE degraded the cAMP produced by sAC in these cells. Insensitivity to IBMX and localization to colonic epithelial cells (6, 18) makes PDE8 and PDE11 candidates for the \( \beta_1 \)-AdrR/\( \beta_2 \)-AdrR complex that signals for K\(^+\).
secretion (Fig. 12). Y2-NpRs also provided a negative regulation of cAMP production from tmAC in the β2-AdrR complex as indicated by sensitivity to PTx (Fig. 4). Proximity of β2-AdrR and Y2-NpR within the signaling complex would allow effective inhibition of the tmACs via Goi, (Fig. 12).

The transition from the basal condition to modulatory secretion involves a concerted activation of the transport proteins required for Cl− and K+ secretion. The basal condition exhibited a negative Isc (Figs. 2–5, 7), consistent with electrogenic K+ secretion that may result from low-level stimulation by agonists remaining in the preparation or through agonist-independent activity of receptors such as β1-AdrR and β2-AdrR. Sensitivity of basal Isc to signaling inhibitors had some similarities with epi-stimulated K+ secretion and several differences. Sensitivity to KH7 (Fig. 2A) indicated that both basal and epi-stimulated K+ secretion required sAC activity. For that reason, the tmAC inhibitor ddAdo might be anticipated to do little to basal electrogenic K+ secretion. Instead, ddAdo stimulated basal Isc and Gs (Fig. 3), supporting an inhibitory role for cAMP generated by tmACs. Inhibition of Isc and Gs by KH7 in the absence of ddAdo (Fig. 3A) further supported sAC as the primary activation pathway. Inhibitory influences of cAMP from tmACs also were apparent in the peak responses to PGE2 and CCh, as seen by the consistently higher maxIsc obtained from the ddAdo concentration dependence compared with control stimulation (Fig. 3, B and C).

PDE control of basal and epi-activated K+ secretion differed, with rolipram and IBMX augmenting basal K+ secretion but failing to enhance sustained K+ secretion (Fig. 5). This divergence of action suggested that PDE4 was near the K+ secretory cAMP pool in basal conditions but not after epi activation, or that the K+ secretory rate during epi activation was maximal such that further increases in cAMP were ineffective. Also, in contrast to the insensitivity of epi-activated K+ secretion to PYY inhibition (74), basal Isc and Gs decreased with PYY in a PTx-sensitive manner consistent with signaling via Goα. The simplest interpretations would be either an as-yet-to-be-reported inhibitory action of Goα on sAC or an involvement of Goα-sensitive tmAC in the sAC-dependent activation of basal K+ secretion.

cAMP-dependent actuators of ion secretion. cAMP stimulates three major protein types that actuate further signaling (7, 8, 10, 26, 59, 64): PKA, cAMP-regulated guanine nucleotide exchange factor (Epac), and cyclic nucleotide-gated ion channels (CNG, HCN). PKA phosphorylates numerous proteins including CFTR, a Cl− channel present in the apical membrane of many Cl− secretory epithelial cells (5, 28). For this reason PKA often plays a major role in activation of electrogenic Cl− secretion. Epac activates Rap1 and Rap2, members of the Ras family of small GTPases, leading to increased activity of various cellular functions in many cell types (59). In the colonic tumor cell line T84, forskolin stimulates a PKA-independent Cl− secretion mediated by Epac (36). This Cl− secretion depends on apical Cl− channels other than CFTR, indicating that each cAMP-dependent actuator likely links to specific transport elements. Cyclic nucleotide-gated channels often occur in nerves and muscle (7, 8), but a CNG appears to mediate Na+ absorption in the inner medullary collecting duct of the kidney (68).

Several cAMP analogs with greater lipid solubility and resistance to PDE degradation than cAMP allow extracellular addition to activate PKA and Epac (35, 55). Relative efficacy of these analogs in stimulating secretory Isc aided identification of signaling pathways (Fig. 9). The selectivity sequence for activating sustained K+ secretion was 8Br-cAMP > 8Pip-cAMP > 8CPT2M-cAMP ≥ 6Bnz-cAMP >> 8MPT2M-cAMP. Since greater lipid solubility should lead to greater penetration into the cell, the relative lipophilicities of the analogs (55) can adjust for this factor, giving a better estimate of actuator selectivity: 8Br-cAMP > 8Pip-cAMP > 6Bnz-cAMP > 8CPT2M-cAMP >> 8MPT2M-cAMP. Differential degradation by PDE would alter the persistence of each analog in the cell also contributing to the observed concentration dependence, and since 6Bnz-cAMP and 8CPT2M-cAMP inhibit PDE activity the most likely effect would be to heighten any efficacy compared with 8Br-cAMP. The relative ineffectiveness of the Epac agonists 8CPT2M-cAMP and 8MPT2M-cAMP and the PKA agonists 8Pip-cAMP and 6Bnz-cAMP suggested that other actuators were involved.

Insensitivity of β-adrenergic activation to PKA inhibitors (Fig. 10) also supported a signaling pathway without a PKA action on either Cl− secretion or K+ secretion (Fig. 12). Each PKA inhibitor was used at concentrations that succeeded at inhibiting responses in cells with PKA as part of the signaling pathway (1, 36, 44, 53). However, each inhibitor exhibits weaknesses that may have limited efficacy in colonic epithelial cells. Rp-8Br-cAMP competes with cAMP for binding, such that it simply may not have reached concentrations sufficient to effectively antagonize PKA activation. A lack of sufficient intracellular accumulation also may have blunted any action of the peptide inhibitor myristoylated PKI (65). Poor penetration into the cell was unlikely to explain the lack of inhibition by KT5720 or H89 since both are very lipid soluble. Also, the minor inhibition by H89 (Fig. 10) was more likely due to antagonism at β1AdrR (54). Thus β-adrenergic activation used a cAMP-dependent actuator other than PKA.

Although Epac can provide a cAMP-dependent activation of the Rap GTPases (10, 26, 59), Epac did not appear to transduce β1AdrR-generated cAMP into Rap1 activation for stimulating secretion (Fig. 11). The basal level of activated Rap2 may have obscured any small epi-induced increase in activated Rap2 (Fig. 11), and if this increase was highly localized near the next step in the signaling pathway, then a chain of activation may have occurred. Small changes in Rap2 have been linked to cell signaling (20, 59), and T84 cells have Epac-dependent increases in Rap2 associated with Cl− secretion (36). A small or localized change in Rap2 activity, therefore, would be the most likely response by a known cAMP-dependent signaling actuator, and either the β2AdrR/β2AdrR-mediated sustained K+ secretion or the β2AdrR-mediated transient Cl− secretion might be stimulated (Fig. 12).

Signaling pathways for flushing and synergistic secretion. The flushing mode of secretion stimulated by PGE2 includes sustained Cl− secretion compared with β-adrenergic activation that only produces transient Cl− secretion (74). The synergistic secretory mode has even higher rates of sustained Cl− secretion. Sensitivity to inhibitors of adenyl cyclase would be expected because of the association of cAMP with Cl− secretion (4, 5). However, the results were mixed. During the flushing mode (Fig. 2B), the sAC inhibitor KH7 apparently inhibited K+ secretion without altering Cl− secretion, and the HCO3−/CO2 sensitivity of sustained K+ secretion fit with this
dependence on sAC (Fig. 7A). Inhibiting tmAC reduced the transient epi response to near zero (Fig. 3, Table 2), but all three components of the flushing and synergistic responses were only partially sensitive, consistent with a substantial contribution by cAMP-independent signaling pathways. Furthermore, insensitivities of flushing and synergistic secretion to PDE inhibitors and PKA inhibitors suggested that the standard model for activating Cl− secretion via cAMP-dependent processes was insufficient to describe these secretory activations.

β-Adrenergic activation of ion secretion in the colonic epithelium has several similarities with β-adrenergic signaling in cardiac muscle cells. In particular, the sequestration of β-AdrR complexes into specialized membrane domains of cardiac cells (38, 75) may be a useful model for the organization of β-AdrRs and signaling components in colonic epithelial cells. Whether the same adaptor proteins are used remains to be determined, but the intricacy of the β-adrenergic response in the colonic epithelium likely will use many similar strategies to produce varied responses to sympathetic input.

ACKNOWLEDGMENTS

The authors thank L. Levin and J. Buck, Weill Medical College of Cornell University, for helpful discussions.

GRANTS

This study was supported by a grant from the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases (DK66845).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


β-ADRENERGIC CAMP SIGNALING FOR K⁺ AND CL⁻ SECRETION