Rutaecarpine Induces Chloride Secretion across Rat Isolated Distal Colon

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ABSTRACT

The present study evaluated the effect of rutaecarpine (Rut) on Cl− secretion across rat distal colonic mucosa. Basolateral application of Rut elicited an increase in short-circuit current (Isc) response in a concentration-dependent manner. Evidence that Rut-stimulated Isc was due to Cl− secretion is based on 1) inhibition of current by bumetanide; 2) Cl− channel blockers diphenylamine-2-carboxylate, 5-nitro-2-(3-phenylpropylamino)benzoic acid, and glibenclamide; and 3) removal of Cl− ions in bath solution. Determination of neurogenic blockers on Rut-induced Isc indicated that pretreatment of tissues with tetrodotoxin or indomethacin, but not atropine or hexamethonium, inhibited Rut-induced response. Treatment with Rut led to release and synthesis of prostaglandin E2 in rat colonic mucosa. Rut-stimulated I sc was markedly reduced by pretreatment with MDL-12,330 A [cis-N-[2-phenylcyclopentyl]-azacyclotridec-1-en-2-amine] and N-[2-[(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), but not with 1,2-bis(2aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid-acetoxyethyl ester, bisindolylmaleimide, and thapsigargin. Elimination of the extracellular Ca2+ also did not alter Rut response. Rut treatment resulted in the increase in intracellular cAMP levels and the activation of protein kinase A. Depolarizing the basolateral membrane with high K+ showed that Rut-stimulated apical Cl− current was largely prevented by cystic fibrosis transmembrane conductance regulator (CFTR) inhibitors. Permeabilizing apical membrane with nystatin revealed that Rut-stimulated basolateral K+ current was specifically inhibited by Ba2+ ions and chromanol 293B. The evidence derived from present study suggests that Rut-stimulated Cl− secretion is mediated by generation of endogenous prostaglandin E2 and that it also involves the stimulation of cAMP and protein kinase A pathways, which subsequently lead to the activation of apical Cl− channels, mostly the CFTR and basolateral cAMP-dependent K+ channels.

Rutaecarpine (Rut) is a quinazolinocarboline alkaloid that has been isolated and purified from the dried, unripe fruit of Evodia rutaecarpa (Juss.) Benth ( Wu-Chu-Yu), a well known traditional Chinese herbal medicine that has widely been used for centuries to treat gastrointestinal disorders, dysmenorrhea, and hypertension (Wang et al., 1999). In addition, Fructus evodiae (fruit of E. rutaecarpa) is also used for the treatment of headache, thoracic abdominal pain, vomiting, colds, and reduced blood circulation (Fei et al., 2003). Pharmacological studies indicate that Rut has various bioactivities, such as causing vasodilatation by mechanisms of an endothelium-dependent manner coupled with the synthesis or release of nitric oxide (Wang et al., 1996), inhibiting vasoconstriction induced by anaphylaxis (Yu et al., 2005), inducing positive inotropic and chronotropic actions (Kobayashi et al., 2001), protecting the myocardium mediated by calcitonin gene-related peptide (Hu et al., 2002; Yi et al., 2004), increasing intracellular Ca2+ concentration in endothelium (Wang et al., 1996), suppressing platelet plug formation in mesenteric venules (Sheu et al., 2000), inhibiting prostaglandin production in RAW264.7 macrophages (Woo et al., 2001),
relaxation of rabbit and human internal anal sphincter (Jiang et al., 2000), gastroprotective effect against injury induced by aspirin and stress (Wang et al., 2005), anti-Helicobacter pylori (Tominaga et al., 2002), blockade of delayed rectifier K+ current in NG108-15 neuronal cells (Tu et al., 2001), inhibiting cytochrome P450 in human liver microsomes (Ueng et al., 2002, 2006; Iwata et al., 2005), and inhibition of COX-2 (Moon et al., 1999).

Electrolyte transport of mammalian colonic epithelium involves both NaCl absorption and Cl− secretion processes. Transport is regulated by a variety of neurotransmitters, hormones, and inflammatory mediators (Kunzelmann and Mall, 2002). NaCl is either absorbed electroneutrally by parallel luminal (apical) Na+−H+ and Cl−−HCO3− exchanges or electrogenically by luminal ENaC channels with Cl− transport passively through the paracellular pathway located in the distal colon (Kötting et al., 2003). Chloride secretion in the mammalian colonic crypt cells is a vectorial transport of Cl− ions from the serosal (basolateral) to luminal compartment; this process requires the coordination of four distinct membrane events: 1) apical Cl− passive diffusion via the cystic fibrosis transmembrane conductance regulator (CFTR) channel; 2) increase of K+ efflux through the basolateral Ba2+-sensitive channels; 3) activation of basolateral bumetanide-sensitive Na+−K+−2Cl−cotransporters; and 4) increase of Na+ efflux by the basolateral Na+−K+−ATPase pump (Gregger, 2000). Cl− secretion stimulated by both Na+−K+−2Cl− cotransporters and Na+−K+−ATPase requires basolateral K+ channel for K+ recycling across the basolateral membrane. Two types of basolateral K+ channels were found to process Cl− secretion via the apical membrane, namely, cAMP-activated KCNQ1 K+ channel and Ca2+-activated small-conductance Ca2+-activated K+ channel (Bleich et al., 1997; Warth, 2003).

Fructus evodiae in Chinese medicine is mainly recommended for treatment of gastrointestinal disorders, such as abdominal pain, acid regurgitation, nausea, diarrhea, and hernia (Yu et al., 2006). Thus, we hypothesize that its major active compound, Rut, may influence gastrointestinal function, especially electrolyte transport across the colonic epithelium. Therefore, the aim of the present study was to elucidate the effect of Rut on the ion transport process in rat distal colon epithelium and to determine the underlying mechanism(s). The information generated from this study demonstrates that Rut stimulates Cl− secretion across rat colonic mucosa, and we suggest that this occurs by generation of endogenous prostaglandin E2 (PGE2) and also involves the stimulation of cAMP and protein kinase A (PKA) pathways.

Materials and Methods

Chemicals. Chemically pure Rut was obtained from the Laboratory of Chemistry (Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China). It was identified on the basis of chemical and spectroscopic evidence. Acetazolamide, amiloride, atropine sulfate, BAFTA-acetoxymethyl ester (AM), bisindolylimidleimide (BIS), bumetanide, charybdotoxin (ChTX), clotrimazole (CLT), 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS), diphenylamine-2-carboxylate (DPC), forskolin, H-89, hexamethonium, indomethacin, 5-isobutyl-1-methylxanthine (IBMX), MDL-12,330A, nystatin, PGE2, and taspisagrin were obtained from Sigma-Aldrich (St. Louis, MO). Chromanol 293B (293B) was purchased from Toeris Cookson Inc. (Ellisville, MO), glibenclamide and tetrodotoxin (TTX) were from BIOMOL Research Laboratories (Plymouth Meeting, PA), and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) was obtained from Calbiochem (San Diego, CA). TTX was dissolved in 0.25% acetic acid, and ChTX was dissolved in an aqueous stock solution containing 0.1% (w/v) bovine serum albumin. Atropine sulfate and hexamethonium were dissolved in aqueous stock solutions diluted in salt buffer just before use. All other drugs were dissolved in dimethyl sulfoxide (DMSO), and the final DMSO concentration was less than 0.1% (v/v). For permeabilization studies, nystatin was used as a 100 mg · mL−1 stock solution in DMSO [final concentration 0.1% (w/v)], and it was sonicated for 30 s just before use. If drugs were dissolved in a solvent rather than aqueous solution, the same volume of solvent was administered to the control tissue.

Solutions. The Parsons solution (Schultheiss et al., 2005) for tissue preparation and Ussing chamber experiments contained 107 mM NaCl, 4.5 mM KCl, 25 mM NaHCO3, 1.8 mM Na2HPO4, 0.2 mM NaH2PO4, 1.2 mM CaCl2, 1.0 mM MgSO4, and 12 mM glucose. The solution was continuously aerated with carbogen (a mixture of 95% O2 and 5% CO2) to maintain the pH at 7.4. In ion substitution experiments, Cl− was replaced with gluconate when Cl−-free solution was used. When HCO3−-free solution was used, HCO3− was replaced by Cl−, and 10 mM HEPES was added to solution. In Cl−-HCO3−-free solution, both Cl− and HCO3− were replaced with gluconate and HEPES. Both HCO3−-free and Cl−-HCO3−-free solutions were gassed with 100% O2. HCO3−-free solution was also used in experiments with BaCl2.

Animals and Tissue Preparation. Male Wistar rats (200–220 g) were obtained from Sino-British SSPR/BK Lab Animal Ltd. (Shanghai, China). This research was approved by the university ethics committee. The animals were maintained on a 12-h light/dark cycle, and they were allowed free access to normal food and water until the day of the experiments. Animals were sacrificed rapidly by stunning and cervical dislocation. A 5-cm segment of the distal colon was removed without distention, and it was suspended in an ice-cold and oxygen-saturated Parsons solution. The colon was opened along the mesenteric border, and it was rinsed free of its fecal contents with Parsons solution. Thereafter, the colon was pinned with the mucosa facing down on a silicon-dissected plate, and then the longitudinal and circular muscles were carefully stripped away with fine forceps. Finally, the isolated mucosal sheet was cut into an appropriate size matched to Ussing chamber size.

Measurement of Electrophysiological Parameters. Freshly isolated rat colonic mucosal sheets were fixed to holding sliders and then they were mounted in modified Ussing chambers (EasyMount chamber; Physiologic Instruments, San Diego, CA), with a window area of 0.5 cm2. Two pieces of mucosal sheets were used from each animal. Tissues were bathed on both the mucosal and serosal sides with 5 ml of Parsons solution maintained at 37°C by heated water jackets; the pH was maintained at 7.4, and the solution was oxygenated with 95% O2 and 5% CO2. The tissues were voltage-clamped at 0 mV to monitor short-circuit current (Isc) using a dual-voltage clamp amplifier (VCC MC2; Physiologic Instruments) connected via a PowerLab 8SP (ADInstruments Pty Ltd., Castle Hill, Australia) to a PC computer. The transepithelial electrical potential was measured by a pair of pipette-shaped voltage-sensing electrodes made of sintered silver-silver chloride wire (Physiologic Instruments) in agar bridges filled with a solution of 4% (w/v) agarose in 3 M KCl solution; the electrical current across mucosae was measured by a pair of pipette-shaped current-passing electrodes made of silver pellet (Physiologic Instruments) in agar bridges filled with a solution of 4% (w/v) agarose in 3 M KCl solution. The current deflection (ΔIsc) was caused by applying a 1-mV pulse for 0.5 s at 60-s intervals under short-circuit condition through the voltage-sensing electrodes. By this procedure, the transepithelial electrical resistance (Rt) was able to be calculated by Ohm’s law (Rt = transepithelial electrical potential/ΔIsc). A positive Isc is referred to as a net flow of anions from the...
basolateral to the apical side, a net flow of cations from the apical to the basolateral side, or a combination.

Measurement of Apical Membrane Cl− Current and Basolateral Membrane K+ Current. The apical membrane Cl− current (I_Cl) was investigated according to Schultheiss et al. (2005). In brief, the basolateral membrane was depolarized with a solution containing high potassium concentration for 40 to 50 min. Thus, apical Cl− current was measured in the presence of a serosal-to-mucosal Cl− gradient with the following bath solution: apical, 107 mM potassium gluconate, 4.5 mM KCl, 25 mM NaHCO3, 1.8 mM Na2HPO4, 0.2 mM NaH2PO4, 5.75 mM calcium gluconate, 1.0 mM MgSO4, and 12 mM glucose; and basolateral, 111.5 mM KCl, 25 mM NaHCO3, 1.8 mM Na2HPO4, 0.2 mM NaH2PO4, 1.25 mM CaCl2, 1.0 mM MgSO4, and 12 mM glucose. This procedure allows measurement of changes in the apical anion conductance, avoiding contaminations in the current response, e.g., by charybdo- to-sensitive apical K+ channel (Schultheiss et al., 2005).

The basolateral membrane K+ current (I_K) was determined after permeabilization of apical membrane with nystatin (100 μg · ml−1) in the mucosal side in the presence of a mucosal-to-serosal K+ gradient (13.5 mM KCl at the mucosal and 4.5 mM KCl at the serosal side) established by the following bath solution: apical, 98 mM N-methyl-D-glucamine-Cl, 13.5 mM KCl, 25 mM choline HCO3−, 1.8 mM Na2HPO4, 0.2 mM NaH2PO4, 1.25 mM CaCl2, 1.0 mM MgSO4, and 12 mM glucose; and basolateral, 107 mM potassium gluconate, 4.5 mM KCl, 25 mM NaHCO3, 1.8 mM Na2HPO4, 0.2 mM NaH2PO4, 5.75 mM calcium gluconate, 1.0 mM MgSO4, and 12 mM glucose. All solutions were adjusted to pH 7.4 at 37°C.

Current.

Results

Effect of Rut on I_SC. After a 90-min equilibration with normal Parsons solution, the transepithelial potential was −3.8 ± 0.1 mV, the basal I_SC was 34.5 ± 0.5 μA · cm−2, and the transepithelial resistance was 111.1 ± 1.1 Ω · cm2 in 409 rat colonic preparations. When added to serosal (basolateral) side of the colonic tissue preparation, Rut (Fig. 1A) induced a rapid increase in I_SC that peaked approximately 2.5 min and subsequently returned to baseline value within approximately 10 min (Fig. 1B). Rut added to mucosal (apical) side of the preparation caused no alteration in I_SC (Fig. 1B). The concentration-dependent response to Rut showed that the amplitude of Rut-induced I_SC increased in a sigmoidal manner over the concentration range between 1.0 and 300 μM (Fig. 1C); the maximal I_SC response to Rut was 67.1 ± 7.9 μA · cm−2, with an EC50 value of 20.0 ± 7.0 μM (Fig. 1D).

Application of 100 μM Rut to serosal side caused an increase in I_SC that was nearly to the maximal response, which was thus used to compare the responses between control and treated samples in the subsequent experiments. Initial experiments showed that second response to Rut was less than the first response, indicating that Rut had a desensitization effect. Therefore, only a single concentration of Rut was applied to one tissue, either in the absence or presence of inhibitors or antagonists. A similar desensitization phenomenon was observed from the alteration of positive inotropic and chronotropic actions induced by Rut in guinea pig isolated right atria (Kobayashi et al., 2001). The current response induced by 100 μM Rut was 63.3 ± 7.3 μA · cm−2, and the corresponding tissue resistance was 105.7 ± 2.8 Ω · cm2 (n = 12), which was not different from its basal value (111.3 ± 2.6 Ω · cm2).
**Ionic Nature of Rut-Induced $I_{SC}$** To determine the ionic nature of Rut-induced $I_{SC}$, ion substitution experiments were performed as follows. When Cl$^-$ was replaced with gluconate in bilateral bath solution, Rut-induced maximal $I_{SC}$ was $15.4 \pm 2.4 \mu A \cdot cm^{-2}$ ($n = 8$), or 23% of the control value ($67.1 \pm 7.9 \mu A \cdot cm^{-2}$; $n = 7$), whereas replacement of HCO$_3^-$ with HEPES did not influence Rut-induced $I_{SC}$. When using a Cl$^-$-HCO$_3^-$-free solution, Rut-induced maximal $I_{SC}$ was $13.6 \pm 1.8 \mu A \cdot cm^{-2}$ ($n = 7$), which was not significantly different from the response obtained in Cl$^-$-free solution (Fig. 2A). Pretreatment of tissues with the carbonic anhydrase inhibitor acetazolamide (100 μM, bilateral) did not influence $I_{SC}$ response induced by Rut (Fig. 2B and C). Rut-mediated peak response was strongly inhibited by pretreatment of tissues with a Na$^+$-K$^+$-2Cl$^-$ cotransporter inhibitor bumetanide (100 μM, basolateral), but not by pretreatment of tissues with an epithelial Na$^+$ channel inhibitoramiloride (100 μM, apical) (Table 1), suggesting that Rut-induced increase in $I_{SC}$ was mainly carried out by Cl$^-$ ions. Furthermore, the effect of Cl$^-$ channel inhibitors on Rut-Induced $I_{SC}$ was compared (Table 1) and showed that preincubation of mucosal membrane with DPC (100 μM) strongly inhibited basal $I_{SC}$ by $19.8 \pm 3.5 \mu A \cdot cm^{-2}$; subsequently, addition of Rut induced a response of $I_{SC}$ by 5.6 ± 3.1 μA cm$^{-2}$, which was 9.5% of control response (59.1 ± 3.8 μA cm$^{-2}$; $n = 9$). In contrast, pretreatment of mucosal membrane with NPPB (100 μM) and glibenclamide (500 μM) did not influence basal $I_{SC}$, but it inhibited the subsequent Rut-induced $I_{SC}$ by $50.3 \pm 10.0$ and $37.8 \pm 6.2 \mu A \cdot cm^{-2}$, compared with their respective control responses. In contrast, the magnitude of Rut-induced $I_{SC}$ was not influenced by prior application of the disulfonic stilbene DIDS (500 μM) to basolateral rather than apical side induced a transient increase in $I_{SC}$ as shown in the original recording traces. Dashed line indicates zero current level. C, original recording traces of a concentration-dependent current response induced by Rut from 1.0 to 300 μM. Each concentration of Rut was added as a single dose to separate tissues. D, summary of concentration-response curve data collected from experiments as shown in C. Each point represented mean ± S.E.M., $n = 7$. The EC$_{50}$ value was calculated to be 20.0 ± 7.0 μM.

**Involvement of Neurogenic Mechanisms in Rut-Induced Increase in $I_{SC}$** To define whether Rut-induced...
I\textsubscript{SC} was involved in activation of submucosal neurons or increase of prostaglandin synthesis, Rut-induced I\textsubscript{SC} was determined after pretreatment with neuronal blockers or indomethacin for 30 min. Figure 3B shows that preincubation of tissues with muscarinic receptor antagonist atropine (1 \textmu M, serosal) did not change the basal I\textsubscript{SC} or subsequent Rut-induced I\textsubscript{SC} (61.4 \pm 2.5 \mu A \cdot cm\textsuperscript{-2}; n = 8) compared with the control value (64.8 \pm 4.5 \mu A \cdot cm\textsuperscript{-2}; n = 8). In addition, preincubating tissues with nicotinic receptor blocker hexamethonium (10 \mu M, serosal) also did not change the basal I\textsubscript{SC} or subsequent Rut-induced I\textsubscript{SC}.

### Table 1

Effect of inhibitors of epithelial Na\textsuperscript{+} channel, Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter, and Cl\textsuperscript{−} channel on Rut-induced increase in I\textsubscript{SC}.

<table>
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<tr>
<th>Inhibitor</th>
<th>– Inhibitor</th>
<th>+ Inhibitor</th>
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<tbody>
<tr>
<td>\mu A \cdot cm\textsuperscript{-2}</td>
<td>n</td>
<td>\mu A \cdot cm\textsuperscript{-2}</td>
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<tr>
<td>Amiloride</td>
<td>57.3 \pm 9.3</td>
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<tr>
<td>Bumetanide</td>
<td>60.4 \pm 8.4</td>
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<tr>
<td>DIDS</td>
<td>66.0 \pm 4.3</td>
<td>7</td>
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<tr>
<td>Glibenclamide</td>
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</tr>
<tr>
<td>NPPB</td>
<td>62.0 \pm 10.0</td>
<td>6</td>
</tr>
<tr>
<td>DPC</td>
<td>59.1 \pm 3.8</td>
<td>9</td>
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** P < 0.01, significantly different from the respective control (– inhibitor).
While pretreatment of basolateral membrane with neuronal blocker TTX (1 µM, serosal) decreased basal $I_{SC}$ by 8.3 ± 3.1 µA · cm⁻² (from 36.3 ± 3.4 to 28.0 ± 6.0 µA · cm⁻²), subsequent addition of Rut induced a response of $I_{SC}$ by 11.3 ± 1.6 µA · cm⁻², which was 17.9% of control response (63.3 ± 4.2 µA · cm⁻²; $n = 8$). Furthermore, pretreatment of tissues with the prostaglandin synthesis inhibitor indomethacin (1 µM, bilateral) slightly decreased basal $I_{SC}$ by 4.5 ± 0.6 µA · cm⁻² (from 39.3 ± 3.2 to 34.8 ± 2.6 µA · cm⁻²), and subsequent addition of Rut induced a response of $I_{SC}$ by 19.6 ± 2.2 µA · cm⁻², which was 30.9% of control response (63.4 ± 3.9 µA · cm⁻²; $n = 8$), indicating that Rut-induced $Cl^-$ secretion was maintained by synthesis of prostaglandins in rat colonic mucosa. Most of the actions of prostaglandins were mediated by intracellular cAMP; therefore, Rut-induced actions of prostaglandins were mediated by intracellular cAMP. Figure 3C showed that Rut evoked an $I_{SC}$ of 56.3 ± 4.6 µA · cm⁻² after intracellular cAMP level that was enhanced by the adenylyl cyclase stimulator forskolin (2 µM, bilateral) in the presence of indomethacin (1 µM, bilateral), which was not statistically different from the control Rut-evoked $I_{SC}$ response (63.8 ± 1.9 µA · cm⁻²). Furthermore, determination of PGE₂ contents in bath solutions and tissues with a competitive enzyme immunoassay kit showed that Rut induced a significant increase in PGE₂ levels in both serosal incubate and colonic mucosa, indicating that Rut-induced $Cl^-$ secretion is involved in prostaglandins release and synthesis in the colonic mucosa (Fig. 4).

**Effect of Rut on Apical $Cl^-$ Current and Basolateral K⁺ Current.** To identify whether the increase of $Cl^-$ secretion by Rut was involved in activation of the apical $Cl^-$ channels, basolateral membrane was depolarized with high K⁺ solution in the presence of a basolateral-to-apical $Cl^-$ gradient. Figure 5A shows that application of Rut induced a detectable outward current (5.8 ± 0.5 µA · cm⁻²; $n = 9$), including a transient peak followed by a plateau phase, which under these conditions represented increased $Cl^-$ efflux across apical membrane down their concentration gradient and showed activation of an apical $Cl^-$ current. Figure 5B shows that Rut-induced $I_{Cl}$ was not affected by pretreatment of tissue with DIDS (5.8 ± 0.7 µA · cm⁻²; $n = 5$), but it was significantly inhibited by pretreatment of tissues with DPC (1.3 ± 0.2 µA · cm⁻²; $n = 6$), glibenclamide (0.7 ± 0.2 µA · cm⁻²; $n = 5$), and NPPB (0.8 ± 0.4 µA · cm⁻²; $n = 5$). These data were similar to that obtained from $I_{SC}$ experiments, indicating that Rut activates a clear and detectable cAMP-dependent $Cl^-$ current in rat colonic mucosa.

$Cl^-$ secretion in polarized epithelia requires parallel activation of apical $Cl^-$ channels and basolateral K⁺ channels to provide the driving force for $Cl^-$ exit into lumen (Mall et al., 2003). To determine the putative effect of Rut on the basolateral K⁺ current, the apical membrane was permeabilized with nystatin in the presence of an apical-to-basolateral K⁺ gradient, with K⁺ as the sole of permeate ion. In the presence of a K⁺ gradient, nystatin (100 µg · ml⁻¹, mucosal) evoked a basal $I_{Cl}$ that was 235.2 ± 6.7 µA · cm⁻² ($n = 27$) in rat colon mucosal sheets (Fig. 6A), which was significantly inhibited by BaCl₂ (84.0 ± 17.1 µA · cm⁻²; $n = 6$), whereas pretreatment with ChTX (100 nM, basolateral; 219.0 ± 17.6 µA · cm⁻²; $n = 6$; 293B (30 µM, basolateral; 264.3 ± 28.5 µA · cm⁻²; $n = 8$) had no significant action on basal K⁺ current (Fig. 6B). When nystatin-evoked basal K⁺ current had reached to the semi-steady-state condition, addition of Rut (100 µM) to basolateral membrane stimulated an increase in outward current by 41.5 ± 1.6 µA · cm⁻² ($n = 34$), corresponding to activation of basolateral K⁺ channel by Rut (Fig. 6A). Rut-evoked basolateral K⁺ current was inhibited by pretreatment with BaCl₂ (0.7 ± 0.4 µA · cm⁻²; $n = 6$), 293B (7.0 ± 1.0 µA · cm⁻²; $n = 8$), and CLT (8.6 ± 1.7 µA · cm⁻²; $n = 7$), but it was barely sensitive to pretreatment with ChTX (44.7 ± 4.7 µA · cm⁻²; $n = 6$) (Fig. 6C).

**Involvement of Ca²⁺- or cAMP-Dependent Signaling Pathway in Rut-Induced Increase in $Cl^-$ Secretion.** To identify the possible involvement of Ca²⁺- or cAMP-dependent signaling pathway in Rut-induced increase in $Cl^-$ secretion, a pharmacological approach was taken. Pretreatment with the adenylyl cyclase inhibitor MDL-12,330A (50 µM, bilateral) or the cAMP-dependent PKA inhibitor H-89 (20 µM, bilateral) markedly inhibited the subsequent Rut-induced $I_{SC}$ compared with their respective control values (Table 2), whereas pretreatment with the Ca²⁺ chelator BAPTA-AM (10 µM, bilateral) or the protein kinase C inhibitor BIS (1.0 µM, bilateral) had no significant effect on the magnitude of the subsequent Rut-induced $I_{SC}$ compared with their respective control values (Table 2). Pretreatment with a sarcoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin

**Fig. 4.** Effect of Rut on PGE₂ synthesis in rat colonic mucosa. A, basolateral application of Rut for 5 min increased release of PGE₂ into the serosal chamber, but it had no effect on PGE₂ levels in the mucosal chamber. B, PGE₂ levels in rat colonic mucosa were significantly increased after stimulation with Rut for 5 min. Each column represents mean ± S.E.M. (n = 6–9). n.s., not significantly different compared with control. *, $P < 0.05$ and **, $P < 0.01$, significantly different from the control.
The intracellular cAMP level in the presence of IBMX (100 μM) increased cAMP level (1582.0 pmol H18528/μg protein) to 303.1 pmol H11005/μg protein (n = 6), which was not markedly different from that induced by Rut.

The elimination of Ca²⁺ from internal calcium stores. When IS_SC had returned to the basal level, addition of Rut to basolateral side induced a response that was not significantly different from that provided by same concentration of Rut without prior treatment with thapsigargin (Table 2). Furthermore, after the elimination of Ca²⁺ from serosal or mucosal side, Rut-induced response had no different effect from their respective control responses (serosal: control IS_SC, 65.3 ± 4.1 μA cm⁻², n = 6; Ca²⁺-free, 65.0 ± 5.3 μA cm⁻², n = 6; and mucosal: control IS_SC, 64.3 ± 3.4 μA cm⁻², n = 6; Ca²⁺-free, 69.7 ± 4.3 μA cm⁻², n = 6). These results suggest that Rut-increased Cl⁻ secretion was mainly mediated by a cAMP-dependent pathway. Intracellular Ca²⁺ seems to play a negligible role in IS_SC response to Rut.

Rut-increased Cl⁻ secretion via a cAMP-dependent pathway was further characterized by measurements of intracellular cAMP level and PKA activity in rat colonic mucosa. As shown in Fig. 7A, the intracellular cAMP level under basal condition was 67.4 ± 14.3 pmol mg⁻¹ protein (n = 7). After preincubation of Rut (100 μM), cAMP level was slightly enhanced to 123.6 ± 18.0 pmol mg⁻¹ protein (n = 7; P < 0.05). The intracellular cAMP level in the presence of IBMX (100 μM, bilateral) was 139.3 ± 18.3 pmol mg⁻¹ protein (n = 7), which was not markedly different from that induced by Rut. However, treatment of preparations with 100 μM Rut in the presence of IBMX increased cAMP level to 303.1 ± 43.3 pmol mg⁻¹ protein (n = 7). There is a significant increase in cAMP level (1582.0 ± 362.3 pmol mg⁻¹ protein) in the presence of both forskolin (1 μM) and IBMX (100 μM), which could not be further increased by Rut (1652.0 ± 326.7 pmol mg⁻¹ protein).

**TABLE 2**

<table>
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<tr>
<th>Inhibitor</th>
<th>μA cm⁻²</th>
<th>n</th>
<th>μA cm⁻²</th>
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<td>59.1 ± 4.2</td>
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<td>13.7 ± 2.6**</td>
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<tr>
<td>H-89</td>
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<td>12.7 ± 2.4**</td>
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<td>BAPTA-AM</td>
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<td>Thapsigargin</td>
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<td>64.9 ± 4.8</td>
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**P < 0.01, significantly different from the respective control (− inhibitor).**
Fig. 7. Effect of Rut on Intracellular cAMP content and PKA activity in rat colonic mucosa. A, cAMP content was accumulated after exposure to Rut (100 μM, basolateral), IBMX (100 μM, bilateral), Rut with IBMX, forskolin (Fors) (1 μM, bilateral) with IBMX, Rut with forskolin, and IBMX for 5 min. B, representative gel electrophoresis for PKA activity assay. Tissues were pretreated with either control alone or 100 μM IBMX for 5 min. B, representative gel electrophoresis for PKA activity assay. The positive and negative controls provided by the assay kit are shown in lanes 4 and 5. C, summary data showing the average PKA activities induced by Rut compared with control level. Each column represents mean ± S.E.M., n = 7 to 9. *, P < 0.05 and **, P < 0.01, significantly different from the control.

PKA activity is assayed using the PepTag nonradioactive cAMP-dependent protein kinase assay (V5340; Promega) that determines phosphorylated Kemptide, a synthetic substrate specific for PKA. As shown in Fig. 7B, phosphorylated peptide migrated toward the negative direction. Application of Rut (100 μM) to basolateral side for 5 and 10 min significantly increased PKA activity by about 21.1 and 39.5%, respectively, compared with control alone (Fig. 7C).

Rut-Induced I_{SC} Response Interacted with cAMP-Dependent Secretagogues. Experiments were performed to examine the effect of Rut on Cl− secretion in presence or absence of cAMP-dependent secretagogues. Addition of Rut to the basolateral side induced an increase in I_{SC} by 63.3 ± 2.1 μA cm⁻² (n = 6); subsequently, addition of cAMP-dependent secretagogues IBMX (100 μM) and forskolin (5 μM) in bilateral sides increased I_{SC} from 22.7 ± 2.3 to 228.7 ± 9.3 μA cm⁻² after Rut-stimulated I_{SC} returned to the basal value (Fig. 8A). In contrast, when the maximal sustained I_{SC} response was elicited by application of 100 μM IBMX and 5 μM forskolin in bilateral sides, addition of Rut (100 μM, basolateral) inhibited I_{SC} from 321.7 ± 8.8 to 295.3 ± 10.6 μA cm⁻² (n = 6) (Fig. 8B). The findings showed that pre-treating tissues with Rut reduces a maximal IBMX and forskolin-stimulated I_{SC} response. In reverse, IBMX and forskolin-stimulated maximal I_{SC} response was inhibited by subsequent addition of Rut. Similar results were observed in the experiment on basolateral K⁺ current. To determine clear effects of Rut or IBMX and forskolin on basolateral K⁺ current, Rut or IBMX and forskolin were administered in the decaying phase of nystatin response (Fig. 8, C and D).

Discussion

The present study showed that Rut stimulated a Cl− secretion (an increase in I_{SC}) in a dose-dependent pattern in rat colonic mucosa. Evidence that Rut-stimulated I_{SC} was due to Cl− secretion is based on 1) inhibition of I_{SC} by Na⁺-K⁺-2Cl⁻ cotransporter bumetanide; 2) inhibition of I_{SC} by cAMP-dependent Cl− channel blockers DPC, NPPB, and glibenclamide; and 3) decrease in I_{SC} by substitution of Cl− with gluconate ions in Parsons solution. Elimination of HCO₃⁻ in Parsons solution or addition of the carbonic anhydrase inhibitor acetazolamide failed to influence Rut-driven I_{SC}. Furthermore, Rut-driven I_{SC} was not caused by activation of electrogenic Na⁺ absorption, because it was not affected by the epithelial Na⁺ channel inhibitor amiloride.

In mammalian colonic crypt cells, Cl− secretion across apical membrane through Cl− channels is predominantly CFTR, which is a major Cl− secretory pathway that greatly contributes to regulating the amount of ions and water in the respiratory and colonic epithelia (Pilewski and Frizzell, 1999; Sheppard and Welsh, 1999). In this study, we find that Rut-induced I_{SC} is inhibited by CFTR inhibitors DPC, NPPB, and glibenclamide, whereas it is not inhibited by Ca²⁺-activated Cl− channel inhibitor DIDS. Therefore, we hypothesize that apical Cl− channels, including CFTR, may be the effective targets for Rut-mediated Cl− secretion. Activation of apical Cl− channels by directly activating CFTR at apical membrane or elevating intracellular cAMP content should be lead to the sustained electrogenic Cl− secretion (Cuthbert et al., 1999). However, Rut mainly induced a transient increase in Cl− secretion. Thus, it is doubtful that Rut-induced transient Cl− secretion is a result of activation of apical CFTR Cl− channel. So, it is necessary to identify whether Rut-induced Cl− secretion is involved in the opening of apical Cl− channels. To reach this end, we determine apical Cl− current via depolarizing the basolateral membrane with high K⁺ in the presence of a basolateral-to-apical Cl− gradient. The findings revealed that Rut induced a detectable sustained Cl− current, which was also inhibited by CFTR inhibitors DPC, NPPB, and glibenclamide, but not by Ca²⁺-dependent Cl− channel inhibitor DIDS. These observations further supported the hypothesis that the apical CFTR channel may be an effective target for Rut-mediated Cl− secretion. The data derived from the present experiment indicate that Rut mostly induced a transient Cl− secretion, whereas a detectable sustained response was only exhibited in the situation depolarized basolateral membrane with high K⁺. In general, cAMP-activated Cl− secretion, including both transient and
sustained phases, is dependent on the intracellular cAMP levels stimulated by secretagogues. In this study, we find that cAMP levels are less with Rut alone versus IBMX and forskolin (Fig. 7), indicating that Rut-generated cAMP levels could not sufficiently maintain a sustained Cl\(^-\) secretory response in contrast to the response obtained by IBMX and forskolin (Fig. 8).

It is essential that the activation of basolateral K\(^+\) channels maintains a membrane potential driving apical Cl\(^-\) secretion. Two types of K\(^+\) channels were reported to locate at the basolateral membrane of rat colon mucosa. One channel is cAMP-activated K\(^+\) channel that is predominantly KvLQT1 (Kunzelmann and Mall, 2002). Another channel is Ca\(^{2+}\)-activated K\(^+\) channel that is mainly ChTX-sensitive small-conductance Ca\(^{2+}\)-activated K\(^+\) channel (Warth et al., 1999; von Hahn et al., 2001). Under permeabilization of the apical membrane with nystatin and application of potassium gradient, Rut activated an outward K\(^+\) current, which was specifically diminished by a nonselective K\(^+\) channel blocker, Ba\(^{2+}\) ions; a KvLQT1 channel blocker, chromanol 293B; and CLT (a dual blocker of Ca\(^{2+}\)-activated and cAMP-activated K\(^+\) channels), but not by a Ca\(^{2+}\)-activated K\(^+\) channel blocker, ChTX. These observations indicate that cAMP-activated K\(^+\) channel is also a potential target for Rut in cAMP-generated Cl\(^-\) secretion.

Cl\(^-\) secretion in colonic mucosa involves the submucosal neural pathway. Pretreatment of tissues with neurotoxin tetrodotoxin inhibited both basal and Rut-stimulated \(I_{sc}\), suggesting that Rut-stimulated Cl\(^-\) secretion was mediated via the activation of submucosal neural plexus. In contrast, pretreatment of tissues with muscarinic receptor antagonist atropine or nicotinic receptor blocker hexamethonium failed to alter Rut-stimulated \(I_{sc}\), indicating that Rut-stimulated Cl\(^-\) secretion was not involved in activation of the acetylcholine-containing submucosal neurons. Further study revealed that the action of Rut to stimulate Cl\(^-\) secretion was mediated by cyclooxygenase-dependent metabolism of arachidonic acid, because indomethacin strongly inhibited Rut-induced \(I_{sc}\). It is noteworthy that Rut-induced \(I_{sc}\) response is restored by the increase in intracellular cAMP level with an adenylate cyclase stimulator forskolin in the presence of indomethacin. The similar result was reported in rat colonic mucosa treated with baicalein (Ko et al., 2002). Determination of PGE\(_2\) with enzyme-linked immunosorbent assay showed that a large part of Rut-mediated stimulation of Cl\(^-\) secretion was occurred through prostaglandins release and synthesis mostly via activation of E-prostanoid receptors. Subsequently, both the apical CFTR and basolateral cAMP-dependent K\(^+\) channels were activated (McNamara et al., 1999).
cAMP and Ca\(^{2+}\) are mostly the important intracellular second messengers in colonic mucosal Cl\(^{-}\) secretion (Cermak et al., 2002). We thus examined the roles of cAMP- and Ca\(^{2+}\)-dependent signaling pathways underlying Rut-induced Cl\(^{-}\) secretion. Rut increased the intracellular cAMP concentration and PKA activity in colonic mucosa, suggesting that Rut may stimulate Cl\(^{-}\) secretion through the cAMP-PKA signal transduction pathway. The involvement of cAMP-PKA is further confirmed by the effectiveness of the adenylate cyclase inhibitor MLD-12,330A and the PKA inhibitor H-89 in reducing the responses to Rut (Table 2). In addition, Rut fails further to enhance cAMP level after the intracellular cAMP concentration is increased to the maximal level stimulated by IBMX and forskolin, which is consistent with the evidence derived from the experiments of \(I_{SC}\) and basolateral K\(^{+}\) current in which Rut decreases rather than increases \(I_{SC}\) and K\(^{+}\) current when a mixture of IBMX and forskolin elicited \(I_{SC}\) or K\(^{+}\) current reaches to the maximal response (Fig. 8). Similar alternations had been reported in T84 cells, in which genistein and baicalein inhibited \(I_{SC}\) response when cAMP-dependent channels were maximally activated by forskolin. This inhibitory effect was considered to be due to the blockage of basolateral K\(^{+}\) channels (Diener and Hug, 1996; Illek et al., 1996; Yue et al., 2004). It has been reported that the action of genistein on CFTR gating is involved in two binding sites in Hi-5 insect cells; genistein increased channel activity that reached maximum at the concentration of 35 \(\mu\)M through decreasing the close rate, whereas it decreased channel activity at higher concentrations through reducing open rate (Wang et al., 1998). Study of CFTR channel function within a bilayer demonstrated that genistein is able to modulate gramacidin channel function, which depends on the degree of hydrophobic mismatch between the bilayer-spanning channels and the host bilayer (Hwang et al., 2003). Therefore, similar to genistein, Rut processes dual effects of both stimulation and inhibition on the overall secretory response in the colonic mucosa.

Unlike cAMP, intracellular Ca\(^{2+}\) seems to play a negligible role in the \(I_{SC}\) response to Rut. Thus, pretreatment of tissue with a cell-permeant Ca\(^{2+}\) chelator, BAPTA-AM, that can chelate intracellular free Ca\(^{2+}\); a sarcoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor, thapsigargin, that can lead to the depletion of Ca\(^{2+}\) stores; or with a PKC inhibitor, bisindolylmaleimide, did not significantly alter the \(I_{SC}\) response to Rut. In contrast to the present experimental results, Rut has been reported to diminish prostaglandin production through inhibition of arachidonic acid release in RAW264.7 macrophages (Woo et al., 2001), and it directly inhibits COX-2 enzyme activity without altering COX-2 protein and mRNA levels in bone marrow-derived mast cells (Moon et al., 1999). It is suggested that many agents seem to exert the opposite pharmacological (excitation or inhibition) profiles acting on various tissues and cells under the different physiological and pathophysiological situations. For example, the flavonol quercetin and baicalein not only stimulated Cl\(^{-}\) secretion in rat colon mucosa under steady-state conditions (Cermak et al., 1998; Yue et al., 2004) but also inhibited Cl\(^{-}\) secretion increased by forskolin in T84 cells (Schuer et al., 2005). Moreover, Rut has been reported to cause vasorelaxation via inhibiting Ca\(^{2+}\) influx and Ca\(^{2+}\) release from intracellular stores in vascular smooth muscle cells and increasing Ca\(^{2+}\) influx in endothelial cells (Wang et al., 1996). Thus, it is not surprising that Rut stimulated Cl\(^{-}\) secretion by PGE\(_2\) generation in rat colon mucosa under physiological conditions and it inhibited prostaglandin production under stimulation of inflammatory factors (Woo et al., 2001).

In summary, the evidence derived from the present study suggests that stimulation of Cl\(^{-}\) secretion by Rut was due to the production of endogenous PGE\(_2\), which in turn activated both apical Cl\(^{-}\) channel, mostly CFTR, and basolateral K\(^{+}\) channel via a cAMP-PKA-dependent mechanism in rat colonic mucosa. Activation of both channel types would concert the secretion response from colonic crypt cells and then would help to lubricate the mucosal surface layer, to flush intestinal content, and to maintain host defense. In addition, Rut could be beneficial for treatment of colonic irritants or constipation associated with physiological and psychological stress.

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