Stimulation of chloride secretion by baicalein in isolated rat distal colon

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Ko, W. H., V. W. Y. Law, W. C. Y. Yip, G. G. L. Yue, C. W. Lau, Z. Y. Chen, and Y. Huang. Stimulation of chloride secretion by baicalein in isolated rat distal colon. Am J Physiol Gastrointest Liver Physiol 282: G508–G518, 2002.—The effect of baicalein on mucosal ion transport in the rat distal colon was investigated in Ussing chambers. Mucosal addition of baicalein (1–100 μM) elicited a concentration-dependent short-circuit current (Isc) response. The increase in Isc was mainly due to Cl– secretion. The presence of mucosal indomethacin (10 μM) significantly reduced both the basal and subsequent baicalein-evoked Isc responses. The baicalein-induced Isc were inhibited by mucosal application of diphenylamine-2-carboxylic acid (100 μM) and glibenclamide (500 μM) and basolateral application of chromanol 293B (30 μM, a blocker of K,LT1 channels and Ba2+ ions (5 mM). Treatment of the colonic mucosa with baicalein elicited a threefold increase in cAMP production. Pretreating the colonic mucosa with carbachol (100 μM, serosal) but not thapsigargin (1 μM, both sides) abolished the baicalein-induced Isc. Addition of baicalein subsequent to forskolin induced a further increase in Isc. These results indicate that the baicalein evoked Cl– secretion across rat colonic mucosa, possibly via a cAMP-dependent pathway. However, the action of baicalein cannot be solely explained by its cAMP-elevating effect. Baicalein may stimulate Cl– secretion via a cAMP-independent pathway or have a direct effect on cystic fibrosis transmembrane conductance regulator.

Ussing chambers; isolated crypts; intracellular cyclic adenosine 5′-monophosphate; colonic mucosa

INTESTINAL FLUID SECRETION is a passive process driven by osmotic forces generated by ion transport. The main determinant of a luminal directed osmotic gradient is the mucosal transport of Cl– into the lumen. Intestinal Cl– secretion is an energy-dependent process that generates an electrical potential difference across the mucosal epithelium (i.e., electrogenic). Cations are drawn into the lumen by the established electrochemical gradient, and water loss is an obligatory consequence of the efflux of salt. If Cl– secretion exceeds the capacity for distal salt and water reabsorption, then diarrhea ensues (7, 8).

The mechanisms of colonic Cl– secretion are well understood. Increase in cAMP level activates Cl– secretion via the luminal cystic fibrosis transmembrane conductance regulator (CFTR) Cl– channel (12). In addition, cAMP also increases basolateral K+ conductance, probably via K,LT1-type K+ channels, which hyperpolarize the membrane and provide driving force for apical Cl– exit. This cAMP-dependent K+ conductance can be blocked by 293B (38). The involvement of Ca2+-activated Cl– channel (CaCC) in Cl– secretion remains controversial in intact enterocytes. However, increase in intracellular free Ca2+ concentration ([Ca2+]i) activates basolateral SK4-type K+ channels (37), which provide additional driving force for luminal Cl– exit through CFTR.

In Japan and China, Scutellariae radix (dried root of Scutellariae baicalensis) has been employed for centuries as an important medicine, which is used as an anti-inflammatory and smooth muscle relaxant. It contains a large amount of flavonoids such as baicalein, baicalin, and wogonin. These flavonoids are known to have multiple biological effects such as anti-inflammatory (22), antitumor (16), and antiproliferative (14) effects. Baicalein and baicalin also potentiate the contractile response in rat mesenteric artery through inhibition of nitric oxide formation and/or release in the endothelium (35). S. radix is often used in combination with Coptidis rhizoma (dried rhizome of Coptis chinensis Franch.) in traditional folk remedies. C. rhizoma has been employed for the treatment of gastroenteritis and secretory diarrhea (32). Berberine is the major active components of C. rhizoma. Recently, the pharmacological effects of berberine on colonic secretion have been extensively studied. Recent reports suggest that its antisecretory activity is due to a direct effect on colonic epithelium via a blockage of K+ channels that are responsible for K+ recycling during Cl– secretion (33). This provides a plausible mechanism to partially explain its therapeutic benefit seen in vivo. In contrast, the effect of baicalein on electrolyte transport processes across colonic mucosa has not been reported. Therefore, the aim of this study was to examine the action of...
baicalein in active ion transport across rat colonic mucosa. Results demonstrate that baicalein stimulates Cl− secretion, probably via a cAMP-dependent pathway. Therefore, in contrast to berberine, it is a secretory compound. The presence of baicalein in S. radix may limit the effectiveness of C. rhizoma in treating secretory diarrhea.

MATERIALS AND METHODS

Ussing chamber experiments. Both male and female Sprague-Dawley rats of −400 g body wt were used. They were killed by CO2 asphyxiation in accordance with a protocol approved by Animal Research Ethics Committee of our university. A segment of the distal colon (~5 cm) was removed and rinsed with ice-cold Krebs-Henseleit (KH) solution. Two pieces of distal colon were used from each animal. The muscle layers were separated from the mucosal sheet as follows. The colon was pinned with the mucosal side facing down on a dissection plate, and then the muscular layer was carefully separated from the mucosa by blunt dissection. Finally, the isolated mucosal sheet was cut into an appropriate size (area of exposed tissue 0.45 cm2) and mounted on a dissection plate, and then the muscular layer was carefully separated from the mucosa by blunt dissection. Isolated crypts were prepared by centrifugation at 200 rpm for 1 min. The isolated crypts were dispersed by treatment with 20 ml of identical KH solution on both sides of the epithelium. The solution was gassed with 95% O2 and 5% CO2 to maintain the pH at 7.4 and to provide adequate agitation. The bath temperature was kept at 37°C by water jackets. An equilibration period of 30 min was given before the experiments.

Electrodes for measuring transepithelial potential difference (PD) and passing current were connected to the chambers. The transepithelial PD was then clamped at 0 mV, and the short-circuit current (Isc) was recorded with MC6-VC4 voltage-current clamp amplifier (Physiologic Instruments, San Diego, CA) and displayed using a chart recorder (Kipp and Zonen, Delft, The Netherlands). A transepithelial PD of 1 mV was applied periodically, and the resultant change in current was used to calculate the transepithelial resistance (Rt) using Ohm’s law. The methodology for tissue preparation and measurement of electrical parameters across the mucosal sheets has been described earlier (15). During the washout treatment, the bathing solution was carefully sucked out by two syringes and replaced with normal KH solution. These maneuvers were repeated seven times. The whole procedure took <15 min.

Measurement of [Ca²⁺]i, in isolated colonic crypts. For the isolation of colonic crypts, the distal colon was exposed to a Ca²⁺-chelating solution [composition (in mM): 96 NaCl, 1.5 KCl, 10 HEPES, 27 Na EDTA, 45 sorbitol, and 28 sucrose] for 30 min at room temperature. A pellet of isolated crypts was formed by centrifugation at 200 rpm for 1 min. The isolated crypts were resuspended in the normal KH solution.

The isolated crypts bathed in normal KH solution were attached to the glass coverslips (Fisher Scientific, Pittsburgh, PA) precoated with Cell-Tak (Becton-Dickinson). The coverslip was then loaded with fura 2-AM, a Ca²⁺-sensitive fluorescent dye, by incubation (45 min, 37°C) in normal KH solution containing 3 μM dye. The coverslip was mounted in a temperature-controlled perfusion chamber (Warner Instrument) on the stage of an inverted microscope (Nikon TE300). Fura 2 fluorescence ratio (340/380 nm) was recorded by the PTI Ratio-Master fluorescence system (Photon Technology International, Lawrenceville, NJ). The cells were continuously superfused with normal KH solution. Changes in [Ca²⁺]i elicited corresponding changes in the fura 2 fluorescence ratio, and this allowed changes in [Ca²⁺], to be monitored using a standard microspectrofluorometric technique (18).

Measurement of cAMP. Cytosolic cAMP concentrations were measured by RIA. After a 30-min period of equilibration in normal KH solution at 37°C, the isolated mucosal sheet was treated with DMSO, IBMX, baicalein, forskolin with IBMX, baicalein with forskolin and IBMX, and PGE2 with IBMX. The mucosal sheets were further incubated for 2 min and then rapidly frozen in liquid nitrogen and stored at −70°C until homogenized in 0.5 ml of ice-cold 6% trichloroacetic acid using a glass homogenizer. The homogenate was centrifuged at 2,000 g for 10 min at 4°C. The supernatant was extracted three times with three volumes of diethyl ether before lyophylization. The amount of cAMP was determined by RIA with a 125I-labeled cAMP RIA kit (Amersham Pharmacia Biotech, Little Chalfont, England). The tissue residue was dissolved in 2 M NaOH, and protein content was determined using a protein assay kit (Sigma, St. Louis, MO) with bovine serum albumin as the standard. The concentration of cAMP was presented as picomoles per milligram of protein.

Solutions. The normal KH solution has the following compositions (in mM): 117 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 24.8 NaHCO3, 2.56 CaCl2, and 11.1 D-glucose. The solution was continuously bubbled with 95% O2 and 5% CO2 to maintain the pH at 7.4. Cl−-free solution was prepared by isosmotically replacing NaCl and KCl with Na gluconate and K gluconate, respectively. CaCl2 was replaced with 11 mM Ca gluconate to counteract the efflux of gluconate anion. NaHCO3 was also replaced by HEPES in the Cl− and HCO3−-free solution. When HCO3−-free solution was used, NaHCO3 was replaced with NaCl and the solution was buffered with 10 mM HEPES at pH 7.4 gassed with 100% O2.

Chemicals. The following drugs were used: baicalein, forskolin, atropine, TTX, carbachol (CCh), IBMX, indomethacin, acetazolamide, ω-conotoxin GVIA (ω-CTX), PGE2, glibenclamide, bumetanide (Sigma), diphenylamine-2-carboxylic acid (DPC), DIDS, thapsigargin (Calbiochem, La Jolla, CA), and Cell-Tak. Chromanol 293B was a generous gift from Dr. H. J. Lang (Aventis Pharma Deutschland, Frankfurt, Germany). Stock solutions of all the chemicals were dissolved in DMSO. Final DMSO concentrations never exceeded 0.1% (vol/vol). Preliminary experiments indicated that the vehicle did not alter any baseline electrophysiological parameters (data not shown).

Data analysis. For Isc measurements, positive currents are defined as those that would be carried by anions moving from the serosal to mucosal compartments and are shown as upward deflections of the traces. Changes in Isc (ΔIsc) were quantified by subtracting the current flowing at the peak of a response from its respective baseline values, which is the current flowing immediately before drug administration.

Student’s paired or unpaired t-test was used when appropriate for data analysis, and P < 0.05 was considered statistically significant. The data were expressed as means ± SE. The values of n refer to the number of experiments undertaken using different tissue preparation.

RESULTS

Effect of baicalein on baseline Isc. The basal Isc and Rt in these tissues were 43.1 ± 3.4 μA/cm2 and 106 ± 6 Ω·cm2 (n = 80), respectively. Subsequent addition of baicalein to the mucosal side induced a triphasic Isc response. As shown in Fig. 1A, 30 μM baicalein stimulated a transient increase in Isc up to 8.5 ± 1.7 μA/cm2 above baseline within one-half minute, but subsequently the Isc returned below prestimulated level with
biphasic response of baicalein (30 μM) stimulated a triphasic increase in short-circuit current (Isc) as shown in the representative trace. Dashed line indicates 0 current level. Transient current followed by a more sustained accumulation of ISc increases were used to compare between control and treated groups. The cumulative concentration-response curves of baicalein-induced ISc are shown in Fig. 1B. The apparent EC50 of baicalein is 16.7 ± 3.0 μM (Fig. 1B).

Effect of TTX and atropine on baicalein-evoked increase in ISc. To test the effect of the neuronal blocker TTX on baicalein-evoked ISc, the colon was first stimulated with 30 μM mucosal baicalein. Afterwards, the ISc was reversed back to basal level on washout of baicalein. The colon was then stimulated again with baicalein in the absence or presence of 1 μM serosal TTX (Fig. 2, A and C). Serosal addition of TTX did not significantly alter basal ISc (Fig. 2C). In control experiments (Fig. 2B), the baicalein-induced increase in ISc did not differ significantly with the second response after washout (first peak, 5.4 ± 1.7 μA/cm²; second peak, 22.5 ± 3.5 μA/cm²; after washout: first peak, 2.9 ± 1.0 μA/cm²; second peak, 33.0 ± 6.2 μA/cm²; n = 10, P > 0.05). In the presence of TTX (Fig. 2D), baicalein induced an increase of ISc (first peak, 2.8 ± 1.3 μA/cm²; second peak, 43.1 ± 13.9 μA/cm²; n = 4), which was not different from the first baicalein-induced ISc responses (first peak, 7.4 ± 1.5 μA/cm²; second peak, 31.0 ± 8.6 μA/cm²; P > 0.05). The TTX-dependent changes of ISc were compared with the second control responses (after mock washout), and there was no statistical significance (P > 0.05) between the two groups of data. In addition, the neuronal Ca2+-channel blocker ω-CTX was used. ω-CTX (0.5 μM) reduced the basal ISc by 7.2 ± 2.2 μA/cm² but did not affect the subsequent baicalein-evoked ISc increase (control: 22.7 ± 2.8 μA/cm², n = 5; after ω-CTX: 20.0 ± 2.4 μA/cm², n = 4; P > 0.05).

Atropine was used to estimate the involvement of the neurotransmitter acetylcholine on baicalein-evoked responses. Pretreatment with the muscarinic receptor antagonist atropine (1 μM, serosal) did not significantly alter basal ISc or reduce baicalein-evoked ISc responses (control ISc, 19.4 ± 4.2 μA/cm²; after atropine, 21.9 ± 2.2 μA/cm²; n = 5, P > 0.05). However, the ISc responses to serosal application of CCh (100 μM) were completely inhibited after preincubation of the colonic mucosa with 1 μM atropine (n = 5). Together,
the data suggest that the response to baicalein in the colon is not mediated by the enteric nervous system or through the neurotransmitter acetylcholine.

**Ionic basis of baicalein-evoked \( I_{sc} \).** The increase in \( I_{sc} \) could be attributed to either enhanced movement of positive charges from the luminal to the serosal side (i.e., \( \text{Na}^+ \)) or movement of negative charges into the lumen (i.e., \( \text{Cl}^- \) and/or \( \text{HCO}_3^- \)). Ion substitution experiments were conducted to determine the ionic basis for the increases in \( I_{sc} \) induced by baicalein, as previously described (19). Fig. 3A shows the concentration-response curves of baicalein when anions in the bathing media were omitted. When \( \text{Cl}^- \) in the bathing solutions was replaced by gluconate, mucosal application of baicalein (100 \( \mu \text{M} \)) only evoked a maximal increase in \( I_{sc} \) up to 6.8 ± 2.2 \( \mu \text{A/cm}^2 \) (\( n = 7 \)). The response that persisted under these conditions was concentration dependent and was not abolished when both \( \text{Cl}^- \) and \( \text{HCO}_3^- \) were removed (\( n = 6 \)) from the bathing solution (Fig. 3A). Removal of \( \text{HCO}_3^- \) from the bathing solution also did not affect the baicalein-evoked \( I_{sc} \) significantly.

As shown in Fig. 3B, blockade of endogenous production of \( \text{HCO}_3^- \) by the carbonic anhydrase inhibitor acetazolamide (90 \( \mu \text{M} \), both sides) did not affect the \( I_{sc} \) induced by baicalein, whereas subsequent application of the \( \text{Cl}^- \) channel blocker DPC completely inhibited the increase in \( I_{sc} \) (\( n = 4 \)). Acetazolamide did not alter the response when it was added before baicalein (control, 27.2 ± 4.8 \( \mu \text{A/cm}^2 \); after acetazolamide, 38.6 ± 5.1 \( \mu \text{A/cm}^2 \); \( n = 6 \), \( P > 0.05 \)). Therefore, carbonic anhydrase has minimal involvement in the secretory response of rat colon to baicalein when bathed in \( \text{HCO}_3^- \)-containing KH solution.

To test whether the baicalein-induced \( I_{sc} \) responses were due to electrogenic \( \text{Na}^+ \) absorption, 0.1 mM amiloride was used as an inhibitor of epithelial \( \text{Na}^+ \) channel (ENaC). A protocol similar to that described in Fig. 2 was employed. The responses to baicalein were
unaffected by mucosal addition of amiloride (control first peak, 8.9 ± 2.7 μA/cm²; second peak, 32.9 ± 4.5 μA/cm²; amiloride first peak, 5.0 ± 1.3 μA/cm²; second peak, 37.4 ± 4.1 μA/cm²; n = 4, P > 0.05).

The sensitivity of the baicalein-induced I_{sc} responses to different Cl⁻-channel blockers was compared. DPC blocks different types of Cl⁻ channels including the CFTR (1), whereas DIDS is more selective on Ca²⁺-activated but not cAMP-dependent Cl⁻ channels (11). Figure 4A shows that pretreating the tissue with mucosal DPC (100 μM) significantly reduced the basal I_{sc} and abolished the subsequent baicalein-induced I_{sc} responses. In contrast, the baicalein-induced I_{sc} responses were completely insensitive to DIDS (Fig. 4B). In the presence of DIDS, addition of mucosal baicalein still elicited an increase in I_{sc}, which could be completely inhibited by apical DPC (100 μM). Therefore, after the colonic mucosa was pretreated with DPC, mucosal addition of baicalein failed to evoke any secretory response (n = 4), whereas the I_{sc} responses were completely insensitive to DIDS (n = 4, as shown in Fig. 4C. Glibenclamide has previously been shown to block CFTR (27, 29). Figure 4D shows that the baicalein-evoked I_{sc} was inhibited by glibenclamide, further indicating that the luminal Cl⁻ exit pathway responsible for this I_{sc} may be CFTR. Serosal application of bumetanide (100 μM) inhibited the baicalein-evoked I_{sc} by 74% (n = 8, Fig. 4D).

In Cl⁻-free solution, mucosal addition of baicalein (300 μM) evoked only a small increase in I_{sc} (3.1 ± 1.1 μA/cm²) and DPC completely inhibited this response (n = 4). Together, the results indicate that the baicalein-induced I_{sc} increase is mainly due to transepithelial Cl⁻ secretion. It is likely that Cl⁻ exits via the luminal eAMP-dependent Cl⁻ channels (i.e., CFTR).

Involvement of prostanoid synthesis. PGs are potent stimulators of intestinal secretion (24). Therefore, the effect of PG synthesis inhibitor indomethacin on baicalein-induced I_{sc} increase was determined. Figure 5A shows that mucosal addition of indomethacin (10 μM) reduced the basal I_{sc} from 26.1 ± 3.3 to 8.9 ± 3.0 μA/cm² (n = 4). The baicalein-evoked first I_{sc} response was absent after indomethacin treatment. Indomethacin also significantly reduced the baicalein-evoked second peak increase in I_{sc} to 3.1 ± 0.7 μA/cm² from the control value of 31.7 ± 6.6 μA/cm² (P < 0.05, Fig. 5B). Indomethacin increased the R_{t} from 87.5 ± 10.2 to 99.0 ± 12.6 Ω·cm² (n = 9). The increase in R_{t} caused by indomethacin was statistically significant (P > 0.05). However, the inhibitory effect of indomethacin could be due to the fact that baicalein depends on a basal activity of CFTR, which is maintained by PGs. In Fig. 5C, after the first baicalein response was washed out, intracellular cAMP level was enhanced by the stimulator of the adenylate cyclase forskolin (0.3, 1, and 3 μM, mucosal) in the presence of indomethacin (10 μM, mucosal). After the cAMP-dependent pathway was activated, the I_{sc} was restored to the level before the first addition of baicalein. Under such conditions, 30 μM baicalein evoked an I_{sc} of 23.9 ± 4.3 μA/cm², which is not statistically significant from the first baicalein-evoked I_{sc} responses (ΔI_{sc} = 16.4 ± 4.9 μA/cm², n = 4, P > 0.05). These data indicate that the inhibitory effect of indomethacin can be overcome by elevating intracellular cAMP. Figure 6A shows that, in the presence of 10 μM indomethacin, exogenous application of PGE2 (100 μM, serosal) stimulated an increase in I_{sc} of 80.0 ± 7.7 μA/cm² (n = 9), which is not statistically different from the control responses without indomethacin (64.0 ± 15.7 μA/cm², n = 7, P > 0.05). After a maximal concentration of PGE2 (ΔI_{sc} = 45.3 ± 7.0 μA/cm²), however, the I_{sc} response to baicalein was reduced significantly from 27.2 ± 4.2 to 13.1 ± 4.8 μA/cm² (Fig. 6B, n = 5, P < 0.05). Therefore, we cannot
Similarly, baicalein-induced I\text{sc} (100 \mu M) caused a further reduction in I\text{sc} of 52.2 ± 10.7 \mu A/cm² (n = 7, Fig. 7A). Serosal addition of chromanol 293B (30 \mu M), a blocker of K\text{LQT1} channels, inhibited the I\text{sc} by 70%, indicating that forskolin activated a 293B-sensitive K⁺ channel. Similarly, baicalein-induced I\text{sc} responses were inhibited by serosal addition of chromanol 293B (Fig. 7B, n = 6) to a similar degree. Figure 7C shows that the baicalein-induced I\text{sc} was completely inhibited by the serosal addition of Ba²⁺ (5 mM, n = 9). When added before baicalein, 293B reduced the basal current by 6.4 ± 1.3 \mu A/cm². Subsequent application of baicalein only evoked an increase in I\text{sc} of 12.6 ± 3.0 \mu A/cm² (n = 6; control, 24.4 ± 2.1 \mu A/cm², n = 5, P < 0.05). In the presence of 293B, baicalein did not evoke any triphasic current response. Two of six responses were biphasic, and the rest were monophasic. On the other hand, the Ca³⁺-activated K⁺-channel blocker charybdotoxin (100 \mu M, serosal) was without effect (n = 6, data not shown). Adding Ba²⁺ (5 mM) to the serosal bath also reduced the baicalein-evoked I\text{sc} from 21.4 ± 2.1 to 5.7 ± 1.0 \mu A/cm² (n = 6). Therefore, activation of Ba²⁺- or 293B-sensitive K⁺ channels is important to maintain Cl⁻ secretion stimulated by baicalein.

Interaction of baicalein with CCh, thapsigargin, and forskolin. As shown in Fig. 8A, serosal application of the calcium secretagogue CCh (100 \mu M) to the rat colon evoked a transient fall in I\text{sc} (ΔI\text{sc} = 82.2 ± 7.2 \mu A/cm²) followed by an increase in positive I\text{sc} (ΔI\text{sc} = 145.2 ± 22.7 \mu A/cm²). Subsequent application of mucosal baicalein (30 \mu M) only evoked a small increase in I\text{sc} of 9.6 ± 5.2 \mu A/cm², whereas application of forskolin (1 \mu M, mucosal) further increased the I\text{sc} by 35.9 ± 9.4 \mu A/cm². In contrast, a maximal dose of forskolin (30 \mu M) elicited an I\text{sc} response of 65.8 ± 14.8 \mu A/cm² (Fig. 8B) and addition of baicalein (30 \mu M) subsequent to forskolin induced a further I\text{sc} of 35.0 ± 8.5 \mu A/cm² (n = 4). The increase in I\text{sc} is not statistically different from the control responses (ΔI\text{sc} = 28.6 ± 4.0 \mu A/cm², n = 4, P > 0.05). In the presence of forskolin, baicalein did not produce any triphasic current response and all the responses were monophasic. Although these results strongly suggest that baicalein and CCh share a common intracellular pathway (i.e., a rise in [Ca²⁺]), Fig. 8C shows that thapsigargin, a microsomal Ca³⁺-ATPase inhibitor, elicited a sustained increase in I\text{sc} (ΔI\text{sc} = 39.1 ± 5.1 \mu A/cm², n = 6). When the current had reached a plateau, mucosal baicalein produced an I\text{sc} response (ΔI\text{sc} = 38.3 ± 3.6 \mu A/cm) that was not significantly different from that produced by the same concentration of baicalein (ΔI\text{sc} = 34.1 ± 3.7 \mu A/cm², n = 6, P > 0.05) without prior treatment of thapsigargin. As shown in Fig. 8D, thapsigargin stimulated a sustained increase in I\text{sc} (ΔI\text{sc} = 57.9 ± 11.0 \mu A/cm²) and inhibited the subsequent CCh-evoked positive I\text{sc} by 80% (ΔI\text{sc} = 25.4 ± 8.0 \mu A/cm²). Afterwards, the I\text{sc} decayed toward its basal level, and the subsequent baicalein-evoked I\text{sc} (ΔI\text{sc} = 4.1 ± 1.3 \mu A/cm²) was inhibited (n = 7, P < 0.05) compared with baicalein-

Fig. 5. Effect of indomethacin on baicalein-evoked I\text{sc}. A: rat colonic mucosa were first challenged with mucosal baicalein (30 \mu M). After baicalein was washed out, mucosal indomethacin (Indo; 10 \mu M) caused a further reduction in I\text{sc}. Subsequently, same concentration of baicalein induced a smaller increase in I\text{sc}. This chart record is representative of 4 different sets of experiments. B: summarized data showing the effect of Indo on baicalein-evoked I\text{sc}. In the presence of Indo, baicalein only evoked a monophasic response, and the maximal increase in I\text{sc} was significantly reduced when compared with the first application of baicalein (*P < 0.05). C: a protocol similar to A was employed. After indomethacin treatment, however, the basal I\text{sc} was restored by mucosal addition of forskolin (0.3, 1, and 3 \mu M). Baicalein (30 \mu M, mucosal) was then added when the I\text{sc} had risen to a stable plateau level after administration of forskolin. This chart record is representative of 4 different sets of experiments. Dashed line indicates 0 current level. Transient current pulses were a result of clamping the potential intermittently at 1 mV.
After incubation with mucosal baicalein (30 μM), forskolin (1 μM, mucosal) and PGE₂ (10 μM, serosal), serving as positive controls, stimulated a rise in cAMP level to 11526.2 ± 949.9 (n = 5) and 11828.3 ± 1304.0 pmol/mg protein (n = 6), respectively. Baicalein cannot produce further increases in cAMP content above forskolin and IBMX. For the increases in $I_{sc}$, the effect of baicalein was potentiated by more than fivefold in the presence of IBMX ($Δ_{sc} = 114.6 ± 14.5 \mu A/cm^2$, $n = 8$ vs. control = 21.8 ± 2.5 μA/cm², $n = 5$; $P < 0.05$). Together, the data suggest that degradation of intracellular cAMP by phosphodiesterase(s) is one of the limiting factors controlling baicalein-evoked current responses.

To address whether [Ca²⁺], is involved in mediating baicalein-evoked Cl⁻ secretion, the colonic crypts from rat distal colon were isolated and loaded with the fluorescent dye fura 2-AM. As shown in Fig. 9B, addition of baicalein (30 μM) to a single colonic crypt did not evoke any discernible increase in the fluorescence 340/380 ratio. However, subsequent application of CCh (100 μM) increased the fluorescence 340/380 ratio by 0.55 ± 0.1 units ($n = 14$).

DISCUSSION

Baicalein is the major constituent and flavonoid derived from S. radix. It has a wide range of pharmacological activities such as anti-inflammatory effects (22), inhibition of platelet lipooxygenase (28), and antitumor effects on human cancer cell lines (16). Recently, it has been shown that baicalein reduces arachidonic acid liberation and PGE₂ release through inhibition of the mitogen-activated protein kinase and cytosolic phospholipase A₂ pathway in rat C6 glioma cells (26). Because S. radix- and C. rhizoma-derived compounds are frequently used together to treat diarrhea diseases in the East and PGE₂ production in the digestive tract is closely associated with the onset of diarrhea (2), it would be expected that baicalein would be an antisecretory agent.

However, the findings of this study indicate that baicalein induced a Cl⁻-dependent secretory response in rat colon in vitro. This is supported by the effect of Cl⁻ ion replacement in the bathing solutions, which greatly attenuated the $I_{sc}$. Removal of both Cl⁻ and HCO₃⁻ did not further reduce the baicalein-induced $I_{sc}$. Addition of acetazolamide, inhibitor of carbonic anhydrase, did not affect the $I_{sc}$ response to baicalein. This is in close agreement with other reports showing that carbonic anhydrase inhibitor has minimal effect on colonic transport in rat (10) and human colon (34). In addition, baicalein stimulated a predominantly bumetanide-sensitive increase in $I_{sc}$, consistent with the stimulation of transepithelial Cl⁻ secretion.

The increase in $I_{sc}$ was not due to electrogenic Na⁺ absorption, because it was not inhibited by the ENaC blocker, amiloride. In this study, the baicalein-evoked Cl⁻ secretion was completely inhibited by DPC but not DIDS, suggesting that CFTR is the likely candidate for apical Cl⁻ exit. DPC is known to have an effect on the cAMP-dependent Cl⁻ channel in various epithelia (9).
Fig. 7. Effect of K⁺-channel blockers on the forskolin- and baicalein-dependent increase in $I_{sc}$ across rat colonic mucosa. A: subsequent to forskolin-induced increase in $I_{sc}$, basolateral addition of chromanol 293B (30 μM) inhibited this $I_{sc}$ ($n = 7$). B: similar to that shown in A, the baicalein-evoked $I_{sc}$ was sensitive to basolateral addition of chromanol 293B (30 μM, $n = 6$). C: application of basolateral Ba$^{2+}$ (5 mM, $n = 9$) completely inhibited the baicalein-induced $I_{sc}$. Dashed line indicates 0 current level. Transient current pulses were a result of clamping the potential intermittently at 1 mV.

Fig. 8. A: the colonic sheet was exposed to serosal carbachol (CCh; 100 μM) followed by mucosal baicalein (30 μM) and forskolin (1 μM). This chart record is representative of 3 different sets of experiments. B: mucosal addition of forskolin (30 μM) induced a sustained increase in $I_{sc}$. The $I_{sc}$ was further increased by the addition of mucosal baicalein (30 μM). The baicalein-evoked $I_{sc}$ after forskolin is not statistically different from the control response. This chart record is representative of 4 different sets of experiments. C: addition of thapsigargin (Tg; 1 μM, both sides) induced a sustained increase in $I_{sc}$. The $I_{sc}$ was further increased by the addition of mucosal baicalein (30 μM). This chart record is representative of 6 different sets of experiments. D: after Tg-induced $I_{sc}$ increase, the colonic sheet was exposed to serosal CCh (100 μM) followed by mucosal baicalein (30 μM). This chart record is representative of 7 different sets of experiments. Dashed line indicates 0 current level. Transient current pulses were a result of clamping the potential intermittently at 1 mV.
It has been shown that up to 500 μM DIDS has no effect on the activity and conductance of CFTR (11). Therefore, the insensitivity of the baicalein responses to 500 μM DIDS (Fig. 4, B and C) is characteristic of CFTR-mediated Cl− secretion, and our data confirm that the involvement of Ca2+‐activated Cl− channels in Cl− secretion in native colonic epithelium is unlikely (12, 30). Application of CFTR blocker glibenclamide inhibited the baicalein‐evoked $I_{sc}$ by 61%, further suggesting that Cl− exit through luminal CFTR.

The activation of basolateral K+ channels is essential in driving apical Cl− exit. The baicalein‐evoked $I_{sc}$ responses were inhibited by a nonspecific K+‐channel blocker, Ba2+, and a specific blocker of the cAMP‐activated K, LQT1 K+ channel, chromanol 293B (23). Chromanol 293B was also effective in blocking forskolin‐evoked $I_{sc}$ responses. Similar findings have been reported in both human (24) and rabbit colon (23). In T84 cells (6) and rat colon (25), 293B selectively blocks basolateral cAMP‐dependent K+ conductance, leading to inhibition of forskolin‐dependent Cl− secretion. Recently, the cAMP‐activated K, LQT1 K+ channel in rat colonic epithelial cells has been cloned. The K+ current activated by forskolin is blocked by 293B (20). Although 293B inhibited a significant portion (70%) of the forskolin‐ and baicalein‐evoked $I_{sc}$, a substantial 293B‐insensitive $I_{sc}$ still remained (Fig. 7, A and B). This may be due to basal Cl− secretion driven by other basolateral K+ conductances similar to that reported in human bronchial epithelia (5). By using the $I_{sc}$ technique, it is difficult to simply use different K+‐channel blockers to delineate which class of K+ channels is activated in each phase of the baicalein responses. Further experiments using patch‐clamp recordings on isolated colonic crypts could be used to answer this question. Moreover, the biphasic response to CCh is consistent with the activation of apical K+ conductance. It is likely that CCh stimulated a rise in [Ca2+]i, and activated the luminal Ca2+-sensitive K+ channels. The transient increase in K+ secretion was then overridden by the apical Cl− conductance. Similar results have been shown in human colon, in which the $I_{sc}$, due to activation of apical K+ conductance, is masked by the parallel activation of luminal Cl− channels, resulting in lumen‐negative $I_{sc}$ (24).

In the present study, the baicalein‐induced $I_{sc}$ increase was not inhibited by the specific neuronal blocker TTX, neuronal Ca2+‐channel blocker ω‐CTX, or the muscarinic acetylcholine‐receptor antagonist atropine, suggesting that in rat, colonic acetylcholine‐containing submucosal neurons are not involved in the mediation of baicalein‐induced secretion. This is in contrast with angiotensin II‐ and substance P‐evoked Cl− secretory response, which involves submucosal cholinergic neurons (13, 21). Therefore, baicalein may act directly on epithelial or subepithelial cells in the mucosa to evoke Cl− secretion, which involves PG synthesis. The present finding that the baicalein‐evoked response was attenuated by indomethacin, a cyclooxygenase inhibitor, suggests the importance of PGs in baicalein‐evoked secretory response. Indomethacin inhibits Cl− secretion in stripped preparations of rat colon (30). In this study, indomethacin reduced the basal $I_{sc}$ by 64% and the baicalein‐evoked $I_{sc}$ by 90% compared with the control. The result suggests that there is an ongoing basal PG tone in the tissue, and the baicalein‐evoked Cl− secretion requires the PG synthesis pathway in rat colon. After addition of indomethacin, there was a fall in $R_{t}$, further indicating a role of basal PG release on Cl− secretory activity of the colon. Pretreating the mucosal layers with a maximal concentration of PGE2 reduced the $I_{sc}$ response to baicalein (Fig. 6B). Therefore, the possibility that the baicalein‐induced secretion is mediated, at least in part, by PG release cannot be excluded.

Although it is likely that the $I_{sc}$ response of the colonic mucosa to baicalein was mediated by an increase in cellular cAMP level (Fig. 9A), which then activated the apical CFTR channels and basolateral K+ conductances, not all of the data were consistent with this hypothesis. Experiments on the additivity of forskolin and baicalein on the cAMP pathway revealed that even after a maximal stimulation of cAMP‐dependent secretion by forskolin, baicalein was still able to further increase the $I_{sc}$ (Fig. 8B). Therefore, the involvement of other second messengers cannot be excluded. Moreover, the inhibitory effect of indomethacin...
on baicalein-evoked $I_{sc}$ could be overcome by increasing the cellular cAMP level by forskolin (Fig. 5C). The effect of baicalein is therefore similar to that of the Ca$^{2+}$-dependent secretagogue CCh. It has been previously reported that the effect of CCh depends on PG synthesis, because the basal activation of CFTR by cAMP is a prerequisite of Cl$^{-}$ secretion (3, 24). Moreover, in the RIA study, baicalein increased cAMP content only in the presence of IBMX. In the basal condition (control) and after a maximal stimulation of adenylate cyclase activity (IBMX and forskolin), addition of baicalein indeed reduced the cAMP levels. At the moment, we do not have an explanation for this observation. The possibility exists that multiple signaling pathways might be involved in mediating the baicalein response and thus permitting the inhibition of adenylate cyclase activity. However, this awaits further investigation. Experiments should be conducted to access whether baicalein exerts control over adenylate cyclase activity and/or phosphodiesterase(s) activity under different conditions. Nonetheless, the data further support that the $I_{sc}$ increase induced by baicalein after forskolin (Fig. 8B) was not due to a rise in cAMP levels (i.e., cAMP-independent effect). However, bear in mind that, in this study, RIA of extracted cAMP at one time point (2-min incubation) was performed. It is difficult to correlate exactly the rise in cAMP content (static accumulated levels) to the increase in $I_{sc}$, which reflects dynamic and continuous changes due to the activation of cAMP-dependent cascades. In addition, baicalein evoked a more sustained increase in $I_{sc}$ (tracings not shown) in the presence of IBMX, suggesting that degradation of cAMP by phosphodiesterase(s) is one of the limiting factors controlling baicalein-evoked current responses. To elucidate the role of Ca$^{2+}$ as a second messenger, the [Ca$^{2+}$]i of the isolated colonic crypts was measured. In contrast to the effect of CCh (Fig. 9B), baicalein did not evoke any discernible increase in [Ca$^{2+}$]i. Charybdoxin, a Ca$^{2+}$-dependent K$^{+}$-channel blocker, also produced no noticeable effect on baicalein-induced Cl$^{-}$ secretion. In addition, the inhibitory effect of CCh on baicalein-induced $I_{sc}$ appears to be independent of [Ca$^{2+}$]i. Thapsigargin, a microsomal Ca$^{2+}$-ATPase inhibitor that leads to the depletion of Ca$^{2+}$ store (31), did not affect the baicalein-induced $I_{sc}$. Thapsigargin, however, disrupted the mechanism permitting receptor-mediated control over [Ca$^{2+}$]i and inhibited the subsequent effect of CCh. The results support the notion that intracellular Ca$^{2+}$ plays a negligible role in the inhibitory effect of CCh. Several laboratories have investigated inhibitory influences of CCh in human colonic epithelial cells (T84). For example, it has been shown that the inhibitory effect of CCh is attributable to a sustained elevation in inositol tetrakisphosphate, which exerts a direct inhibitory effect on the open probability of CaCC (17, 36). However, our data do not support the existence of CaCC in the rat colon. Therefore, it is unlikely that CCh inhibited thapsigargin-evoked $I_{sc}$ increase through this mechanism. Another possibility is that CCh and baicalein may share a common and yet unidentified intracellular second-messenger pathway. Together, there is no direct evidence to suggest that the effect of baicalein is mediated through an increase in [Ca$^{2+}$]i. The mechanism underlying the inhibitory effect of CCh on baicalein-evoked $I_{sc}$ remains to be elucidated and awaits further investigation. Another possibility of a cAMP-independent action of baicalein is its direct effect on CFTR. It has been suggested that certain flavonoids such as quercetin may directly activate CFTR in rat colon independent of intracellular cAMP level (4).

In traditional remedies, C. rhizoma and S. radix are commonly used together to treat gastrointestinal diseases such as diarrhea. Recently, it has been shown that the antisecretory mechanism is due to the blocking effect of berberine, which is the major constituent of C. rhizoma, on basolateral K$^{+}$ conductance in colonic epithelia (33). It is interesting that baicalein, in contrast to berberine, is a prosecretory compound. Although the presence of baicalein may counteract and limit the effectiveness of berberine in the treatment of diarrhea, baicalein may have modulatory effects on the antisecretory action of berberine-containing herbs. This may be important to maintain a basal Cl$^{-}$ secretion for lubrication of the mucosal surface layer and for the flushing of intestinal contents during host defense against microbial invasions or artificial irritants.

In summary, the present study has demonstrated that Cl$^{-}$ secretion across the rat colonic mucosa could be stimulated by mucosal baicalein. Mechanisms involve luminal cAMP-dependent Cl$^{-}$ channels and serosal 293B-sensitive K$^{+}$ channels. However, the action of baicalein cannot be solely explained by its cAMP-elevating effect. Baicalein may stimulate Cl$^{-}$ secretion via a cAMP-independent pathway or have a direct effect on CFTR.

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REFERENCES


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