Involvement of Endogenous Prostaglandin in Emodin-Evoked Rat Colonic Anion Secretion

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It has been reported that emodin is able to promote gastrointestinal motility and stimulate large intestinal water secretion; however, the mechanism is still not clear. The aim of the present study is to examine the effects of emodin on the rat colonic transepithelial ion transport and the underlying mechanism. The study was carried out by means of the short circuit current (I_{SC}) recording. Basolateral application of emodin induced a concentration-dependent I_{SC} increase, and the EC_{50} was $76.0 \mu\text{mol/l}$. Pretreatment with epithelial Na^+ channel blocker, amiloride ($10 \mu\text{mol/l}$), did not affect the I_{SC} responses elicited by emodin, but removal of extracellular Cl^- or apical pretreatment with Cl^- channel blocker, glibenclamide (1 mmol/l) inhibited emodin-elicited I_{SC} responses by 76.3% and 83.8% respectively. Inhibiting basolateral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter (NKCC) with bumetanide ($100 \mu\text{mol/l}$) decreased emodin-induced I_{SC} from $118.1 \pm 6.7 \mu\text{A/cm}^2$ to $16.7 \pm 2.0 \mu\text{A/cm}^2$, which was reduced by 85.9%. Basolateral pretreatment with neuronal Na^+ channel blocker tetrodotoxin (TTX) ($1 \mu\text{mol/l}$) did not affect emodin-induced I_{SC} increase, but pretreatment with indomethacin ($10 \mu\text{mol/l}$) alone or with both TTX and indomethacin significantly decreased emodin-induced I_{SC} increase by 88.4 and 81.2%, respectively. The present study demonstrated that emodin was able to stimulate rat colonic epithelial Cl^- secretion, which was predominantly mediated by endogenous prostaglandin release.

Key words emodin; colon; ion secretion; short-circuit current

The mammalian colon is the final station of the gastrointestinal (GI) tract, where the organism has the last opportunity to modify the electrolyte contents in the feces with balanced absorptive and secretory activities. Colonic epithelium, a typical electrolyte-transporting epithelium, lines the surface of both crypts and villi, and plays an important role in the maintenance of water and electrolyte balance.

Anthraquinones are present in the roots, bark or leaves of numerous plants such as senna, cascara, aloe, frangula and rhubarb.¹⁾ 1,3,8-Trihydroxy-6-methylanthraquinone (*rhubarb-emodin*; emodin, for shortened form), an anthraquinone derivative from rhubarb (*Rheum tanguticum* MAXIM. ex. BALF.), plays an important role in brain protection against disturbances induced by severe cerebral injury,^{2,3)} antiviral activity against enveloped viruses⁴⁾ and GI motility.⁵⁾ Emodin has been reported to be able to trigger acetylcholine release and cause muscle contraction by binding with muscarinic receptors.^{6,7)} The cellular signaling mechanism underlying emodin action has been reported to increase intracellular $[\text{Ca}^{2+}]$ of gastrointestinal smooth muscle cells,^{8,9)} inhibition of the activation of p38 MAPK pathway and downregulation of the expression of fibronectin of renal dysfunction in diabetic nephropathy rats,¹⁰⁾ and vasorelaxant effect due to cGMP accumulation.⁷⁾ Aloe-emodin-9-anthrone (AE-anthrone), produced from barbaloin, caused not only an increase in the intestinal water content but also mucus secretion in the colon at its 1/2 dose.¹¹⁾ Further, synergistic purgative effect of aloe-emodin anthrone and rhein anthrone in mice was resulted from synergistic stimulation of large intestinal transit and large intestinal water secretion,¹²⁾ emodin, as an anthraquinone derivative from rhubarb, was demonstrated to tend to enhance the purgative activity in mice.¹³⁾ However, the effect of the emodin on anion secretion and its underlying mechanism has not been illuminated.

Fluid secretion plays a key role in intestinal physiology. Under normal conditions, the intestine carries out the absorp-

tion of luminal fluid, electrolytes and nutrients. A small amount of basal secretion facilitates hydration of the intestinal mucosa and mixing of intestinal contents. Fluid secretion is driven by active Cl^- transport from the basolateral to the apical side of enterocytes.

Secretagogue-induced GI Cl^- secretion is important for the lubrication of intestinal contents during regular bowel movements or the flushing of microbial organisms or artificial irritants in host defense responses¹⁴⁾; epithelial Cl^- channels, especially the cAMP-activated Cl^- channels, play an important role in regulating and maintaining the normal physiological functions of the GI tract. The abnormal regulation of Cl^- channels may result in diarrhea or constipation.¹⁵⁾ Therefore Cl^- channels are very likely to be the target for pharmacological intervention. Using rat colonic epithelium and the short-circuit current technique, we undertook the present study to examine whether emodin exerted any effect on Cl^- secretion and to investigate the underlying cellular signaling mechanism.

MATERIALS AND METHODS

Emodin Extraction and Identification The root of *Polygonum multiflorum* Rhubarb was purchased from Beijing-tongrentang, Beijing, China, and authenticated by Dr. Wen Wang. The air-dried root material was powdered and extracted with 80% ethanol. The combined solution was concentrated to afford a residue. After dissolving the residue in water, the aqueous solution was extracted with *n*-butanol. The extract was concentrated and chromatographed on a silica gel column using petroleum ether, EtOAc (10:1—0:10), to give nine fractions. Fraction 3 was re-chromatographed on a silica gel column using petroleum ether, EtOAc (7:1) as eluent to afford the product as yellow needles, which was identified by comparing its data with those of an authentic sample. Its purity, determined by HPLC method, was 98.1%.

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Fig. 1. (a) Structure of the Emodin, (b) Standard Sample by HPLC and (c) Purified Emodin by HPLC

HPLC conditions: An YMC C18 column, a mixture of methanol 0.1% phosphoric acid (85:15) as mobile phase, and UV detection at 254 nm.

Materials Hank's balanced salt solution (HBSS), glibenclamide, amiloride hydrochloride, bumetanide, indomethacin, tetrodotoxin (TTX) were from Sigma (St. Louis, MO, U.S.A.). And stock solutions of some chemicals (glibenclamide, indomethacin, TTX, and bumetanide) were prepared in dimethylsulphoxide (DMSO). Final concentrations of DMSO never exceeded 0.1% (v/v). Preliminary experiments indicated that the vehicle did not alter any baseline electrophysiological parameters.

Solutions Krebs–Henseit (K–H) solution had the following composition (mM): NaCl, 117; KCl, 4.5; CaCl₂, 2.5; MgCl₂, 1.2; NaHCO₃, 24.8; KH₂PO₄, 1.2; glucose, 11.1. In Cl[−]-free solution, NaCl, KCl and CaCl₂ were replaced by sodium gluconate, potassium gluconate and calcium gluconate, respectively. The solution was gassed with 95% O₂–5% CO₂, pH 7.4, and temperature at 37 °C.

Tissue Preparation Animal protocols followed guidelines established by the NIH and were approved by Animal Care and Use Committee, Capital Medical University. Adult male Sprague-Dawley rats (Laboratory Animal Services Center, Capital Medical University) ranging in weight from 200 to 300 g had free access to standard rodent laboratory food and water until the day of the experiments. The animals were killed by cervical dislocation. The distal colon was removed and defined as the *ca.* 7 cm long segment proximal to the lymph node (typically situated 3 cm apart from the anus). Then the distal colon was divided into 4 segments, which were cut along the mesenteric border into a flat sheet and flushed with ice-cold Krebs–Henseit solution (K–HS). The tissue was pinned flat with the mucosal side down in a Sylgard-lined petri dish containing ice-cold oxygenated solution. The serosa, submucosa, and muscular layer were stripped away with fine forceps to obtain a mucosa preparation.

Short-Circuit Current Measurement The short-circuit current was measured *in vitro* in Ussing chambers. Flat sheet of colonic mucosa preparations were mounted between two halves of modified Ussing chambers, in which the total cross-sectional area was 0.5 cm². The mucosal and serosal surfaces of tissue were bathed with 5 ml K–HS by recirculation from a reservoir maintained at 37 °C during the experiments. The K–HS was bubbled with 95% O₂ and 5% CO₂ to maintain the pH of the solution at 7.4. Drugs could be added directly to the apical or basolateral side of epithelium. Responses were continuously recorded by computer. Transepithelial potential difference for every colonic mucosa was

measured by the Ag/AgCl reference electrodes (Physiologic Instruments, P2020S) connected to a preamplifier that was in turn connected to a voltage-clamp amplifier VCC MC6 (Physiologic Instruments). The change in I_{SC} was calculated on the basis of the value before and after stimulation and was normalized as current per unit area of epithelial ($\mu A \cdot cm^{-2}$), which allowed the curve area for 15 min to be calculated ($\mu A \cdot min$). The change in current in response to the applied potential was used to calculate the transepithelial resistance of the monolayer by Ohm's Law. Experiments were normally repeated in different batches of colon mucosa to ensure that the data were reproducible.

Statistics The results were given as arithmetic means $\pm$ standard error of the mean (S.E.M.); “*n*” refers to the number of rats. EC₅₀ was calculated using the GraphPad Prism software 4.0 package. Statistical analyses were performed by Student's paired or unpaired *t*-test. “*p*” values less than 0.05 were assumed to denote a significant difference.

RESULTS

Structure of emodin was shown in Fig. 1a and its purity, determined by HPLC, was 98.1% (Fig. 1c) compared with a standard sample (Fig. 1b).

Emodin-Induced I_{SC} Responses in Different Concentrations After an equilibration time of 30 min, emodin was added to the apical (mucosal) side or serosal (basolateral) side to investigate its effect on the transepithelial ion transport (I_{SC}) in freshly isolated rat colonic mucosa. The results indicated that addition of emodin into the mucosal side produced I_{SC} increase was much smaller than that of application of it into the basolateral side at the same higher dose (100 $\mu mol/l$) (Fig. 2a), suggesting that the effect of emodin on the colonic ion transport might be first acted on the serosal side but not luminal side. The following results therefore came from basolateral addition of emodin. The basolateral emodin induced I_{SC} response was concentration-dependent (Fig. 2b). When the dose of emodin was 1.0, 10, 100, 500 and 1000 $\mu mol/l$, the I_{SC} increase evoked by emodin was $17.8 \pm 3.7 \mu A/cm^2$ ($n=4$), $52.5 \pm 3.5 \mu A/cm^2$ ($n=7$), $124.9 \pm 12.0 \mu A/cm^2$ ($n=10$), $169.0 \pm 21.4 \mu A/cm^2$ ($n=12$) and $251.8 \pm 36.2 \mu A/cm^2$ ($n=9$), respectively, and the total charge transferred for 15 min was $132.2 \pm 64.4 \mu A \cdot min$ ($n=6$), $597.9 \pm 86.3 \mu A \cdot min$ ($n=7$), $1233.7 \pm 68.7 \mu A \cdot min$ ($n=4$), $1522.0 \pm 101.0 \mu A \cdot min$ ($n=4$) and $1734.7 \pm 101.2 \mu A \cdot min$ ($n=6$), respectively, with an apparent EC₅₀ of about 76.0 $\mu mol/l$ (Fig. 2c). The transepithelial resistance wasn't significantly affected when the concentration of emodin was lower (1, 10, 100 $\mu mol/l$), but when the dose of emodin was

Fig. 2. Concentration-Response Curve for Emodin-Induced I_{sc} Response

(a) The I_{sc} responses evoked by luminal and basolateral addition of emodin (100 $\mu\text{mol/l}$). (b) Representative I_{sc} recordings in response ($\mu\text{A}/\text{cm}^2$) to basolateral addition of emodin (1, 10, 100, 500, 1000 $\mu\text{mol/l}$). Arrowheads indicate the time of emodin addition. (c) The concentration-response curve for emodin-induced total charge transferred in 15 min ($\mu\text{A} \cdot \text{min}$). Each data point was obtained from at least 4 individual experiments. (d) The effect of different concentration of emodin on the transepithelial resistance. Higher dosage of emodin (500, 1000 $\mu\text{mol/l}$) significantly reduced colonic transepithelial resistance. Values are means $\pm$ S.E.M.; * $p < 0.05$, ** $p < 0.01$.

Fig. 3. Effect of Cl^- Replacement and Transporter Inhibitors on the Emodin-Induced I_{sc} Response

(a) Representative I_{sc} recording with arrows indicating the response to emodin (100 μmol) in normal and Cl^- -free K-H solutions; as well as in the pretreatment with Na^+ channel blocker, amiloride (10 $\mu\text{mol/l}$). (b) Summary of the effects of removal of extracellular Cl^- and apical addition of amiloride (10 $\mu\text{mol/l}$) on the basolateral application of emodin-induced I_{sc} response. Values are mean $\pm$ S.E.M.; *** $p < 0.001$.

heightened to 500 $\mu\text{mol/l}$ and 1.0 mmol/l, the resistance was significantly reduced to $26.3 \pm 2.5 \Omega \cdot \text{cm}^2$ from 41.2 ± 4.4 ($n=6$, $p < 0.05$), and to $23.4 \pm 2.3 \Omega \cdot \text{cm}^2$ from 44.5 ± 5.4 ($n=6$, $p < 0.01$) (Fig. 2d), respectively.

Anion Dependence of Emodin-Induced I_{sc} In order to study the ion species involved in mediating the emodin-induced I_{sc} , Cl^- was removed from the bathing solution. The emodin (100 $\mu\text{mol/l}$)-induced response was significantly decreased by Cl^- removal (Fig. 3a), from $119.5 \pm 7.9 \mu\text{A}/\text{cm}^2$ ($n=4$) to $28.4 \pm 6.54 \mu\text{A}/\text{cm}^2$, about 76.3% reduction ($n=8$, $p < 0.001$, Fig. 3b), indicating a Cl^- dependence of the emodin-induced current. The effect of Na^+ channel blocker, amiloride, and Cl^- channel blocker, glibenclamide, on the emodin-induced I_{sc} response was also examined. The apical addition of amiloride (10 mM) was found to have no effect on

emodin (100 $\mu\text{mol/l}$)-induced I_{sc} ($n=6$, $p < 0.05$, Fig. 3). Apical-applied glibenclamide (1 mM) abolished the emodin-induced I_{sc} response by 83.8%, from $150.8 \pm 14.6 \mu\text{A}/\text{cm}^2$ to $24.4 \pm 3.9 \mu\text{A}/\text{cm}^2$ ($n=6$, $p < 0.001$). Bumetanide (100 mM), a strong inhibitor of the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter, added to the basolateral side, resulted in a more than 85.9% reduction, from $118.1 \pm 6.7 \mu\text{A}/\text{cm}^2$ to $16.7 \pm 2.0 \mu\text{A}/\text{cm}^2$ ($n=7$, $p < 0.001$) in the I_{sc} induced by emodin (100 $\mu\text{mol/l}$) as compared to control (Figs. 4a, b).

Involvement of Prostaglandin-Dependent Pathway As shown in Fig. 5b, basolateral application of TTX (1 $\mu\text{mol/l}$), a neuronal Na^+ channel blocker, had no effect on the emodin-induced increase in I_{sc} , from $111.4 \pm 11.8 \mu\text{A}/\text{cm}^2$ to $121.5 \pm 6.2 \mu\text{A}/\text{cm}^2$ ($n=4$, $p > 0.05$). However, basolateral pretreatment with indomethacin (10 $\mu\text{mol/l}$), a cyclo-oxyge-

Fig. 4. Effect of CFTR Channel Inhibitors and $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ Cotransporter Inhibitors

(a) Representative I_{sc} recording with arrows indicating the time for the apical application of glibenclamide (1 mmol/l) or basolateral application of bumetanide (100 $\mu\text{mol}/\text{l}$), respectively, and basolateral addition of emodin (100 $\mu\text{mol}/\text{l}$). (b) Summary of the effects of glibenclamide (1 mmol/l, apical), bumetanide (100 $\mu\text{mol}/\text{l}$, basolateral) on emodin-induced ΔI_{sc} . Values are means $\pm$ S.E.M.; *** $p < 0.001$.

Fig. 5. Effect of a Cyclo-Oxygenase (COX) and Neuronal Na^+ Channel on the Emodin-Induced ΔI_{sc}

(a) Representative I_{sc} recording with arrows indicating the time for the basolateral application of indomethacin (10 $\mu\text{mol}/\text{l}$), a cyclo-oxygenase (COX) inhibitor, TTX (1 $\mu\text{mol}/\text{l}$), a neuronal Na^+ channel blocker as well as emodin (100 $\mu\text{mol}/\text{l}$). (b) Summary of the effects of indomethacin, TTX (1 $\mu\text{mol}/\text{l}$) alone, and both TTX and indomethacin. Values are means $\pm$ S.E.M.; *** $p < 0.001$.

nase (COX) inhibitor, significantly decreased the emodin-induced I_{sc} response by more than 88.4% (Fig. 5a), from $155.2 \pm 19.7 \mu\text{A}/\text{cm}^2$ ($n=12$) to $18.1 \pm 3.8 \mu\text{A}/\text{cm}^2$ ($n=6$, $p < 0.001$, Fig. 5b) which was similar to that pretreatment with both TTX and indomethacin, emodin-induced I_{sc} response was reduced by 81.2% (Fig. 5a), from $110.1 \pm 13.6 \mu\text{A}/\text{cm}^2$ ($n=10$) to $20.7 \pm 2.8 \mu\text{A}/\text{cm}^2$ ($n=7$, $p < 0.001$, Fig. 5b).

DISCUSSION

Pharmaceutical purgative preparations contained from *Rheum palmatum* have been widely used in China for hundreds of years to treat gastrointestinal disorders.¹⁶⁾ Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an anthraquinone derivative isolated from *Rheum palmatum*.¹⁷⁾ The reported biological effects of emodin include anti-tumor, antibacterial, and anti-inflammatory actions.^{18–20)} Apart from its beneficial effects on overall body functions, emodin has also been described in the improvement of digestion and gastrointestinal (GI) function.⁵⁾ It is reported that emodin is able to enhance the purgative action by injected directly into the caecum.¹³⁾ The present study further demonstrated the stimulatory effect of emodin on the colonic epithelial Cl^- secretion. Epithelial Cl^- channels have been known to play an important role in regulating and maintaining the normal physiolog-

ical functions of the GI tract.²¹⁾ In this epithelium, Cl^- secretion is mediated by two steps, *i.e.*, the accumulation of cytosolic Cl^- by $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporters in the basolateral membrane and then the exit of Cl^- through Cl^- channels in the apical membrane.^{22,23)} The supporting evidence for the stimulatory effect of emodin on Cl^- secretion includes (1) emodin-induced I_{sc} increase was insensitive to the Na^+ channel blocker; amiloride, but sensitive to Cl^- channel blocker, glibenclamide, and removal of extracellular Cl^- ; (2) the response was inhibited by the inhibitor of $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporters, bumetanide. Thus a stimulation of Cl^- secretion by emodin is readily confirmed. Furthermore, since Cl^- secretion requires both the basolateral accumulation of Cl^- by $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter and apical exit through Cl^- channels, at this point basolateral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter would be the primary target of emodin since apical, but not basolateral, addition of emodin couldn't evoke an evident I_{sc} increase. However, the current results did provide evidence for the activation of apical Cl^- channels, since apical addition of emodin decreased membrane resistance. The present study has also suggested that emodin-induced Cl^- secretion is mediated by endogenous prostaglandin, since pretreatment of the tissue with cyclo-oxygenase (COX) inhibitor, indomethacin (10 $\mu\text{mol}/\text{l}$) blocked emodin-induced I_{sc} increase by more than 88.4%. Taken together, the present results have demonstrated that emodin has a stimulatory ef-

fect on gastrointestinal Cl^- secretion by predominantly activating basolateral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter and apical Cl^- channels. The ability of emodin stimulating Cl^- secretion may contribute to its beneficial effects on GI tract, such as smoothing bowel movement and enhancing fluid clearance during host defense response. Nevertheless, the current study has established a model for the quantitative measurement of emodin effect on the GI tract and for further screening of its possible underlying mechanism. The differential sensitivity of the emodin-induced current response to bumetanide, or glibenclamide, and the reduction of the current by COX inhibitor, indomethacin, raised the possibility that emodin may activate cAMP-dependent Cl^- channel, CFTR (cystic fibrosis transmembrane conductance regulator). It would be worthwhile to further explore the therapeutic potential of emodin for the clinic gastrointestinal disorders.

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