Barakol Extracted from *Cassia siamea* Stimulates Chloride Secretion in Rat Colon

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**ABSTRACT**

Barakol is a purified extract of *Cassia siamea*, a plant that has been used as a laxative in traditional medicine. In this study, the effect of barakol on anion transport across the rat colon epithelium was investigated. Colonic epithelium was mounted in Ussing chambers and bathed with Ringer’s solution. Addition of 1 mM barakol to the basolateral solution produced a slow increase in short-circuit current (Isc) in proximal colon and distal colon by 24.5 ± 2.2 and 24.2 ± 1.4 μA/cm², respectively. Barakol increased Isc in a concentration-dependent manner with an EC₅₀ value of 0.4 mM. The barakol-stimulated increase in Isc was inhibited by subsequent treatment with 500 μM diphenylamine-2-carboxylic acid or 400 μM glibenclamide added to the apical solution and 200 μM bumetanide added to the basolateral solution. Pretreatment of the tissues with 200 μM bumetanide, but not 10 μM amiloride, completely abolished the barakol-increased Isc. Ion substitution experiments showed an inhibition of barakol-stimulated Isc in chloride-free solution but not in bicarbonate-free solution. In addition, pretreatment of tissues with 10 μM tetrodotoxin or 10 μM indo-methacin, but not 1 μM atropine or 10 μM hexamethonium, partially inhibited the Isc response by barakol. The present results demonstrated the stimulatory effect of barakol on the bumetanide-sensitive chloride secretion in rat colon. The effect of barakol was partly mediated by the stimulation of submucosal nerves and through the release of cyclooxygenase metabolites. These findings thus provide an explanation for the underlying mechanism of barakol as a secretagogue in mammalian colon.

Barakol is a biologically active compound extracted from leaves and flowers of *Cassia siamea*, a plant that has been traditionally used for the treatment of fever, skin disease, constipation, diabetes, hypertension, and insomnia (Kinhorn and Balandrin, 1992). It was originally extracted by Hassanali-Walji et al. in 1969. Its chemical structure was identified as 3α,4-dihydro-3α,8-dihydroxy-2,5-dimethyl-1,4-dioxaphenalene (C₁₃H₁₂O₄) or 2,5-dimethyl-3αH-pyrano [2,3,4-de]-1-benzopyran-3α,8-diol (Fig. 1), and a proposed synthetic procedure was described in 1970 (Bycroft et al., 1970). In animal model studies, barakol has been shown to possess hypotensive activity (Suwan et al., 1992) and serotonergic receptor antagonist activity (Tongroach et al., 1992), and it appears to function as an anxiolytic in exploratory behavioral activities (Thongsaard et al., 1996). Further in vitro studies in the central nervous system have found that barakol inhibits K⁺-stimulated dopamine release from striatal slices of rat brain (Thongsaard et al., 1997). Recent studies in our laboratory have shown that barakol increases smooth muscle contraction in the isolated rat ileum under basal conditions and during electrical field stimulation. In these studies, barakol was found to inhibit norepinephrine-suppressed smooth muscle contraction, suggesting a role for barakol as a prokinetic drug (Poonyachoti et al., 2002).

The colonic epithelium plays an essential role in the absorption and secretion of water and electrolytes. Its transport function is regulated by a variety of neurotransmitters, hormones, and inflammatory mediators. Under basal conditions, the colonic epithelium absorbs fluid from the lumen into the circulation, and this process is driven by energy-dependent Na⁺ transport. In contrast, colonic secretion is activated by secretagogues that enhance Cl⁻ transport mechanisms,
which in turn create electrochemical and osmotic driving forces for passive cation and water movement into the lumen (Kunzelmann and Mall, 2002). Decreased secretory function or increased fluid absorption by the colon is typically associated with constipation. Since *Cassia siamea* has been used as a laxative, we hypothesized that its active ingredient, barakol, may exert a laxative effect by stimulating chloride secretion and/or inhibiting NaCl absorption across the colonic epithelium. Therefore, the aim of the present study was to investigate the effect of barakol on ion transport across the rat colon epithelium and to determine the mechanisms involved in barakol action.

**Materials and Methods**

**Plant Extraction.** Barakol was extracted and purified from *Cassia siamea* by a method modified in our laboratory (Thongsarda et al., 2001). Briefly, fresh young leaves and flowers of *Cassia siamea* were obtained from Ladkrabang, Bangkok, Thailand. The herbarium specimens were authenticated, deposited, and given the voucher specimen number A001432 by the Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. They were cut into small pieces and boiled in 0.5% sulfuric acid for 30 min. The mixture was blended, filtered, and alkalized with concentrated NaHCO₃ and then extracted with chloroform. The chloroform extract was further concentrated with 5% acetic acid and neutralized with 25% ammonium hydroxide. The crude barakol was obtained as greenish crystallized yellow needles with a 0.3% yield. Concentrated hydrochloric acid was finally added to obtain barakol hydrochloride, and the mixture was dried by vacuum filtration to form yellowish crystallized anhydrobarakol hydrochloride. The compound was shown to be a single chemical using thin-layer chromatography on silica gel, and the identification was confirmed by nuclear magnetic resonance. Barakol was dissolved in distilled water immediately before testing its activity. When anhydrobarakol hydrochloride is dissolved in water, the reaction is reversed, and the product used in the anion replacement compartments or movement of cations from the mucosal to serosal compartments or a combination of both. In the anion replacement experiments, gluconate salts were substituted for chloride, and 20 mM HEPES buffer was substituted for HCO₃⁻. Experiments under HCO₃⁻-free and Cl⁻-HCO₃⁻-free conditions were performed in the presence of 100 μM acetazolamide and bubbled with 100% O₂.

**Chemicals.** Tetrodotoxin, atropine sulfate, hexamethonium, amiloride, glibenclamide, diphenylamine-2-carboxylic acid (DPC), 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS), bumetanide, dihydropyridine, indomethacin, acetazolamide, and high-purity grade salts were obtained from Sigma-Aldrich (St. Louis, MO). All chemicals were made in aliquots and kept at −20°C before use. Some chemicals were dissolved in dimethyl sulfoxide, the final dimethyl sulfoxide concentration of which was less than 0.1% (v/v).

**Animals and Tissue Preparation.** Male Wistar rats (250–300 g) were obtained from National Animal Center, Mahidol University, Bangkok, Thailand. They were housed in stainless steel cages in a room with a 12-h light/dark cycle and allowed free access to food and water. All animals were taken care of in accordance with the National Guiding Principles for Biomedical Research Involving Animals provided by the National Research Council of Thailand.

For the experiment, rats were sacrificed with a small animal decapitator (Harvard, Kent, UK). After a laparotomy incision, the whole colon was removed, rinsed, and placed in an ice-cold oxygenated Ringer’s solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 0.5 mM MgCl₂, 25 mM NaHCO₃, 1.0 mM NaH₂PO₄, and 11 mM D-glucose; pH 7.4). The colon was longitudinally cut close to the mesentery, and the serosal muscle layers were carefully stripped away by blunt dissection to obtain a mucosa-submucosal preparation. The appearance of palm-like foldings was used to distinguish between the distal and proximal colon.

**Measurement of Electrical Parameters.** The mucosa-submucosal preparation was mounted in Ussing chambers (0.62 cm²) bathed on the apical and basolateral sides with identical Ringer’s solutions at 37°C and gassed with 95% O₂ and 5% CO₂. Transepithelial potential difference (PD) and short-circuit current (Isc) were measured with the use of an EVC-4000 voltage-clamp amplifier (World Precision Instruments, Inc., Sarasota, FL), using Ag/AgCl₂ electrodes connected to the bathing solution via agar bridges. Tissue conductance (G) was calculated using Ohm’s law (G = Isc/PD). The tissues were continuously short-circuited, except for a brief interval of open-circuited readings before and after adding any chemicals. The data from the voltage clamp was connected to a MacLab 48 A/D converter and recorded with a 400-MHz PowerPC Macintosh. After mounting, the tissues were equilibrated for at least 30 min to achieve a stable Isc before the addition of chemicals. Positive Isc corresponded to the movement of anions from the serosal to mucosal compartments or movement of cations from the mucosal to serosal compartments or a combination of both. In the anion replacement experiments, gluconate salts were substituted for chloride, and 20 mM HEPES buffer was substituted for HCO₃⁻. Experiments under HCO₃⁻-free and Cl⁻-HCO₃⁻-free conditions were performed in the presence of 100 μM acetazolamide and bubbled with 100% O₂.

**Data Analyses.** All values were expressed as means and standard error of mean (S.E.M.); n was the number of different tissue preparations, and N was the number of animals in each experiment. The increase in Isc was quantified by subtracting the peak of an Isc response from its respective baseline value before drug administration. The statistical differences between control and treatment means were analyzed using Student’s paired or unpaired t test when appropriate. The differences among groups were analyzed using a one way analysis of variance followed by Dunnett’s multiple comparison. A p value of less than 0.05 was considered statistically significant.

**Results**

Under basal conditions, after an equilibration period of 30 to 45 min, the distal colon showed average Isc, PD (lumen negative), and G values of 35.5 ± 2.4 μA/cm², −3.7 ± 0.3 mV, and 11.0 ± 0.8 pS/cm² (n = 44 tissues, N = 17 rats), respectively. The proximal colon exhibited average Isc, PD, and G values of 39.6 ± 5.1 μA/cm², −2.7 ± 0.3 mV, and 15.3 ± 1.5 pS/cm² (n = 17 tissues, N = 12 rats), respectively.

**Effect of Barakol on Isc.** Addition of 1 mM barakol to the basolateral solution produced a slow increase in Isc, which peaked in 5 to 10 min and was sustained for 30 to 45 min (Fig. 2A) before it gradually returned to the baseline level. The maximal increase in Isc produced by barakol was 24.4 ± 2.0 μA/cm² (n = 33 tissues, N = 18 rats) in distal colon and 24.0 ± 4.6 μA/cm² (n = 10 tissues, N = 7 rats) in proximal colon. The tissue conductance at the maximal barakol response was not different from its corresponding baseline value (control, 13.3 ± 1.0 pS/cm²; after barakol, 12.8 ± 1.0 pS/cm², n = 35 tissues, N = 18 rats). Addition of 0.01 M
hydrochloric acid in equal volume to barakol solution (200 μl) did not alter any baseline electrophysiological parameters. Since the tracings and maximal Isc responses to barakol in the distal and proximal colon were not different, the rest of the experiments were performed using distal colon unless otherwise stated. Barakol (10 μM–3 mM) induced a cumulative concentration-dependent increase in Isc with an apparent EC₅₀ value of 0.4 mM (n = 5 tissues, N = 5 rats; Fig. 2B).

The barakol-induced increase in Isc was completely inhibited by the Cl⁻ channel blocker DPC (500 μM; n = 4 tissues, N = 3 rats; Fig. 3A) and partially inhibited by glibenclamide (400 μM) added to the apical solution. Glibenclamide produced a 52.6 ± 11.0% decrease in barakol-stimulated Isc (n = 5 tissues, N = 4 rat; Fig. 3B). In contrast, the barakol-stimulated increase in Isc was not affected by a subsequent addition of DIDS to the apical solution (n = 6 tissues, N = 4 rats).

Baseline addition of bumetanide (200 μM), a Na⁺-K⁺-2Cl⁻ cotransporter inhibitor, completely suppressed the barakol-stimulated Isc (n = 5 tissues, N = 4 rats; Fig. 3C).

The Isc response to barakol did not significantly change in the presence of the Na⁺ channel blocker amiloride (10 μM; Fig. 4A) in the apical solution (control, 33.5 ± 6.8 μA/cm², n = 6 tissues, N = 4 rats; after amiloride, 32.5 ± 10.1 μA/cm², n = 3 tissues, N = 3 rats). In contrast, pretreatment of tissues with bumetanide (200 μM; Fig. 4B) abolished the barakol-induced increase in Isc, from 33.5 ± 6.8 μA/cm² (n = 6 tissues, N = 4 rats) to 1.0 ± 3.6 μA/cm² (n = 3 tissues, N = 3 rats, p < 0.01; Fig. 4C).

**Effect of Ion Substitution on Barakol-Increased Isc.**

Ion substitution experiments were performed to determine the ionic basis of the Isc response induced by barakol. In normal Ringer’s solution, barakol at a concentration of 1 mM produced a mean increase in Isc of 31.1 ± 2.8 μA/cm² (n = 24 tissues, N = 13 rats). Replacement of Cl⁻ in both apical and basolateral solutions significantly inhibited the maximal Isc response to barakol (7.8 ± 0.6 μA/cm², n = 8 tissues, N = 6 rats, p < 0.05), whereas replacement of HCO₃⁻ had no effect on the maximal barakol-induced Isc (29.4 ± 2.6 μA/cm², n = 5 tissues, N = 4 rats). Replacement of both Cl⁻ and HCO₃⁻ significantly inhibited the maximal Isc response to 2.6 ± 0.7 μA/cm² (n = 5 tissues, N = 4 rats, p < 0.05; Fig. 5).
Effects of Submucosal Neuronal Blockers and Indomethacin on Barakol-Increased Isc. To assess whether barakol activation of Cl⁻ secretion was mediated by submucosal neurons and prostaglandin synthesis, the tissues were pretreated with neuronal blockers or indomethacin for 5 min followed by basolateral addition of barakol (1 mM). The effect of barakol alone on control tissues is shown in Fig. 6 (30.4 ± 3.2 μA/cm², n = 22 tissues, N = 11 rats). Tetrodotoxin (TTX; 10 μM) significantly decreased the basal Isc from 51.9 ± 13.0 to 33.2 ± 9.9 μA/cm², and the subsequent addition of barakol resulted in an Isc response that was 60% lower than that in control tissues (11.8 ± 4.2 μA/cm², n = 5 tissues, N = 4 rats, p < 0.05). In contrast, pretreatment with the muscarinic receptor antagonist atropine (1 μM) in the basolateral solution did not significantly change the basal Isc or reduce the barakol-induced increase in Isc (24.7 ± 6.4 μA/cm², n = 4 tissues, N = 4 rats). In addition, hexamethonium pretreatment (10 μM) did not significantly alter the barakol-stimulated Isc (n = 5 tissues, N = 4 rats; data not shown). Pretreatment of tissues with the cyclooxygenase inhibitor indomethacin (10 μM) in both apical and basolateral solutions significantly reduced the barakol-stimulated Isc response by 60% (12.3 ± 7.1 μA/cm², n = 7 tissues, N = 6 rats, p < 0.05). The barakol-stimulated Isc response was decreased by 90% in the presence of indomethacin and TTX (4.95 ± 1.1 μA/cm², n = 4 tissues, N = 4 rats, p < 0.05; Fig. 6). In addition, pretreatment with 10 μM dihydralazine, a histamine (H₁) receptor blocker, did not change the barakol-stimulated Isc (n = 3 tissues, N = 3 rats; data not shown).

Discussion

In the present study, the direct effect of barakol on the ion absorption and secretion was studied in vitro using serosal muscle-stripped colonic epithelium. Barakol was shown to increase Isc in both proximal and distal colon by the same magnitude. The increased Isc was due to an activation of Cl⁻ secretion. This was supported by the findings that Cl⁻ channel blockers, DPC and glibenclamide, inhibited the barakol-induced increase in Isc. In ion substitution experiments, the barakol-stimulated Isc was abolished in Cl⁻-free solution and further inhibited in Cl⁻ and HCO₃⁻-free solutions. In addition, pretreatment with the loop diuretic bumetanide completely inhibited the barakol-stimulated Isc. All of these findings were consistent with stimulation of transepithelial Cl⁻ secretion in human and rat colon (Dharmasathaphorn et al., 1985; Ko et al., 2002; Kunzelman and Mall, 2002). The finding that the increase in Isc by barakol was not affected by the presence of the Na⁺ channel blocker amiloride indicates that barakol did not stimulate electrogenic Na⁺ absorption.

Cl⁻ secretion in mammalian colon involves Cl⁻ uptake across the basolateral membrane by a Na⁺-K⁺-2Cl⁻ cotransport mechanism and subsequent efflux across the apical membrane through Cl⁻ channels. CFTR is the predominant Cl⁻ channel that plays a role in Cl⁻ secretion in many epithelia, including the colonic epithelium (Kunzelmann and Mall, 2002). CFTR is a cAMP-mediated Cl⁻ channel that has previously been shown to be inhibited by DPC and glibenclamide (Sheppard and Welsh, 1992). On the other hand, DIIDS has been shown to block Ca²⁺-activated Cl⁻ channel but had no effect on the activity and conductance of CFTR (Anderson et al., 1992; Schultz et al., 1999). Since the present study showed that the barakol-induced increase in Isc was completely inhibited by DPC and partly inhibited by gliben-
compared with the control value.

pathway for barakol-stimulated Cl
activity of the barakol response to DIDS further confirmed the
inhibiting Na+
Isc and TTX (Ind + TTX). Values were mean ± S.E.M. *, P < 0.05 when compared with the control value.

cell, it is most likely that CFTR was the apical Cl− exit pathway for barakol-stimulated Cl− secretion. The insensitivity of the barakol response to DIDS further confirmed the involvement of CFTR-mediated Cl− secretion and suggested that the Ca2+-activated Cl− channel was not involved in the Isc response to barakol. The barakol-stimulated increase in Isc was also abolished by bumetanide, an inhibitor of Na+/K+/2Cl− cotransporter, suggesting that barakol-stimulated Cl− uptake was mediated by this mechanism. Moreover, the alleviation of barakol-stimulated Cl− secretion by CFTR Cl− channel blockers and bumetanide indicated that the cellular mechanism of barakol may involve the cAMP-dependent pathway. Our finding was consistent with Cl− secretion induced by other plant-derived bioactive compounds, especially flavonoid baicalein. Baicalein has been shown to stimulate Cl− secretion across rat colonic epithelium by the activation of the cAMP-dependent apical Cl− channel and the basolateral K+ channels (Ko et al., 2002). From studies in human colonic cancer cells (T84 cells), baicalein was found to potentiate the Ca2+-mediated Cl− secretion via an accumulation of cAMP and activation of protein kinase A activity (Yue et al., 2004). However, the signaling mechanisms involving either intracellular Ca2+ or cAMP and protein kinase A in the mediation of barakol-stimulated Cl− secretion could not be ruled out and are subject to further investigation.

Most of the naturally occurring laxatives exert their effects on the colonic epithelium by stimulating Cl− secretion and/or inhibiting Na+ absorption, resulting in an accumulation of fluid and subsequent increased colonic motility. The increased Cl− secretion by anthranoid laxatives, chuanquimone and sennosides is due to disruption of epithelial tight junctions, leading to increased permeability of the epithelium (Ewe, 1980; Wanitschke, 1980). Their chemical structures related to junctional disruption have not been indicated. Although the direct effect of barakol on tight junction permeability was not examined in the present study, an increase in junctional permeability was unlikely to account for barakol-stimulated Cl− secretion since changes in tissue conductance were relatively small. The suppressive actions of anthranoid laxatives on Na+ absorption could result from decreased ATP production or a direct inhibition of the Na+/K+−ATPase activity in the basolateral membrane (Wanitschke, 1980; Wanitschke and Karbach, 1988). Reduced Na+/K+−ATPase activity would, in turn, decrease net Na+ absorption across the epithelium. In this study, the barakol-induced increases in Isc were not affected by amiloride, indicating that the barakol effect was not due to activation of epithelial Na+ channels. This finding may be due to the fact that Na+ channels normally play a relatively minor role in Na+ absorption across the proximal and distal colon in rats (Kunzelmann and Mall, 2002). However, a question still remains as to whether barakol may have some effects on Na+/H+ or Cl−-HCO3− exchange, since the activities of these electroneutral transporters could not be detected by measurement of Isc.

To identify whether the barakol response involved neurotransmitter release from nerves within the submucosal plexus or was the result of a direct effect on the epithelium, the tissues were pretreated with tetrodotoxin to block the neuronal Na+ channel activity and inhibit action potential propagation. A substantial portion of basal Isc (36%) was inhibited following the addition of tetrodotoxin, suggesting that it was sustained by endogenous release of neurotransmitters from the submucosal plexus. Pretreatment with tetrodotoxin inhibited the barakol-induced increase in Isc by 48%, confirming that barakol-stimulated Cl− secretion was partially mediated through the activation of submucosal nerves. In contrast, the lack of effect of the muscarinic cholinergic receptor blocker atropine or the nicotinic receptor blocker hexamethonium on the barakol-stimulated Isc response argued against involvement of the acetylcholine-containing submucosal neurons in barakol action on Cl− secretion in the rat. The tetrodotoxin-insensitive barakol response suggested that barakol may act directly on the colonic epithelial cells to stimulate Cl− secretion.

To further test whether the barakol activation of Cl− secretion was due to prostaglandin release, tissues were pretreated with the cyclooxygenase inhibitor indomethacin to block the synthesis of prostaglandins. Prostaglandins are normally released in response to a variety of stimuli. They have been known to activate Cl− secretion directly via prostanoïd receptors located in rat colonic epithelial cells (Brown et al., 1992). The present findings demonstrated that indomethacin inhibited the effect of barakol on Isc by 62%, suggesting that its sustained stimulatory effect on Cl− secretion was partially mediated through the release of prostaglandins. The combined presence of indomethacin and tetrodotoxin nearly abolished (90%) the stimulatory effect of barakol on Cl− secretion, indicating that the direct interaction of barakol with epithelial cells may account in part for its response.

The synthesis and release of prostaglandins are known to be part of the mechanisms of the anthranoid laxatives. Damage to epithelial cells caused by anthranoids induces the release of histamine and serotonin from monocytes, mast cells, and other intestinal monocytes, leading to increased biosynthesis of prostaglandin (Yagi et al., 1988; Nijss et al., 1992). Prostaglandin release, in turn, accelerates the large intestine transit and alters fluid absorption and secretion (Leng-Peschlow, 1986). In addition, the release of inflammatory mediators, especially histamine, is known to stimulate Cl− secretion in colonic epithelium (Traynor et al., 1993; Yue et al., 2004). However, inflammatory mediators were unlikely to be responsible for the actions of barakol since the histamine antagonist diphenhydramine did not alter the Isc response to barakol.

From the present findings, it seemed that barakol response was substantially mediated by enteric nerves within the submucosal plexus. Being insensitive to atropine and hexame-
thonium, barakol actions may be mediated by other neurotransmitter substances of the noncholinergic pathways. Candidates include vasoactive intestinal peptide, substance P, and calcitonin gene-related peptide (CGRP), which are among several neurotransmitters present in enteric nerves and sensory neurons of the small and large intestine (Cooke, 1994). These substances have been shown to be capable of inducing Cl⁻-dependent secretion (Traynor et al., 1991; Brown et al., 1992; Riegler et al., 1999; Esfandyari et al., 2000). The effect of substance P involved in barakol response is presently under investigation. Possible involvement of excitatory, noncholinergic transmitters in barakol action is also a subject worthy of future study.

The fact that a decrease in Cl⁻ secretory activity in the colon is one explanation for the increased incidence of constipation in the elderly; therefore drugs that increase fluid secretion through modulating ion channel activity could be beneficial for the treatment of constipation. Barakol, shown by the present study to have a stimulatory effect on electrolyte and water secretion, could thus be therapeutically useful for the management of age-related changes in colonic function or constipation associated with physiological or psychological stress. By increasing the Cl⁻ secretion in both proximal and distal colon, barakol could work as a laxative by increasing colonic volume and motility.

In conclusion, we have shown in rat colonic epithelium that barakol stimulated chloride secretion without affecting electrolytic sodium absorption. The transport mechanisms involved basolateral Na⁺⁻K⁺⁻2Cl⁻ cotransporters and apical Cl⁻ channels. Although not specifically addressed in this study, some increase in K⁺ channel activity was also likely as a means to sustain the electrical driving force for Cl⁻ exit across the apical membrane. The barakol-stimulated Iₛcl was partially controlled by enteric nerves within the submucosal plexus and partially mediated by the release of cylooxygenase metabolites. Our findings provided a mechanistic explanation for the action of the active compound barakol and the laxative effect of Cassia siamea plant extract.

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