Cellular Signaling Mechanisms Underlying Pharmacological Action of Bak Foong Pills on Gastrointestinal Secretion

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Abstract: Bak Foong Pills (BFP, also known as Bai Feng Wan) is an over-the-counter traditional Chinese medicine that has long been used for treating gynecological disorders and improving overall body functions, including gastrointestinal (GI) function. However, the cellular signaling mechanism underlying BFP action, especially on the GI tract, has not been elucidated. In the present study, the human colonic epithelia cell line T84 was used as a model to investigate the effect of BFP ethanol extract on ion transport in conjunction with the short-circuit current (Isc) technique. The results showed that the apical addition of BFP extract produced a concentration-dependent (10–1,000 μg/ml, EC50=120 μg/ml) increase in Isc. The maximal response was observed at 500 μg/ml with an increase in Isc of 24.4±2.3 μA/cm² and apical conductance. The BFP-induced Isc was not observed when extracellular Cl⁻ was replaced or when treated with bumetanide (100 μM), an inhibitor of the Na⁺-K⁺-2Cl⁻ cotransporter. The BFP-induced Isc was insensitive to the Na⁺ channel blocker, amiloride, but partially inhibited by the Cl⁻ channel blocker, DIDS (100 μM), and completely blocked by DPC (2 mM) or glibenclamide (1 μM) with a significant reduction in the apical conductance. The BFP-induced Isc could be mimicked by forskolin (10 μM), but inhibited by a pretreatment of the cells with adenylate cyclase inhibitor, MDL-12330A (10 μM). Pretreatment with EGTA (5 mM) and thapsigargin (10 μM) decreased the BFP-induced Isc by 10%. These results demonstrated that BFP ethanol extract exerted a stimulatory effect on gastrointestinal Cl⁻ secretion by predominantly activating adenylate cyclase and apical cAMP-dependent Cl⁻ channels, with minor contributions from calcium-dependent Cl⁻ channels. The effect of BFP may be explored to treat GI disorders such as constipation. [Japanese Journal of Physiology, 52, 129–134, 2002]

Key words: Bak Foong Pills, Bai Feng Wan, T84 cells, Cl⁻ secretion, cAMP.

Bak Foong Pills (BFP, also known as Bai Feng Wan) is an over-the-counter traditional Chinese medicine (China registration No. Z980035) that has long been used for treating gynecological disorders and improving overall body functions, including gastrointestinal (GI) function [1]. However, the cellular signaling mechanism underlying BFP action, especially on the GI tract, has not been elucidated.

Secretagogue-induced Cl⁻ secretion by the GI tract is important because Cl⁻ secretion provides an essential driving force for the lubrication of intestinal contents during regular bowl movements or the flushing of microbial organisms or artificial irritants in host defense responses [2, 3]. 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, such as by cholera toxin,
may result in diarrhea [4–6] or constipation [7, 8] with severe pathological consequences. Therefore Cl⁻ channels are very likely to be the target for pharmacological intervention.

Using human colonic T₈₄ cells and the short-circuit current technique, we undertook the present study to examine whether BFP exerted any effect on Cl⁻ secretion and to investigate the cellular signaling mechanism underlying its action.

Materials and Methods

Materials. Dulbecco’s Modified Eagle’s medium (DMEM)/F12, Hank’s balanced salt solution (HBSS), and fetal bovine serum was from Gibco Laboratories (New York, NY). 4,4’-Diisothiocyanostilbene-2,2’-disulfonic acid (DIDS), Forskolin, and glibenclamide were from Sigma (St. Louis, MO). BFP was purchased from Eu Yan Sang Ltd. (Hong Kong). Diphenylamine-2,2’-dicarboxylic acid (DPC), was obtained from Riedel-de Haen Chemicals (Hannover, Germany). Calbiochem (San Diego, CA) was the source for the adenylate cyclase inhibitor MDL-12330A, amiloride hydrochloride, bumetanide, and thapsigargin.

BFP extraction. We put 500 g of BFP powder in 70% ethanol at a ratio of 1 to 10 (g/ml) into a round-bottom flask and boiled it under reflux for 2 h. The mixture was filtered and the residue of BFP was subjected to the same treatment for a second time. The filtrates from the two treatment procedures were collected and put into the vacuum rotary evaporator for concentration. The extract was collected and lyophilized by freeze dryer.

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. The solution was gassed with 95% O₂/5% CO₂, and the pH 7.4. In Cl⁻-free K-H solution, ambient Cl⁻ in K-H solution was removed by substituting with gluconate.

Cell culture. Human colonic T₈₄ was purchased from American Type Culture Collection (Rockville, MD). The cells were grown in DMEM/F12 with 10% fetal bovine serum. The cells (1.0×10⁶/ml) were plated onto each floating permeable support, which was made of a Millipore filter with a silicone rubber ring attached to its top for confining the cells (0.45 cm²). The cultures were incubated at 37°C in 95% O₂/5% CO₂ for 4 d before the experiments.

Short-circuit current measurement. The measurement of Iₛᶜ has been described previously [9]. Monolayers grown on permeable supports were clamped vertically between two halves of the Ussing chamber. The monolayers were bathed in both sides with Krebs-Henseit solution, which was maintained at 37°C by a water jacket enclosing the reservoir. The solution was bubbled with 95% O₂/5% CO₂ to maintain its pH at 7.4. Drugs could be added directly to the apical or the basal side of the epithelium. Usually the epithelium exhibited a basal transepithelial potential difference for every monolayer examined, which was measured by the Ag/AgCl reference electrodes (World Precision Instruments, Inc., Sarasota, FL, USA) connected to a preamplifier that was connected to a voltage-clamp amplifier (World Precision Instruments, Inc., DVC-1000). In most of the experiments, the change in Iₛᶜ was defined as the maximal rise in Iₛᶜ following agonist stimulation, and it was normalized to current change per unit area of the epithelial monolayer (μA/cm²). In each experiment, a transepithelial potential difference of 0.1 or 1.0 mV was applied. 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 culture to ensure that the data were reproducible.

Data analysis. Results were expressed as mean±standard error of the mean (SEM). The number of experiments represents independent measurements on separate monolayers. Comparisons between groups of data were made by Student’s unpaired t-test. A p value of less than 0.05 was considered statistically significant.

Results

BFP-induced Iₛᶜ response. The apical addition of BFP extract produced a sustained increase in Iₛᶜ response, which lasted for at least 30 min. The effect of BFP extract was concentration-dependent (Fig. 1) with an apparent EC₅₀ of about 120 μg/ml (Fig. 2). The BFP-induced Iₛᶜ was accompanied with a significant decrease in transepithelial resistance, to 344.8±27.1 Ω cm², from 423.2±42.3 Ω cm² (n=7, p<0.001), indicating the activation of apical conductance.

Anion dependence of the BFP-induced Iₛᶜ. To study the ion species involved in mediating the BFP-induced Iₛᶜ, Cl⁻ was removed from the bathing solution. The BFP-induced response was completely abolished by Cl⁻ removal (Fig. 3), indicating a Cl⁻ dependence of the BFP-induced current. The effect of an Na⁺ channel blocker, amiloride, and a couple of Cl⁻ channel blockers, DPC or glibenclamide, on the BFP extract-induced Iₛᶜ was also examined. The apical addition of amiloride (10 μM) was found to have no effect on the basal current as well as the BFP-induced Iₛᶜ (Fig. 4A), excluding the involvement of
Na⁺ absorption. Apical-applied DPC (2 mM) (Fig. 4A) or glibenclamide (1 mM) could completely abolish the response (Fig. 4B) with a significant increase in the transepithelial resistance back to a value similar to that before stimulation, 441.5 ± 37.9 V cm⁻², from 344.8 ± 27.1 V cm⁻². Bumetanide (100 μM), a strong inhibitor of the Na⁺-K⁺-2Cl⁻ cotransporter, added to the basolateral side, resulted in a more than 90% reduction in the I_SC induced by BFP extract (p < 0.001 as compared to control, Fig. 4B).

**Involvement of adenylate cyclase.** The BFP-induced I_SC could be mimicked by adenylate cyclase activator, forskolin (10 μM), in terms of current magnitude, activation kinetics, and blocker sensitivity. The subsequent addition of forskolin or BFP extract after activation by BFP extract (600 μg/ml) or forskolin (100 μM), respectively, could not produce an additive effect on the current (Fig. 5), indicating a similar signaling pathway that possibly involved cAMP. To test this, an adenylate cyclase inhibitor, MDL-12330A, was added 10 min before the addition of BFP extract (Fig. 6). The treatment with MDL-12330A 10 and 20 μM inhibited the I_SC induced by BFP extract (300 μg/ml) by 60.52% (p < 0.01) and 80.95% (p < 0.001), respectively. The concentration-dependent inhibition of the BFP-induced current by MDL-12330A suggested the involvement of adenylate cyclase and cAMP.

**Involvement of Ca²⁺-dependent Cl⁻ channel.** The addition of EGTA (5 mM), which depleted extracellular Ca²⁺, and thapsigargin (10 μM), a vesicular Ca²⁺ pump inhibitor for emptying intracellular Ca²⁺ store, resulted in a 10% (n = 4, p < 0.01, data not shown) reduction in the BFP-induced I_SC. The apical application of DIDS (100 μM), which is known to block Ca²⁺-dependent Cl⁻ channels, also inhibited the BFP-induced current response by about 9.0 ± 3.7% (Fig. 7), indicating a minor involvement of Ca²⁺ and Ca²⁺-dependent Cl⁻ channels in the BFP-induced I_SC.

**Discussion**

BFP has long been used for the treatment of gynaecological and obstetrical diseases, since the 17th century. Apart from its well-known effect on the female reproductive tract, BFP is also considered to have beneficial effects on overall body functions, including the improvement of digestion and GI tract function [1]. The present study is the first to provide scientific evidence for the pharmacological action of BFP on the GI tract. The present results have demonstrated that BFP ethanol extract stimulated Cl⁻ secretion in the human colonic epithelial cell line T₈⁴. In this epithelium, Cl⁻ secretion is mediated by 2 steps, i.e., the accumulation of cytosolic Cl⁻ by Na⁺-K⁺-2Cl⁻ cotransporters in the basolateral membrane and then the exit of Cl⁻ through Cl⁻ channels in the apical membrane. The supporting evidence for an effect of BFP on Cl⁻ secretion includes the following: (1) A BFP-induced response was insensitive to the Na⁺ channel blocker;
(2) The response was completely inhibited by a replacement of extracellular \( \text{Cl}^- \) with impermeable gluconate; (3) The response was substantially inhibited by the inhibitor of Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporters and Cl\(^-\) channel blockers. Thus a stimulation of Cl\(^-\) secretion by BFP is readily confirmed.

The stimulation of Cl\(^-\) secretion by BFP appears to involve mainly the cAMP-dependent pathway. This was first suggested by the mimicking of BFP-induced current response by adenylate cyclase activator, forskolin, in terms of current magnitude, activation kinetics, and blocker sensitivity. Furthermore, no additive effect was observed for BFP and forskolin, regardless of the order of drug addition, indicating that the two share a similar signaling pathway, namely the cAMP-dependent one. This notion was further borne out by the result obtained by the use of adenylate cyclase inhibitor, MDL-12330A, because the BFP-in-
duced current was suppressed by the inhibitor in a concentration-dependent manner. Because only 10% of the BFP-induced current could be blocked by pretreatment with EGTA and thapsigargin or a known Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel blocker, DIDS, a major role of Ca\textsuperscript{2+} in mediating the BFP response is excluded. Instead, the observation that the current could be completely blocked by DPC or glibenclamide indicates the predominant involvement of the cAMP-dependent pathway, since CFTR, a cAMP-activated Cl\textsuperscript{−} channel abundantly expressed in several epithelial cells, including T84 cells [10–12], is known to be blocked by DPC or glibenclamide, but not by DIDS [13–15].

Since Cl\textsuperscript{−} secretion requires both the basolateral accumulation of Cl\textsuperscript{−} by Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter and an apical exit through Cl\textsuperscript{−} channels, it is not clear at this point which site would be the primary target of BFP. The current results did provide evidence for the activation of apical Cl\textsuperscript{−} channels, since an increase in apical conductance during stimulation and a return of the conductance to basal level upon the addition of channel blockers were observed. It is possible that BFP may also activate the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter, since it has been reported that the cotransporter could be activated by cAMP-dependent mechanism [16, 17].

Taken together, the present results have demonstrated that BFP ethanol extract exerted a stimulatory effect on gastrointestinal Cl\textsuperscript{−} secretion by predominantly activating adenylate cyclase and apical cAMP-dependent Cl\textsuperscript{−} channels, namely CFTR and possibly the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter. The ability of BFP to stimulate Cl\textsuperscript{−} secretion in the GI tract may contribute to its beneficial effects, such as smoothing bowel movement and enhancing fluid clearance during host defense response.

At the moment, it is uncertain whether the stimulatory effect of BFP on Cl\textsuperscript{−} secretion is due to the collective effect of all the constituent herbal components or some active ingredients contained in the 70% ethanol extract. Nevertheless, the current study has established a model for the quantitative measurement of BFP effect on the GI tract and for further screening of its possible active ingredients. This effect may be explored to improve GI disorders, such as constipation, a condition commonly associated with aged people [18, 19].

The similarity between forskolin and BFP-induced currents, the differential sensitivity of the BFP-induced current response to DIDS and DPC, or glibenclamide, and the reduction of the current by the adenylate cyclase inhibitor raised the possibility that BFP may activate CFTR. It would be worthwhile to pursue further the activation mechanism to explore the therapeutic potential of BFP or its active ingredients for the treatment of cystic fibrosis, since an alternative activation mechanism of CFTR by a plant-derived compound, genistein, has been reported [20, 21]. It is also interesting to note that our previous studies have demonstrated an upregulation of the CFTR expression in mice endometrial epithelial cells by feeding the animals with BFP for 12 d [22]. The previously observed genomic effect of BFP on CFTR expression and the presently observed possible nongenomic effect of BFP on CFTR warrant a further exploration of BFP as a therapeutic agent for cystic fibrosis.
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REFERENCES


