

Research Article

Butylidenephthalide Blocks Potassium Channels and Enhances Basal Tension in Isolated Guinea-Pig Trachea

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Butylidenephthalide (Bdph, 30~300 μ M), a constituent of *Ligusticum chuanxiong* Hort., significantly enhanced tension in isolated guinea-pig trachea. In this study, we investigate the mechanism(s) of Bdph-induced contraction in the tissue. Isolated trachea was bathed in 5 mL of Krebs solution containing indomethacin (3 μ M), and its tension changes were isometrically recorded. Cromakalim (3 μ M), an ATP-dependent K⁺ channel opener, significantly antagonized the Bdph-induced enhancement of baseline tension. Bdph (300 μ M) also significantly antagonized cromakalim-induced relaxation. Bdph (300 μ M) did not significantly influence the antagonistic effects of glibenclamide (GBC, 1 μ M) and tetraethylammonium (TEA, 8 mM) against the cromakalim-induced relaxation. However, Bdph (300 μ M) and 4-aminopyridine (4-AP, 5 mM), a blocker of K_v1 family of K⁺ channels, in combination significantly rightward shifted the log concentration-relaxation curve of cromakalim. The antagonistic effect of the combination almost equals the sum of the individual effects of Bdph and 4-AP, suggesting that the antagonistic mechanism of Bdph may be similar to that of 4-AP. All calcium channel blockers influenced neither the baseline tension nor antagonistic effect of Bdph against cromakalim. In conclusion, Bdph may be similar to 4-AP, a blocker of K_v1 family of K⁺ channels, to enhance the baseline tension of guinea-pig trachea.

1. Introduction

The rhizomes of *Ligusticum chuanxiong* Hort. (previously named *L. wallichii* Franch.) and *Angelica sinensis* Diels. (Apiaceae) have been used by the Chinese for several thousand years. In ancient medical literature, such as Shen-Nung-Pen-Tsao-Ching, the rhizome of *L. chuanxiong* Hort. was delineated to prevent and restore stroke-induced dyskinesia. We reported that butylidenephthalide (Bdph), a neutral oil constituent of the rhizome, inhibited cyclooxygenase to have antiplatelet effects [1]. Other investigators reported that shimotsu-to, a prescription of traditional Chinese medicine (TCM), had antiproliferative effects in primary cultures of mouse aorta smooth muscle cells [2], mainly due to *Cnidium* rhizome-derived phthalides, such as senkyunolide, ligustilide, and Bdph [3]. Both antiplatelet and antiproliferative effects of these crude drugs benefit to prevent stroke.

To recover from stroke-induced dyskinesia the damaged nerve cells need to be repaired mainly by themselves. The vasodilating effects of Bdph [4–6] improve the circulation and may partially benefit this restoration. Recently, Bdph was reported to provide neuroprotection by reducing the release of various proinflammatory molecules from activated microglia [7]. It is also reported to maintain stem cell pluripotency by activating the Jak2/Stat3 pathway and increasing the efficiency of induced pluripotent stem cells generation [8]. These results highlight the ability for these crude drugs to aid in the recovery from dyskinesia. Interestingly, Bdph was also reported to inhibit growth of malignant brain tumor [9], lung adenocarcinoma [10], and glioblastomas [11] with a high therapeutic ratio [12].

Bdph (50~250 μ M) was reported to noncompetitively inhibit ACh-, KCl-, and BaCl₂-induced contractions in guinea-pig ileum [13]. Bdph (30~300 μ M) was also reported

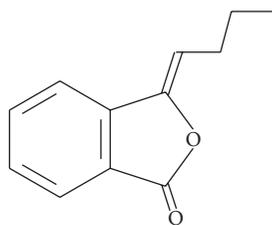


FIGURE 1: The chemical structure of butyldenephthalide (Bdph, mol. wt. 188.23).

to noncompetitively inhibit phenylephrine- and KCl-induced contractions in rat aortic rings [6]. However, in preliminary test, we found that Bdph (30~300 μM) failed to, except at very high concentrations (600~1000 μM), inhibit histamine (10 μM)-induced contraction in isolated guinea-pig trachea. More surprisingly, we found that Bdph (30~300 μM) alone can significantly induce enhancement of baseline tension in the tissue. Therefore we are interested in investigating the mechanism(s) of Bdph-induced contraction in the tissue.

2. Methods and Materials

2.1. Drugs and Animals. Bdph was synthesized and yielded a light yellow oily substance according to the previously described method [14]. Its purity (99.8%) was analyzed by using high performance liquid chromatography and the structure is shown in Figure 1 [15]. 4-Aminopiridine (4-AP), atropine sulfate, α -chymotrypsin, diltiazem, glibenclamide (GBC), histamine diphosphate, indomethacin, nifedipine, nifedipine (Nif), pyrilamine maleate, tetraethylammonium bromide (TEA), and verapamil (Vrp) were purchased from Sigma-Aldrich, St. Louis, MO, U.S.A. Papaverine was purchased from Narcotics Bureau, Taipei, Taiwan. Cromakalim, methysergide, and FPL 557121 were gifts from SmithKline Beecham Pharmaceutical, U.K., Sandoz, Swiss, and Fisons, U.K., respectively.

Male Hartley guinea-pigs (250~400 g) were obtained from the Animal Center of the National Science Council (Taipei, Taiwan). The animals were housed in ordinary cages at $22 \pm 1^\circ\text{C}$ with a humidity of 50%~60% under a constant 12/12-h light/dark cycle and provided with food and water *ad libitum*. Under a protocol approved by the Animal Care and Use Committee of Taipei Medical University, the following *in vitro* experiments were performed.

2.2. Tracheal Preparation. The guinea-pigs were sacrificed by cervical dislocation after anesthesia, and their tracheas were removed. Each trachea was cut into six segments. Each segment consisted of three cartilage rings. All segments were cut open opposite the trachealis. The segments were randomized to minimize regional variability. Each segment was tied at one end to a holder via silk sutures, placed in 5 mL of Krebs solution containing indomethacin (3 μM) throughout the entire experiment, bubbled with a 95% O_2 and 5% CO_2 mixture at 37°C , and attached by the other end to a force displacement transducer (Grass FT03) for the isometric

recording of tension changes on a polygraph (Gould RS3200). The composition of the Krebs solution was (mM): NaCl 120, KCl 4.7, MgSO_4 0.5, KH_2PO_4 1.2, CaCl_2 2.5, NaHCO_3 25, and dextrose 11.0. The tissues were suspended under an initial tension of 1.5 g and allowed to equilibrate for 1 h with washing at 15-min intervals. After equilibration, the following experiments were performed.

2.3. Bdph Enhanced Baseline Tension but Relaxed Precontraction at High Concentrations. Bdph (30~1000 μM) or its vehicle (0.03~1% ethyl alcohol) was cumulatively added to examine the tension change of baseline. After the histamine (10 μM)-induced precontraction reached steady state, Bdph (30~1000 μM) or its vehicle was cumulatively added to the organ bath. At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of Bdph for both baseline tension and relaxation were constructed.

2.4. Cromakalim Antagonized Bdph-Induced Enhancement of Baseline Tension. In order to examine the possible transmitter(s) or mediators which may enhance the tracheal baseline tension, some pharmacological agents, such as 1 μM atropine (a cholinergic antagonist), 1 μM FPL 55712 (a leukotriene receptor antagonist) [16], 1~10 μM pyrilamine (a histamine receptor antagonist), 1 μM methysergide (a serotonin receptor antagonist), and 2 u/mL α -chymotrypsin (a neuropeptidase), were added 30 min prior to the cumulative addition of Bdph (30~300 μM). However, 1~3 μM cromakalim (an ATP-sensitive K^+ channel opener) was pretreated for only 10 min which was enough to reach equilibration [17].

2.5. Bdph Also Antagonized Cromakalim-Induced Relaxation. After preincubation of Bdph (30~300 μM) or its vehicle for 20 min, histamine (10~30 μM) was added to reach a half-maximal contraction and then cumulatively added cromakalim (0.1~10 μM). At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of cromakalim in the absence and presence of Bdph were constructed.

2.6. Interaction between Bdph and Other K^+ Channel Blockers to Antagonize Cromakalim-Induced Relaxation. After preincubation of Bdph (100 or 300 μM) and other K^+ channel blockers, such as GBC (1 μM), TEA (8 mM), and 4-AP (5 mM) alone or combination for 20 min, histamine (10~30 μM) was added to reach a half-maximal contraction, and then cromakalim (0.1~10 μM) was cumulatively added. At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of cromakalim in the absence and presence of drug(s), such as Bdph and other K^+ channel blockers, alone or combination, were constructed.

2.7. Interaction between Bdph and Other Ca^{2+} Channel Blockers to Antagonize Cromakalim-Induced Relaxation. First,

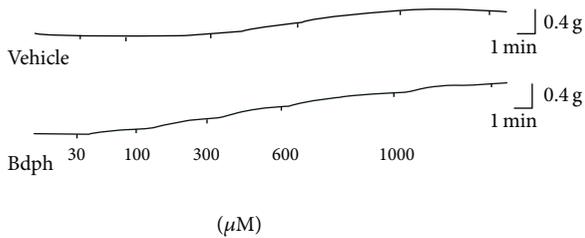


FIGURE 2: Typical tracing of baseline tension change induced by cumulative butyldenephthalide (Bdph, lower panel) compared to its vehicle (0.03~1% ethyl alcohol, upper panel) in isolated guinea-pig trachea. The vertical line indicates tension change.

Vrp, Nif, diltiazem, nicardipine, or their vehicles were cumulatively added to examine the tension change of baseline in the isolated trachea. Second, after preincubation of Bdph (300 μM) or its vehicle for 20 min, histamine (10~30 μM) was added to reach a half-maximal contraction, and then Vrp (0.01~10 μM) or Nif (0.001~1 μM) was cumulatively added. At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of Vrp and Nif in the absence and presence of Bdph were constructed. Third, after preincubation of Bdph (300 μM), Vrp (1 μM), and Nif (0.1 μM) alone or combination for 20 min, histamine (10~30 μM) was added to reach a half-maximal contraction, and then cromakalim (0.1~10 μM) was cumulatively added. At the end of experiment, papaverine (0.1 mM) was added to maximally relax the tissue and to standardize the relaxation (100%). The log concentration-response curves of cromakalim in the absence and presence of drugs, such as Bdph, Vrp, and Nif, alone or combination were constructed.

2.8. *Statistical Analysis.* The tracheal contraction was expressed as percentage of maximal contraction (100%), with some exceptions expressed as tension. However, the tracheal relaxation was expressed as percentage of maximal relaxation induced by papaverine (100%) at the end of experiment. All values are expressed as mean \pm SEM, n is the number of experiment. Student's unpaired t -test was used for statistical analysis between test and control with P values < 0.05 being regarded as significant.

3. Results

3.1. *Effects of Bdph on Baseline and Histamine-Induced Precontraction.* The effect of Bdph (30~1000 μM), compared to its vehicle, on the baseline tension in isolated guinea-pig trachea is shown in Figures 2 and 3(b). Bdph (30~300 μM) did not significantly relax the histamine (10 μM)-induced precontraction, except at higher concentrations of 600~1000 μM in the tissue (Figure 3(a)). In contrast, Bdph (30~300 μM) significantly enhanced its baseline tension (Figure 3(b)).

3.2. *Cromakalim Antagonized Bdph-Induced Enhancement of Baseline Tension.* Atropine (1 μM), FPL 55712 (1 μM),

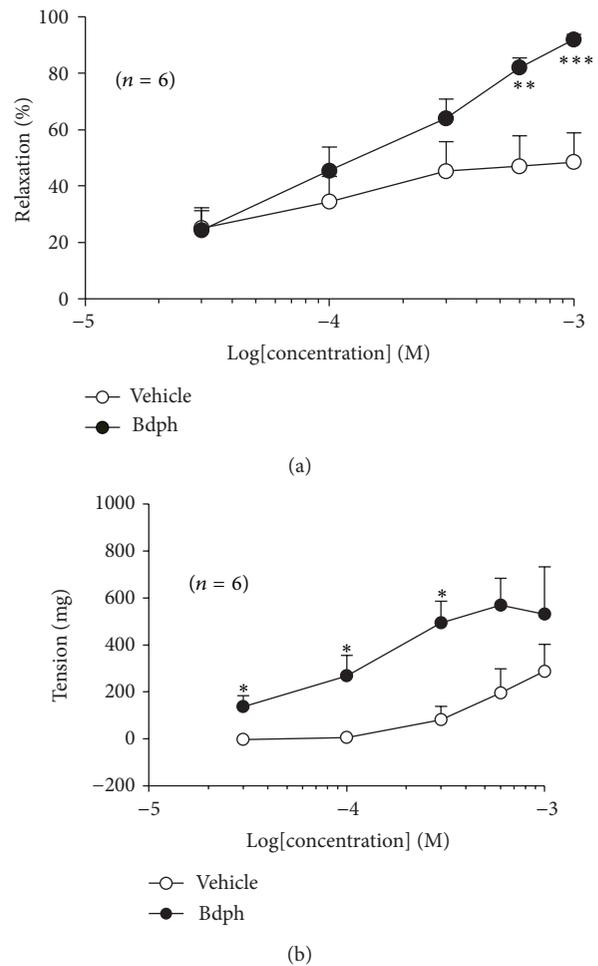


FIGURE 3: Log concentration relaxant (a) and contractile (b) response curves of butyldenephthalide (Bdph) in isolated guinea-pig trachea. The relaxant and contractile effects of cumulative Bdph (30~1000 μM) on histamine (10 μM)-induced precontraction and on baseline tension were performed as method described, respectively. All values are shown as mean \pm SEM, and n is the number of experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to its vehicle.

pyrilamine (1 and 10 μM), methysergide (1 μM), or α -chymotrypsin (2 u/mL) did not significantly influence the Bdph-induced enhancement of baseline tension (Figure 4). However, cromakalim (3 μM) significantly antagonized the Bdph-induced enhancement of baseline tension (Figure 5).

3.3. *Bdph Also Antagonized Cromakalim-Induced Relaxation.* Bdph (300 μM) significantly antagonized cromakalim-induced relaxation (Figure 6).

3.4. *Interaction between Bdph and Other K^+ Channel Blockers to Antagonize Cromakalim-Induced Relaxation.* Bdph at concentrations of 100 μM and 300 μM did not significantly influence the antagonistic effects of GBC (1 μM) against the cromakalim-induced relaxation (Figure 7). Bdph (300 μM) never influenced the antagonistic effects of TEA at 8 mM

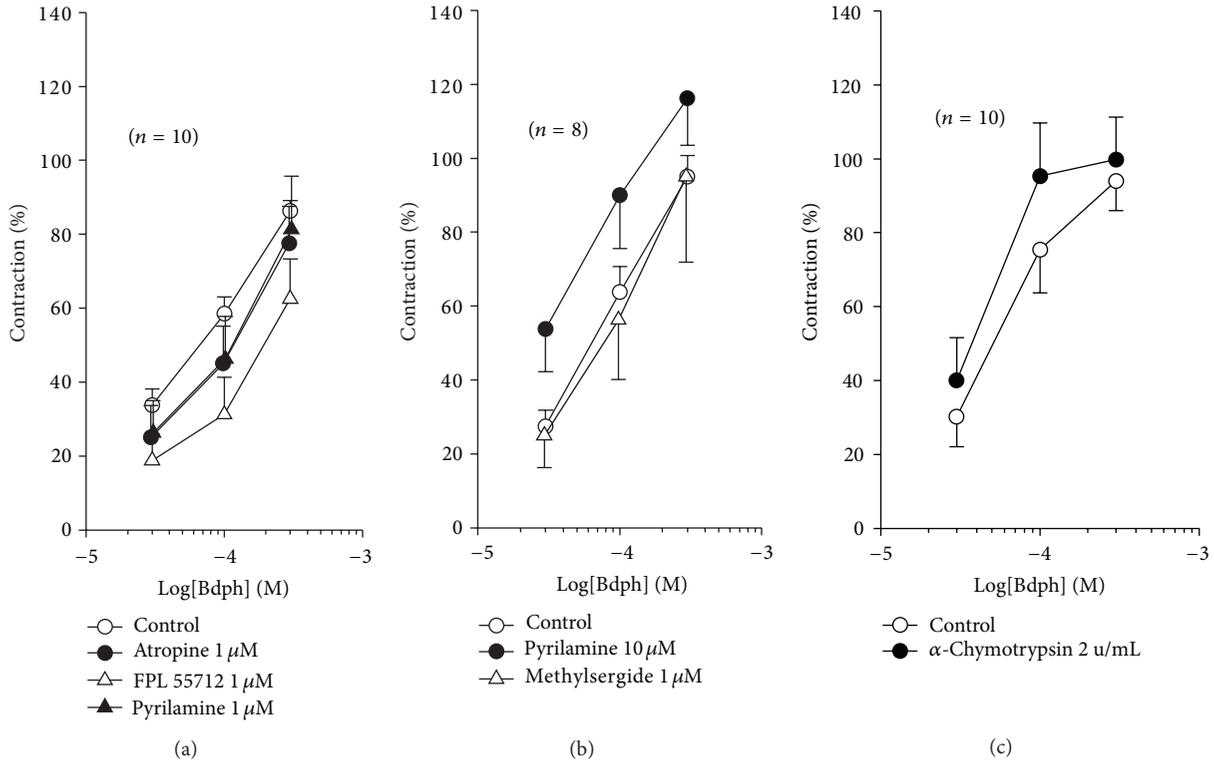


FIGURE 4: Inhibitory effects of atropine, FPL 55712, or pyrilamine 1 μM (a), as well as pyrilamine 10 μM or methysergide (b) and α -chymotrypsin (c) on cumulative butyridenephtalide- (BdpH-) induced contraction of baseline tension in isolated guinea-pig trachea. All values are shown as mean \pm SEM, and n is the number of experiments. There is no significant difference between test and respective control.

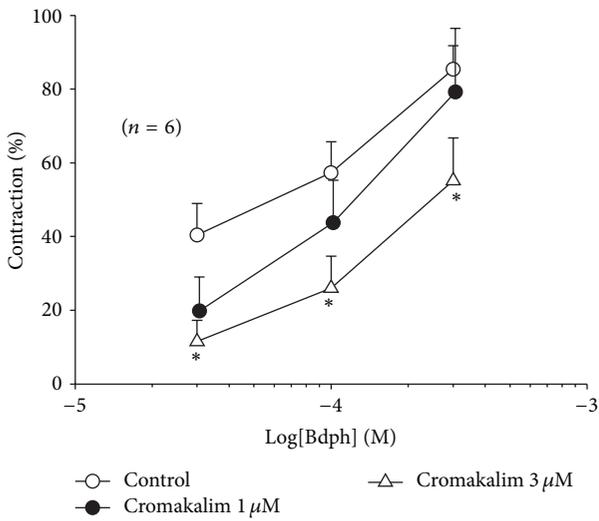


FIGURE 5: Inhibitory effect of cromakalim on cumulative butyridenephtalide (BdpH)-induced contraction of baseline tension in isolated guinea-pig trachea. All values are shown as mean \pm SEM, and n is the number of experiments. * $P < 0.05$ compared to its vehicle.

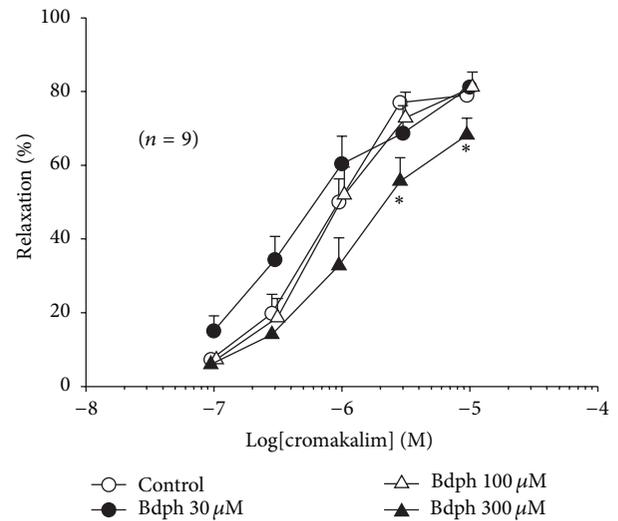


FIGURE 6: Inhibitory effect of butyridenephtalide (BdpH) on cumulative cromakalim-induced relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean \pm SEM, and n is the number of experiments. * $P < 0.05$ compared to its vehicle.

(Figure 8(a)). However, BdpH (300 μM) and 4-AP (5 mM) in combination significantly antagonized the cromakalim-induced relaxation, compared to the individual effects on

the relaxation, and rightward shifted the log concentration-response curve of cromakalim. The antagonistic effect of the

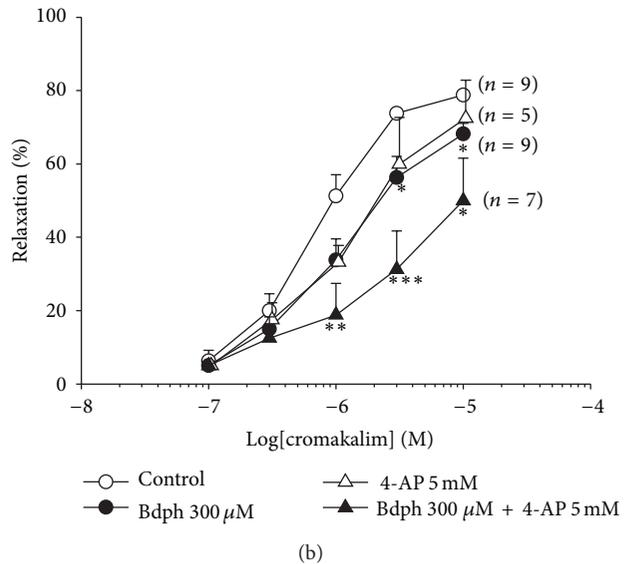
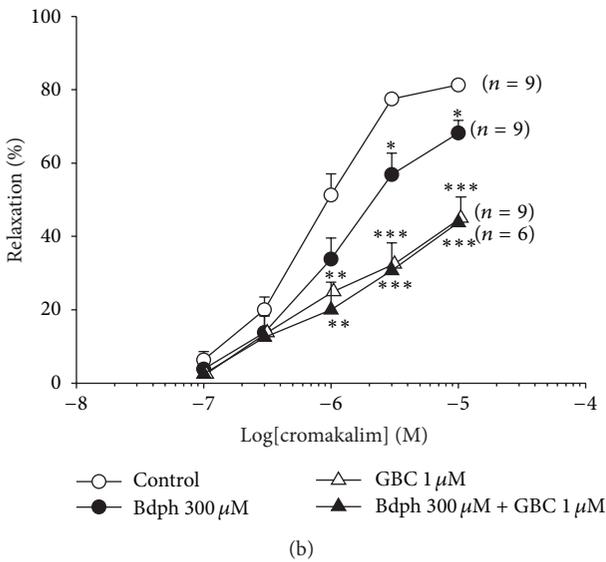
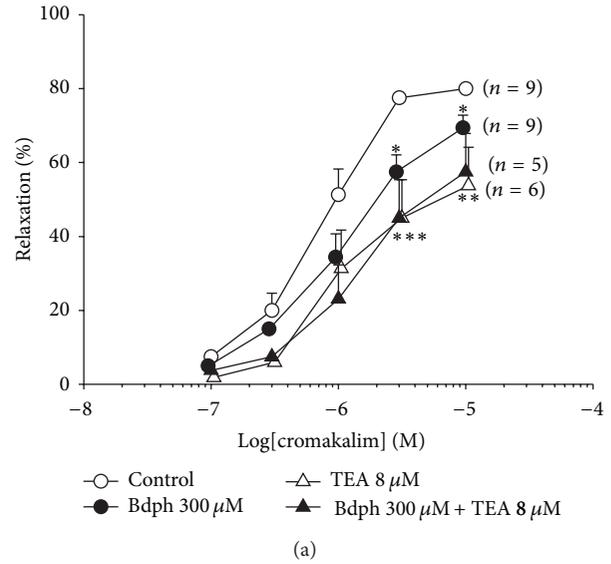
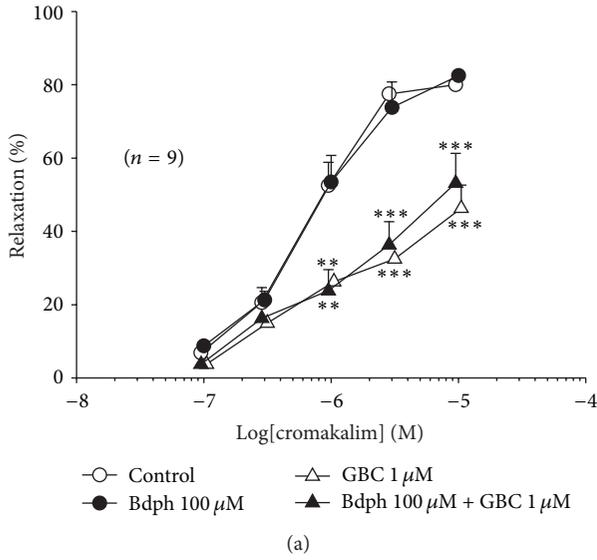


FIGURE 7: Influences of glibenclamide (GBC) to the antagonistic effects of butylidenephthalide (Bdph) 100 μM (a) and 300 μM (b) on cumulative cromakalim-induced relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean \pm SEM, and n is the number of experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to its vehicle.

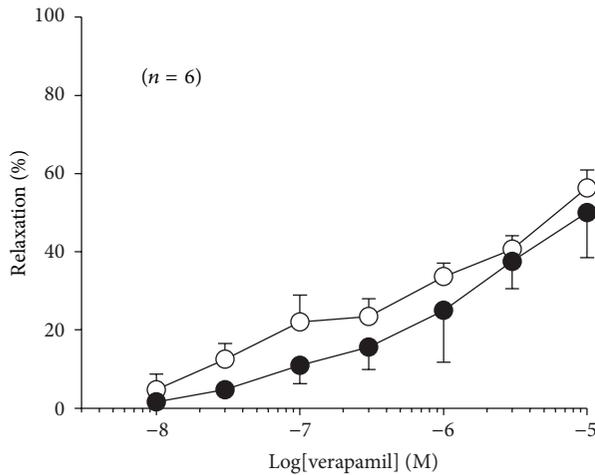
FIGURE 8: Influences of tetraethylamine (TEA, (a)) and 4-aminopyridine (4-AP, (b)) to the antagonistic effect of butylidenephthalide (Bdph) on cumulative cromakalim-induced relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean \pm SEM, and n is the number of experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to its vehicle.

combination is almost equal to the sum of individual effects (Figure 8(b)).

3.5. Interaction between Bdph and Other Ca^{2+} Channel Blockers to Antagonize Cromakalim-Induced Relaxation. All Ca^{2+} channel blockers used did not enhance or reduce the baseline tension of the isolated guinea-pig trachea (data not shown). Bdph did not influence the relaxant effects of Vrp (Figure 9(a)) and Nif (Figure 9(b)) on the histamine-induced precontraction. Vrp (1 μM) and Nif (0.1 μM) also did not influence the antagonistic effect of Bdph (300 μM) against the cromakalim-induced relaxation (Figure 10).

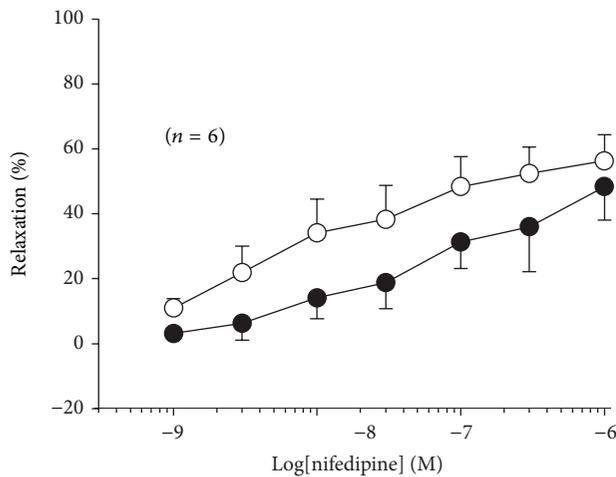
4. Discussion

The present results suggest that the enhancement of basal tension by Bdph is unrelated to the release of cholinergic transmitter, leukotrienes, histamine, serotonin, and neuropeptides [18]. It is also unrelated to the release of prostaglandins, as the experiment was conducted throughout in the presence of indomethacin. However, the enhancement was antagonized by cromakalim (3 μM), an ATP-sensitive K^+ channel opener [17], which may increase outflux of K^+ and hyperpolarize the membrane of tracheal smooth muscle cells and cause relaxation. Furthermore, Bdph (300 μM) also antagonized



○ Control
● Bdph 300 μM

(a)



○ Control
● Bdph 300 μM

(b)

FIGURE 9: Antagonistic effects of butylidenephthalide (Bdph) on cumulative verapamil-induced (a) and nifedipine-induced (b) relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean \pm SEM, and n is the number of experiments. There is no significant difference between test and respective control.

and rightward shifted the log concentration-relaxation curve of cromakalim on histamine-induced precontraction in the isolated guinea-pig trachea (Figure 6). Thus, Bdph may be a kind of K^+ channels blockers, which have been reviewed to have a potential clinical use for Alzheimer disease [19]. Indeed, Bdph have been reported to reverse the deficits of inhibitory avoidance performance and improve memory in rats [20]. GBC ($1 \mu\text{M}$), a specific ATP-sensitive K^+ channel blocker [17], effectively antagonized and rightward shifted the curve of cromakalim. Bdph neither at $100 \mu\text{M}$ nor at $300 \mu\text{M}$ influenced the antagonistic effect of GBC. Also, Bdph at

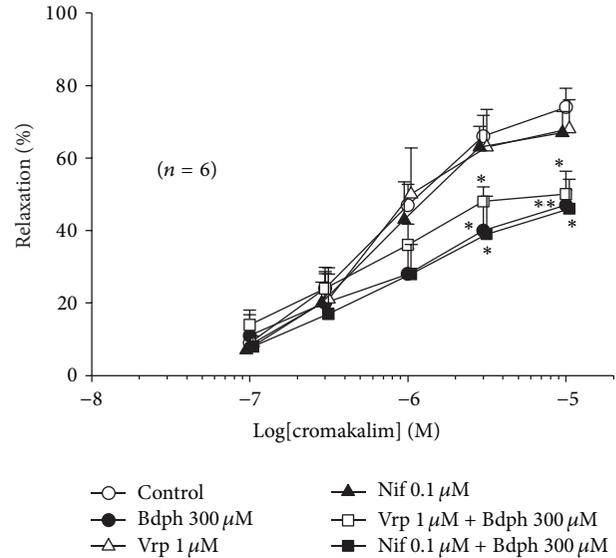


FIGURE 10: Influences of verapamil (Vrp) and nifedipine (Nif) to the antagonistic effect of butylidenephthalide (Bdph) on cumulative cromakalim-induced relaxant response to histamine-induced precontraction in isolated guinea-pig trachea. All values are shown as mean \pm SEM, and n is the number of experiments. * $P < 0.05$ and ** $P < 0.01$ compared to its vehicle.

$300 \mu\text{M}$ did not affect the antagonistic effect of TEA (8 mM), a nonselective big (BK_{Ca}) and intermediate (IK_{Ca}) conductance Ca^{2+} -activated K^+ channels blocker [21]. However, Bdph at $300 \mu\text{M}$ significantly enhanced the antagonistic effect of 4-AP (5 mM) and rightward shifted the curve in the combination. The antagonistic effect of the combination was almost the sum of individual effects (Figure 8(b)). This result strongly suggests that the mechanism of Bdph may be similar to that of 4-AP to antagonize cromakalim. The mechanism of Bdph was unrelated to Ca^{2+} -dependent K^+ channels, as all Ca^{2+} channel blockers did not influence the antagonistic effect of Bdph against cromakalim.

Episodic ataxia type 2 (EA2) is a form of hereditary neurological disorder caused by cerebellar malfunction and is characterized by interictal ataxia and frequent attacks of dyskinesia, vertigo, and imbalance [22]. Recently, 4-AP was reported to treat EA2 [23, 24]. The targets of 4-AP are K_v1 family of K^+ channels, possibly the $\text{K}_v1.5$ subtype [25]. Further investigation is needed to determine whether Bdph is useful in treating EA2.

In conclusion, Bdph ($30\text{--}300 \mu\text{M}$) concentration-dependently evoked an enhancement of baseline tension in isolated guinea-pig trachea. The enhancement was antagonized by cromakalim, and Bdph ($300 \mu\text{M}$) also antagonized cromakalim-induced relaxation. Furthermore, Bdph ($300 \mu\text{M}$) and 4-AP (5 mM) in combination rightward shifted the log concentration-response curve of cromakalim and significantly antagonized the cromakalim-induced relaxation. The antagonistic effect of the combination is almost equal to the sum of individual effects. Therefore, Bdph may be similar to 4-AP, a blocker of K_v1 family of

K⁺ channels, to enhance the baseline tension of guinea-pig trachea.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgment

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