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Effect of Butylidenephthalide on Calcium Mobilization in Isolated Rat Aorta

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Abstract

Butylidenephthalide (Bdph), an antispasmodic compound originally isolated from the rhizome of *Ligusticum chuaxiong*, has a selective anti-anginal effect without changing blood pressure. Experiments have been performed to determine the mechanism of this action.

Synthetic *Z*-butylidenephthalide concentration-dependently relaxed phenylephrine (1 μM)- or KCl (60 mM)-induced precontractions of intact and denuded rat aorta rings. The relaxation induced by Bdph was endothelium-independent. Bdph (30–300 μM) concentration-dependently reduced cumulative phenylephrine- and KCl-induced contractions of intact rat aortic rings and non-competitively inhibited their log concentration–response curves. The pD_2' values of Bdph for phenylephrine- and KCl-induced contraction were $3{\cdot}66 \pm 0{\cdot}13$ ($n = 8$) and $3{\cdot}71 \pm 0{\cdot}07$ ($n = 8$), respectively, which were not significantly different from each other. Bdph also concentration-dependently reduced cumulative Ca^{2+}-induced contractions of intact rat aortic rings in high-KCl (60 mM) Ca^{2+}-free physiological salt solution and non-competitively inhibited its log concentration–response curve. The pD_2' value of Bdph for the Ca^{2+}-induced contractions was $3{\cdot}21 \pm 0{\cdot}01$ ($n = 7$) which was significantly different from the pD_2' value obtained from the cumulative KCl-induced contractions.

These results suggest that Bdph inhibits calcium release from calcium stores more selectively than calcium influx from extracellular space via voltage-dependent calcium channels. The inhibition by Bdph of calcium release from KCl-sensitive calcium stores might be similar to its inhibition of calcium release from phenylephrine-sensitive calcium stores. However, because phenylephrine generates inositol-1,4,5-trisphosphate (IP_3) whereas KCl does not, the inhibitory effect of Bdph might not be related to IP_3 production.

The rhizome of *Ligusticum chuaxiong* Hort. (*Ligusticum wallichii* Franch., Umbelliferae), has been used in China for several thousand years to relieve the symptoms of many illnesses including cerebral thrombosis, stroke, headache, spasm, abdominal pain, and gynaecological disease. We have previously isolated and purified three antispasmodics, butylidenephthalide (Bdph), ligustilide and butylphthalide from the neutral oil of the crude drug (Ko et al 1977, 1978). Of these Bdph has proven to be the most active and we have reported its inhibition of calcium influx and calcium release in the guinea-pig ileum (Ko 1980; Ko et al 1997). Our studies have also shown that it increases the rate of perfusion in rabbit ears (Ko et al 1994) and guinea-pig hearts (Ko et al 1992) in vitro, as a result of dilation of blood vessels, and that it relaxes isolated rabbit aortic strips (Ko et al 1994), dilates dog coronary blood vessels in-vivo, and has an antianginal effect (Ko et al 1994). We recently reported that it has a selective anti-anginal effect without changing blood pressure in conscious rats (Ko et al 1998). The mechanism of Bdph-induced dilatation of blood vessels is still unknown. This study sought to determine the nature of this mechanism.

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Materials and Methods

Drugs

Bdph was synthesized and purified according to the method described by Lin et al (1984). Purified Z-Bdph was identified by gas chromatography–mass spectrometry and nuclear magnetic resonance spectrometry. The structure of Z-Bdph is shown in Figure 1. L-phenylephrine hydrochloride, methacholine chloride and papaverine hydrochloride were purchased from Sigma (St Louis, MO). Other reagents, including KCl, were analytical grade.

Preparation

Wistar male rats, 200–300 g, were killed by a blow on the head. The descending thoracic aorta was rapidly dissected and placed in physiological salt solution (PSS) of composition (mM): NaCl 118·3, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4$ 1·2, NaH_2PO_4 1·2, $NaHCO_3$ 25 and dextrose 11·1. After removal of excess fat and connective tissue, the aorta was cut into rings approximately 2 mm long. These were mounted between two stainless steel wire hooks in 5-mL organ baths containing PSS at 37°C which was gassed continuously with 95% O_2–5% CO_2. After equilibration for 1 h under an initial tension of 1 g, the basal tension was approximately 0·5 g. To observe the inhibitory effect of Bdph on cumulative phenylephrine-, KCl- and Ca^{2+}-induced contractions (van Rossum & van den Brink 1963), the rings were pre-incubated with Bdph in normal or in high-KCl (60 mM), Ca^{2+}-free PSS for 5 min before the first addition of the contractile agents. The contraction was measured by use of a force–displacement transducer (Statham UC2) and isometrically recorded on a two-channel polygraph (Gould, OH). When Ca^{2+} was used as the contractile agent the high-KCl (60 mM)-Ca^{2+} free PSS was prepared by replacing an equimolar concentration of NaCl from Ca^{2+}-free PSS; Ca^{2+}-free PSS was prepared by omitting $CaCl_2$ from normal PSS. To determine whether the relaxant responses of Bdph to phenylephrine (1 μM)- and KCl (60 mM)-induced precontractions were endothelium-dependent, aortic rings were denuded by gentle rubbing with a PSS-wetted cotton applicator. No relaxation was induced by methacholine (3 μM) in the denuded rings after precontraction by phenylephrine or KCl (Furchgott & Zawadzki 1980). The concentrations of Bdph (30–300 μM) were cumulatively added when the relaxant response reached a steady state. At the end of the experiment papaverine (0·1 mM) was added to relax the rings maximally. The relaxant effect of Bdph was expressed as a percentage of the relaxation induced by papaverine (100%).

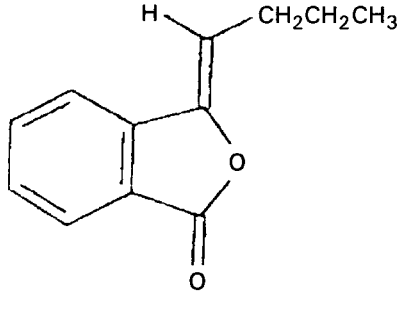

Figure 1. The structure of z-butylidenephthalide.

Statistical analysis

The $-\log IC50$ value was considered to be equal to the negative logarithm of the molar concentrations of Bdph at which a half-maximum inhibitory effect was observed. Calculation of the pD_2' value was performed according to the method described by Ariëns & van Rossum (1957). Statistical significance ($P < 0.05$) was determined by the use of Student's *t*-test.

Results

Effects of Bdph on phenylephrine- and KCl-induced precontractions

Bdph concentration-dependently relaxed the phenylephrine (1 μM)- or KCl (60 mM)-induced precontractions of intact and denuded rat aortic rings (Figure 2). The $-\log IC50$ values of Bdph for intact or denuded preparations were 3·90 ± 0·09 (n = 8) and 3·92 ± 0·08 (n = 8), respectively for

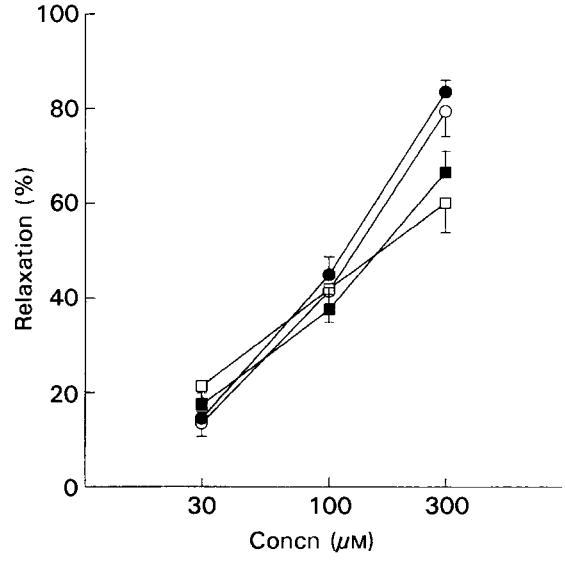

Figure 2. Log concentration–relaxation effect of Bdph on KCl (60 mM; ○, ●)- and phenylephrine (1 μM; □, ■)-induced precontractions of intact (○, □) and denuded (●, ■) rat aortic rings. Each point represents the mean ± s.e.m. of results from eight experiments.

phenylephrine-induced contractions and 3·66± 0·14 (n = 8) and 3·67±0·16 (n = 8), respectively, for KCl-induced contractions; these values were not significantly different. Therefore, the relaxation induced by Bdph was endothelium-independent. The induced relaxations were also not significantly different from phenylephrine- and KCl-induced precontractions in the same kind of preparation.

Inhibitory effects of Bdph on cumulative phenylephrine- and KCl-induced contractions

Bdph (30–300 μM) concentration-dependently reduced cumulative phenylephrine-induced (Figure 3) and KCl-induced (Figure 4) contractions of intact rat aortic rings and non-competitively inhibited their log concentration–response curves. The pD_2' were 3·66±0·13 (n = 8) and 3·71±0·07 (n = 8), respectively, not significantly different from each other. This suggests that the inhibitory ability of Bdph on calcium release from phenylephrine-sensitive calcium stores might be similar to that on KCl-sensitive calcium stores.

Effects of Bdph on Ca^{2+}-induced contractions in depolarized preparations

Bdph also concentration-dependently reduced cumulative Ca^{2+}-induced contractions of intact rat aortic rings in high-KCl (60 mM)-Ca^{2+}-free PSS (Figure 5) and non-competitively inhibited its log concentration–response curve. The pD_2' value of Bdph was 3·21±0·01 (n = 7). This was significantly different from the inhibitory effect of Bdph on cumulative KCl-induced contractions.

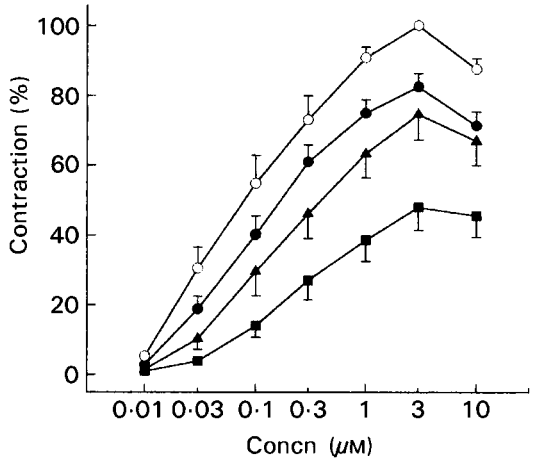

Figure 3. Inhibitory effect of Bdph (●, 30 μM; ▲, 100 μM; ■, 300 μM) on phenylephrine-induced contractions of rat aortic rings. Maximum contraction in the control (○, vehicle, 0·1% alcohol) was set as 100%. Each point represents the mean ± s.e.m. of results from eight experiments.

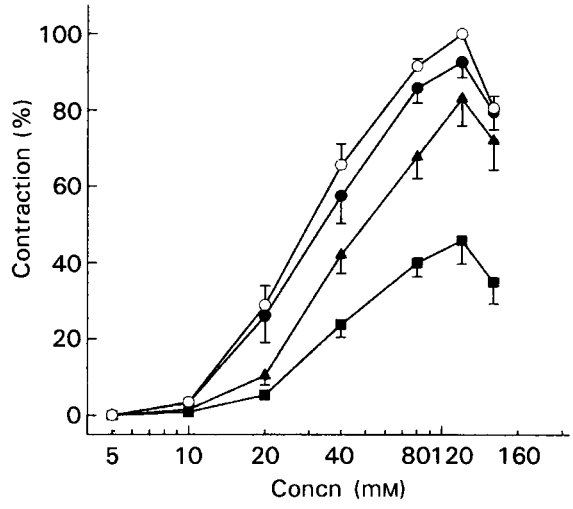

Figure 4. Inhibitory effect of Bdph (●, 30 μM; ▲, 100 μM; ■, 300 μM) on KCl-induced contractions of rat aortic rings. Maximum contraction in the control (○, vehicle, 0·1% alcohol) was set as 100%. Each point represents the mean ± s.e.m. of results from eight experiments.

This suggests that Bdph inhibited calcium release from calcium stores more selectively than it inhibited calcium influx from extracellular space via voltage-dependent calcium channels.

Discussion

Furchgott & Zawadzki (1980) first reported that endogenous vasodilators such as acetylcholine do

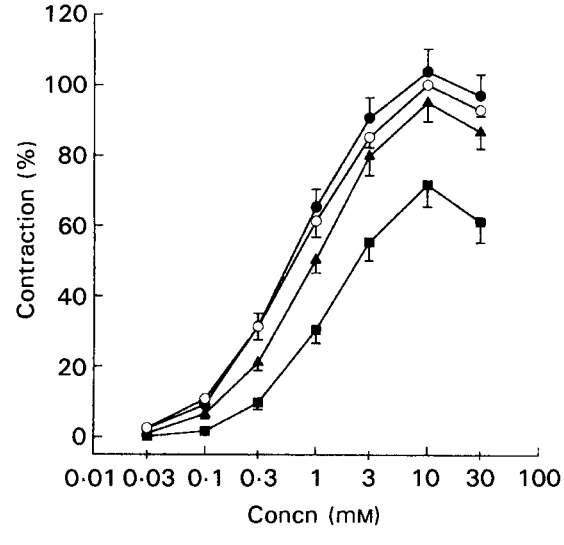

Figure 5. Inhibitory effect of Bdph (●, 30 μM; ▲, 100 μM; ■, 300 μM) on Ca^{2+}-induced contractions of depolarized (KCl, 60 mM) rat aortic rings. Maximum contraction in the control (○, vehicle, 0·1% alcohol) was set as 100%. Each point represents the mean ± s.e.m. (standard error of the mean) of results from seven experiments.

not act directly on vascular smooth muscle but instead act on endothelial cells, causing them to release a labile factor that diffuses into the overlying smooth muscle and induces relaxation by activating guanylate cyclase within the muscle. It has been suggested (Ignarro et al 1987; Palmer et al 1987) that nitric oxide (NO), which is synthesized from L-arginine by a citrulline-forming enzyme known as NO synthase (Palmer et al 1988) is the endothelium-derived relaxing factor. The above results suggest that Bdph elicits endothelium-independent relaxation and relaxes both phenylephrine- and KCl-induced precontraction with equal potency (Figure 2). There is, therefore, no relationship between NO and the Bdph-induced relaxation which acts on vascular smooth muscle itself.

Phenylephrine, a selective α_1 (both α_{1a} and α_{1b})-adrenoceptor agonist, induces two distinct components of contractile responses (Timmermans & Thoolen 1987; Oriowo & Ruffolo 1992)—a transient phasic contraction which is dependent on the liberation of intracellular calcium stores, and a sustained tonic contraction which is maintained by the influx of extracellular calcium through dihydropyridine-sensitive (voltage-dependent) and insensitive (receptor-operated) calcium channels via activation of α_{1a} and α_{1b} receptors, respectively (Minneman 1988). Stimulation of α_1-adrenoreceptors results in the regulation of at least four effector systems (Lomasney et al 1991; Bylund 1992). The primary mode of signal transduction involves the mobilization of intracellular calcium from endoplasmic reticulum, a major intracellular calcium store. This increase of intracellular calcium is currently believed to result from activation of phospholipase C_β isoforms through the G_q family of G proteins (Lefkowitz et al 1996). The hydrolysis of membrane-bound polyphosphoinositides via phospholipase C results in the generation of two second messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 stimulates the release of Ca^{2+} from intracellular stores via a specific receptor-mediated process, whereas DAG is a potent activator of protein kinase C (Berridge 1993). The increased concentration of intracellular calcium ($[Ca^{2+}]_i$) ultimately causes contraction as a result of activation of calcium-sensitive protein kinase, such as the calmodulin-dependent myosin light-chain kinase, and phosphorylation of the light chain (Stull et al 1990). Bdph non-competitively and concentration-dependently inhibits the cumulative phenylephrine-induced contractions (Figure 3) which were mainly phasic in nature (van Rossum & van den Brink 1963). Therefore, Bdph might inhibit calcium release from intracellular calcium stores. Bdph also concentration-dependently relaxes the phenylephrine-induced precontractions (Figure 2) which were mainly tonic in nature and might, therefore, inhibit calcium influx from extracellular space via voltage-dependent and receptor-operated calcium channels.

KCl, a non-selective and depolarizing contractile agent which does not invoke IP_3 production (Chiu et al 1987), also induces two contractile responses, transient phasic and sustained tonic contractions, in isolated rat aortic rings (Yamashita et al 1994). It has been suggested that with K^+-depolarization, calcium is directly released from intracellular stores, but that the marked and sustained elevation of $[Ca^{2+}]_i$ observed in the presence of extracellular calcium is mainly a result of influx of extracellular Ca^{2+} (Kobayashi et al 1985). The magnitude of $[Ca^{2+}]_i$ elevation induced by K^+-depolarization in the absence of extracellular calcium was much less than that observed in the presence of extracellular calcium (Kanaide 1990). In other words, calcium release from the intracellular stores might be less than calcium influx from extracellular space by K^+-depolarization. Bdph, therefore, more selectively inhibits calcium release than calcium influx. However, the strength of the inhibitory effects of Bdph on calcium release from KCl-sensitive calcium stores might be similar to that from phenylephrine-sensitive calcium stores (see Results). This suggests that the inhibitory effects of Bdph on calcium release from intracellular calcium stores might be unrelated to the production of IP_3.

Acknowledgement

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