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Stereoselectivity of Butylidenephthalide on Voltage-dependent Calcium Channels in Guinea-pig Isolated Ileum

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Abstract

Two geometric isomers, the *Z*- and the *E*- forms, can be separated from synthetic mixtures of butylidenephthalide (Bdph).

Z-Bdph (50–100 μM) non-competitively inhibited Ca^{2+}-induced contractions in depolarized (K^+, 60 mM) guinea-pig ileum longitudinal smooth muscle, with a pD_2' value of 3·88 ± 0·20 (n = 5). However, *E*-Bdph (20–100 μM) competitively inhibited these contractions with a pA_2 value of 4·56 ± 0·18 (n = 5) which was significantly ($P < 0·05$) greater than the pD_2' value of *Z*-Bdph. In contrast, the two isomers had no stereoselective inhibitory action on Ca^{2+} influx through pre- or post-junctional membranes of cholinergic nerve endings from which the transmitter acetylcholine is released or on Ca^{2+} release from intracellular stores.

Therefore, the *trans-Z* and *cis-E* forms of Bdph might have geometric stereoselectivity for voltage-dependent calcium channels (VDC) in guinea-pig longitudinal smooth muscle. Both isomers might inhibit more selectively the contractile twitch responses evoked by electrical stimulation than by cumulative acetylcholine- or carbachol-induced transient contractions in guinea-pig ileum longitudinal smooth muscle.

Much evidence indicates that isomers of some 1,4-dihydropyridine derivatives have stereoselective effects on voltage-dependent calcium channels of vascular smooth muscle (Sanguinetti & Kass 1984; Triggle 1990; Poulsen et al 1992). The calcium agonism and antagonism properties might reside in either the (+) or the (−) isomer of a single chiral 1,4-dihydropyridine molecule (Franckowiak et al 1985; Hof et al 1985). For example, (+)-202-791 is a calcium-channel activator, whereas the (−) isomer is a calcium-channel inhibitor (Wei et al 1986). However, some 1,4-dihydropyridine isomers, such as both (+)-*S*-12967 and (−)-*S*-12968, have a dual action of agonism and antagonism at lower and higher concentrations, respectively (Poulsen et al 1992). Recently, Vo et al (1995) reported a 2-pyridinyl isomer (±)-12 that acts as a calcium-channel agonist on the guinea-pig left atrium and a calcium channel antagonist in guinea-pig ileum longitudinal smooth muscle. In contrast, the 3-pyridinyl ((±)-13) and 4-pyridinyl ((±)-14) isomers act as calcium-channel agonists on both guinea-pig left atrium and guinea-pig ileum longitudinal smooth muscle. The (+)-2-pyridinyl enantiomer (+)-12A has agonist activity on both guinea-pig ileum longitudinal smooth muscle and guinea-pig left atrium, whereas the (−)-2-pyridinyl enantiomer (−)-12B has agonist activity on guinea-pig left atrium and antagonist activity on guinea-pig ileum longitudinal smooth muscle. Not all 1,4-dihydropyridine optical-isomer-pairs have quantitative enantioselective properties, and many optical isomers differ only in their potency of action—e.g. both enantiomers of PN200-110 are calcium inhibitors, but they differ in their potency and thus have marked quantitative stereoselective properties (Morel & Godfraind 1987).

Butylidenephthalide (Bdph), isolated from *Ligusticum chuaxiong* Hort. (*Ligusticum wallichii* Franch.; Ko et al 1977, 1978), has been reported to inhibit calcium influx and calcium release from guinea-pig ileum longitudinal smooth muscle (Ko 1980) and to have an antianginal effect (Ko et al 1992, 1994, 1996). In contrast with 1,4-dihydropyridine, Bdph has a planar olefin double bond and therefore has two geometric isomers, *Z*- and *E*-Bdph (Fig. 1). The aim of this study was to investigate whether or not Bdph was stereoselective in its inhibition of voltage-dependent calcium channels in depolarized guinea-pig ileum longitudinal smooth muscle.

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Materials and Methods

Preparation

Approximately 2 cm of ileum was removed 10 cm above the caecum of male Hartley guinea-pigs, 300–400 g, and mounted in 5 mL normal Locke-Ringer's solution (LRS), oxygenated with 95% O_2–5% CO_2 and maintained at 32°C, and equilibrated for 1 h under an initial tension of 1 g. To observe the inhibitory effect of *Z*- or *E*-Bdph on cumulative carbachol- or acetylcholine-induced contractions (van Rossum & van den Brink 1963), the ileum was preincubated with *Z*- or *E*-Bdph in normal LRS for 10 min before the first addition of carbachol or acetylcholine. According to the method described by Paton (1955), electrical coaxial stimulation (60 V, 1 ms, 0·2 Hz) was derived from a Grass S-88 stimulator and applied to the tissues. The twitch responses induced by electrical stimulation and contractile responses to carbachol were isometrically (Grass FT ·03) recorded on a two-channel polygraph (Gould, OH). After equilibration of the control twitch tension, either isomers *Z*- or *E*-Bdph or their vehicle (0·1–0·6% alcohol) were cumulatively added to the organ bath to inhibit the twitch responses. The concentrations of *Z*- or *E*-Bdph which inhibited the control twitch tension by half (IC50) were calculated. To observe the inhibitory effects of *Z*- or *E*-Bdph on Ca^{2+} influx across the junctional membranes in the electrically-stimulated and depolarized (high K^+, 60 mM) guinea-pig ileum, normal

LRS was replaced by Ca^{2+}-free LRS containing ethylenediaminetetraacetic acid (EDTA) (0·1 mM). Before preincubation with *Z*- or *E*-Bdph, the tissues were washed for 10 min with Ca^{2+}-free LRS containing ethyleneglycoltetraacetic acid (EGTA) (0·1 mM) until there was no response to electrical stimulation and no spontaneous contraction without electrical stimulation. The bath solution was then replaced with Ca^{2+}-free or high-K^+ (60 mM) Ca^{2+}-free LRS without EGTA, and Ca^{2+} cumulatively added until the response reached its maximum. The maximum response of the control was taken as 100%. *Z*-Bdph, *E*-Bdph, or their vehicle (0·1% alcohol) was applied 10 min before the cumulative addition of Ca^{2+} to construct log concentration–response curves. Normal LRS consisted of (mM): NaCl 154, KCl 5·6, $CaCl_2$ 2·2, $MgCl_2$ 2·1, $NaHCO_3$ 5·9 and dextrose 2·8. Ca^{2+}-free LRS was prepared by omitting $CaCl_2$ from normal LRS and high-K^+ (60 mM), Ca^{2+}-free LRS was prepared by replacing an equimolar concentration of NaCl from Ca^{2+}-free LRS with KCl from Ca^{2+}-free LRS. Each preparation was preincubated at only one concentration of *Z*- or *E*-Bdph.

Drugs

Bdph was prepared via Perkin synthesis and was mainly the thermodynamically stable *Z*-isomer, but it also partly transformed into the *E*-form. They were chromatographed over silica gel by step-wise elution with 1%–50% gradients of an ether–methanol mixture (98 : 2) in *n*-hexane. The purified *Z*- and *E*-Bdph isomers were identified by GC-MS and by NMR spectrometry (Gijbels 1983); their structures are shown in Fig. 1. Tetrodotoxin (TTX), atropine sulphate, nifedipine and carbachol chloride were purchased from Sigma (St Louis, MO). Acetylcholine chloride was purchased from Daiichi Pharmaceutical (Tokyo, Japan). Other reagents were analytical grade.

Statistical analysis

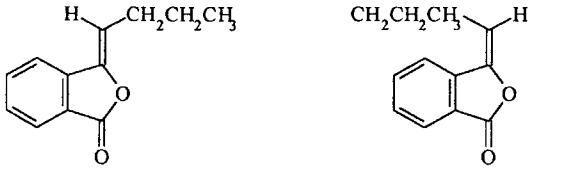
The −log IC50 value was considered to be equal to the negative logarithm of the molar concentrations of Bdph at which a half-maximum inhibitory effect was observed. Calculation of pA_2 or pD_2' values was performed according to the method described by Ariëns & van Rossum (1957). Statistical significance ($P < 0.05$) was determined by use of Student's *t*-test.

Results

The effect of Bdph on twitch responses evoked by electrical stimulation

The twitch responses to electrical stimulation of guinea-pig ileum longitudinal smooth muscle were completely abolished by 1 μM TTX or 1 μM atropine, but were resistant to nifedipine (data not shown) and therefore should have been elicited as a result of cholinergic excitation. The control twitch tension before the addition of *Z*- or *E*-Bdph was 1·64 ± 0·08 g (n = 16) and 1·54 ± 0·06 g (n = 18), respectively. They reduced concentration-dependently and almost abolished the twitch response at 100 μM (Fig. 2). Their −log IC50 values were 4·70 ± 0·06 (n = 8) and 4·77 ± 0·09 (n = 9), respectively, which were not significantly different. However, 0·6% alcohol only slightly inhibited twitch tension by 11·1 ± 4·7% (n = 17).

The cumulative Ca^{2+}-induced twitch responses in electrically stimulated guinea-pig ileum longitudinal smooth muscle were calcium-concentration-dependent. The maximum response was at 4·4 mM Ca^{2+} (Fig. 3). The maximum twitch tension before preincubation of *Z*- or *E*-Bdph was 1·80 ± 0·14 g (n = 21) and 2·02 ± 0·08 g (n = 24), respectively, which were not significantly ($P > 0.1$) different. The cumulative Ca^{2+}-induced twitch responses were also abolished by 1 μM TTX or 1 μM atropine (data not shown), but were resistant to nifedipine. Nifedipine at concentrations of 1 and 10 μM, inhibited the maximum control twitch tension by only 13·7 ± 4·4% (n = 6) and 49·3 ± 4·7% (n = 6), respectively. This result was consistent with the observation of Cousins et al (1993). *Z*- or *E*-Bdph non-competitively inhibited the cumulative Ca^{2+}-induced twitch responses and shifted the log concentration–response curves to the right with pD_2' values of 4·40 ± 0·12 (n = 7) and 4·19 ± 0·06 (n = 6), respectively. There was no significant difference between these two values ($P > 0.1$).



Z-Butylidenephthalide *E*-Butylidenephthalide

FIG. 1. Structures of the *Z*- and *E*-stereoisomers of synthetic butylidenephthalide.

Effects of Bdph on cumulative carbachol- and acetylcholine-induced transient contractions

Z- and *E*-Bdph concentration-dependently reduced the cumulative carbachol-induced transient contractions and non-competitively inhibited its log concentration–response curves, respectively (Fig. 4). Their respective pD_2' values were

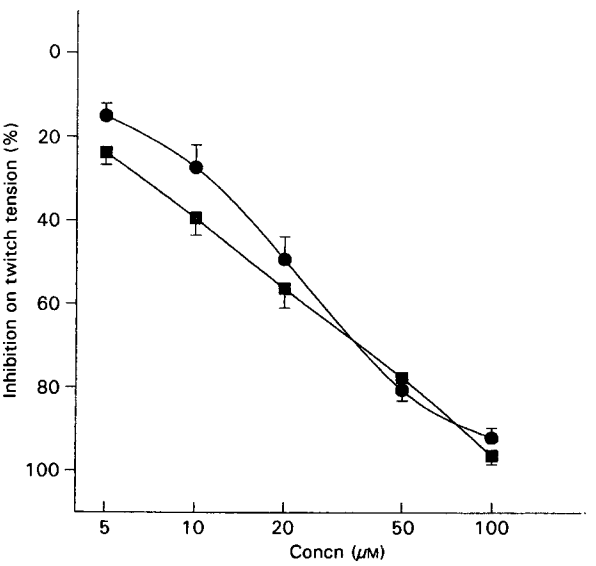


FIG. 2. Log concentration-inhibition on twitch tension curves of *Z*- (●) and *E*- (■) Bdph in guinea-pig ileum. There was no significant difference between the −log IC50 values of these two isomers. The inhibitory effects of *Z*- and *E*-Bdph do not include those of the vehicle (0·1–0·6% alcohol). Each point and −log IC50 value represents the mean ± s.e.m. of results from 8 or 9 experiments.

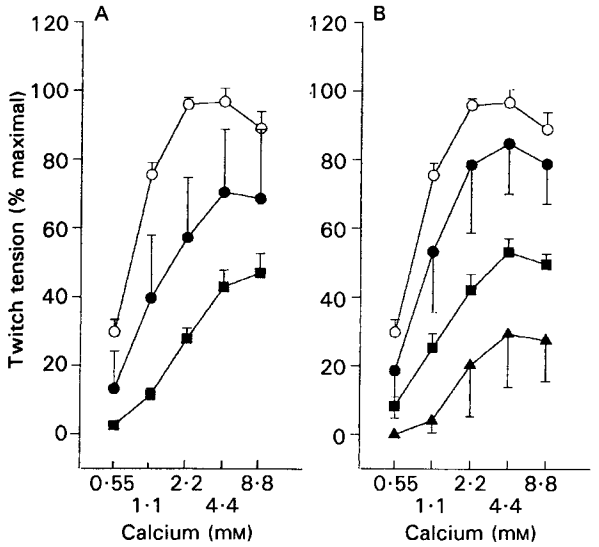


FIG. 3. A. Effects of *Z*- (● 50 μM, ■ 100 μM) and B. *E*- (● 20 μM, ■ 50 μM, ▲ 100 μM) Bdph on Ca^{2+}-induced twitch responses evoked by electrical stimulation in guinea-pig ileum. Ca^{2+} was cumulatively added to the Ca^{2+}-free bathing solution. The vehicle (○) was 0·1% alcohol. Each point and pD_2' value represents the mean ± s.e.m. of results from 6 or 7 experiments.

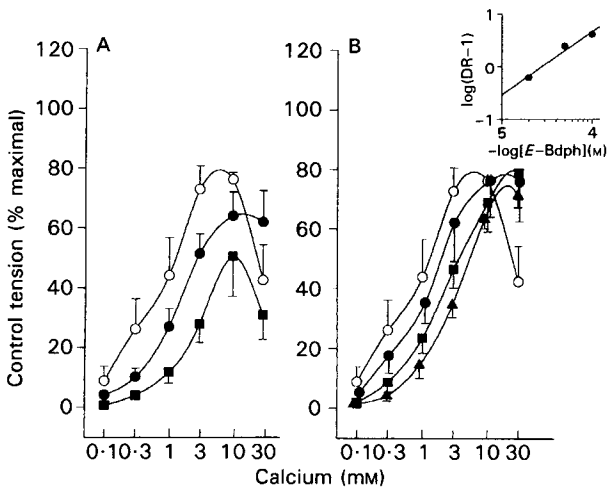


FIG. 5. A. Effects of *Z*- (● 50 μM, ■ 100 μM) and B. *E*- (● 20 μM, ■ 50 μM, ▲ 100 μM) Bdph on Ca^{2+}-induced contractions in depolarized (K^+, 60 mM) guinea-pig ileum. Ca^{2+} was cumulatively added to a high-K^+ (60 mM), Ca^{2+}-free bathing solution. The vehicle (○) was 0·1% alcohol. Each point and pD_2' and pA_2 value represents the mean ± s.e.m. of results from five experiments. The relationship between −log concentration of *E*-Bdph and log(DR-1), where DR is the dose ratio, is shown in the inset. The Schild regression equation for *E*-Bdph is Y = 5·48 − 1·20X (r = 0·985).

3·19 ± 0·13 (n = 5) and 3·79 ± 0·23 (n = 6) which were also not significantly different. Both isomers also non-competitively inhibited the cumulative acetylcholine-induced contractions and rightwards shifted the log concentration–response curve (figure not shown). Their pD_2' values were 4·12 ± 0·08 (n = 5) and 3·96 ± 0·11 (n = 5), respectively, not significantly different from each other.

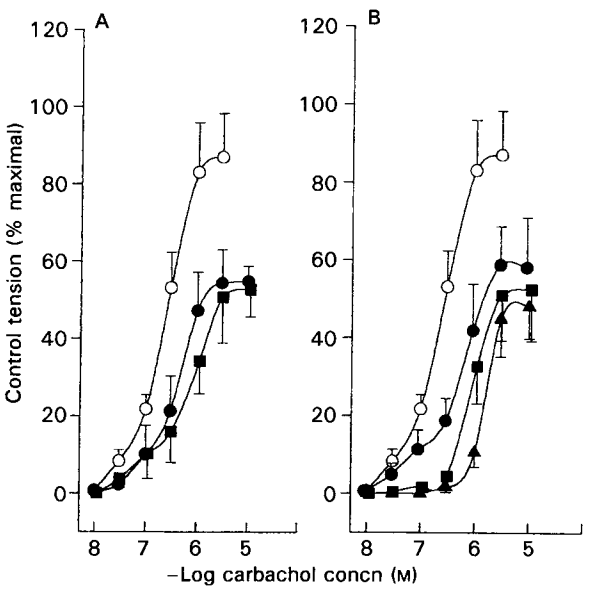


FIG. 4. A. Effects of *Z*- (● 50 μM, ■ 100 μM) and B. *E*- (● 20 μM, ■ 50 μM, ▲ 100 μM) Bdph on cumulative carbachol-induced transient contractions in the guinea-pig ileum. The vehicle (○) was 0·1% alcohol. Each point and pD_2' value represents the mean ± s.e.m. of results from 5 or 6 experiments.

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

In high K^+ (60 mM), Ca^{2+}-free LRS, however, *E*-Bdph (20–100 μM) competitively inhibited the cumulative Ca^{2+}-induced contractions, and shifted the log concentration–response curves parallel to the right (Fig. 5B), although Z-Bdph (50–100 μM) inhibited them non-competitively (Fig. 5A). Z-Bdph shifted them to the right and concentration-dependently inhibited the maximum contractions with a pD_2' value of 3·88 ± 0·20 (n = 5), whereas *E*-Bdph did not inhibit the maximum contractions (pA_2 value 4·56 ± 0·18; n = 5). The Schild regression equation for *E*-Bdph was Y = 5·48 − 1·20X (r = 0·985) (Fig. 5 inset).

Discussion

Most of the excitatory neurones innervating guinea-pig ileum longitudinal smooth muscle are thought to release acetylcholine which interacts with a muscarinic receptor to trigger excitatory junctional potentials and contractions (Kapilta & Triggle 1983; Cousins et al 1993). Activation of the muscarinic receptor causes liberation from the plasma membrane of the second messenger D-*myo*-inositol-1,4,5-trisphosphate, which in turn leads to the release of stored Ca^{2+} (Bolton & Lim 1989; Komori & Bolton 1990, 1991). Therefore twitch–contractile responses evoked by electrical stimulation are the final results of a cascade of the processes promoting increased contraction of free cytoplasmic Ca^{2+} in the vicinity of contractile proteins. From the results of this study, the twitch responses to electrical stimulation at a normal Ca^{2+} concentration or at various concentrations of Ca^{2+} (0·55–8·8 mM) added exogenously to Ca^{2+}-free medium, were insensitive to nifedipine. These results are consistent with the findings of other investigators (Kapilta & Triggle 1983; Bauer et al 1991; Cousins et al 1993). It has been suggested that nifedipine acts mainly at post-

Table 1. Comparison of the inhibitory effects of *Z*- and *E*-butylidenephthalide in isolated guinea-pig ileum.

Method	*Z*-Butylidenephthalide	*E*-Butylidenephthalide
Twitch responses to electrical stimulation	$-\log IC50 = 4.77 \pm 0.09$ (9)	$-\log IC50 = 4.70 \pm 0.06$ (9)
Cumulative Ca^{2+}-induced twitch responses to electrical stimulation	$pD_2' = 4.40 \pm 0.12$ (7)†	$pD_2' = 4.19 \pm 0.06$ (6)‡
Contractile responses to acetylcholine	$pD_2' = 4.12 \pm 0.08$ (5)‡	$pD_2' = 3.96 \pm 0.11$ (5)‡
Contractile responses to carbachol	$pD_2' = 3.91 \pm 0.13$ (5)‡	$pD_2' = 3.79 \pm 0.23$ (6)‡
Cumulative Ca^{2+}-induced contractions in depolarized (K^+, 60 mM) preparation	$pD_2' = 3.88 \pm 0.20$ (5)*‡	$pA_2 = 4.56 \pm 0.18$ (5)

Values are the means ± s.e.m. of results from n experiments, where n is the number in parentheses. *$P < 0.05$ when compared with pA_2 for *E*-Bdph and †$P < 0.05$, ‡$P < 0.001$ when compared with their corresponding −log IC50 values.

synaptic, but not presynaptic, action sites on the guinea-pig ileum longitudinal smooth muscle membrane (Kažič & Miloslavljevič 1980; Kapilta & Triggle 1983). In contrast, Ca^{2+}-induced contractions in high-K^+ medium and sustained (tonic) contractions induced by agonists are more sensitive to the action of Ca^{2+}-channel blockers than transient (phasic) contractions induced by agonists and nerve stimulation (Bolton 1979; Karaki & Weiss 1984; Varagič et al 1984; Karaki & Mitsui 1988; Bauer et al 1991; Cousins et al 1993). It is generally assumed that the phasic response to agonists involves the release of stored Ca^{2+} and the tonic response is the result of an increased influx of Ca^{2+} across the membrane through receptor-operated calcium channels (Urakawa & Holland 1966; Imai & Takeda 1967; Bolton 1979; Godfraind 1981; Karaki & Weiss 1984, 1988; Michelson & Ziegler 1984; Ahn et al 1988). Under conditions of membrane depolarization, Ca^{2+} influx occurs through voltage-dependent calcium channels, and nifedipine (3–30 nM) markedly and non-competitively inhibits Ca^{2+}-induced contractions in guinea-pig ileum longitudinal smooth muscle (Bauer et al 1991), although it has been suggested that the calcium entry blocker might block Ca^{2+}-induced contractions of smooth muscle by competitive antagonism (Triggle 1981). The results of this study show that *Z*-Bdph (50–100 μM) non-competitively inhibited Ca^{2+}-induced contractions with a pD_2' value of 3.88 ± 0.20 ($n = 5$; Fig. 5A). However, *E*-Bdph (20–100 μM) competitively inhibited the contractions with a pA_2 value of 4.56 ± 0.18 ($n = 5$; Fig. 5B) which was significantly ($P < 0.05$) greater than the pD_2' value for *Z*-Bdph (Table 1). Though *E*-Bdph at concentrations above 100 μM might inhibit these contractions in a non-competitive, but not competitive, manner, both isomers at the same concentration (100 μM) showed different properties. Therefore, the *trans Z*- and *cis E*-forms of Bdph might have stereoselective properties on voltage-dependent calcium channels in guinea-pig ileum longitudinal smooth muscle. In contrast, these two isomers might have no stereoselective inhibitory property on Ca^{2+} influx through pre- or post-junctional membranes of cholinergic nerve endings from which the transmitter acetylcholine is released. From these results it is suggested that both isomers non-competitively inhibit Ca^{2+}-induced twitch responses to electrical stimulation and that there is no significant difference between their pD_2' values (Fig. 3). Also, these two isomers non-competitively inhibit cumulative acetylcholine- or carbachol (Fig. 4)-induced contractions and again their pD_2' values are not significantly different from each other (Table 1). This suggests that there might be no stereoselective inhibitory action on Ca^{2+} release from intracellular Ca^{2+} stores.

In contrast with nifedipine, which is insensitive to inhibiting-twitch responses evoked by electrical stimulation and similar to the inhibition of the phasic components of acetylcholine-induced contractions (Kapilta & Triggle 1983), both *Z*- and *E*-Bdph isomers might inhibit the twitch responses more selectively. From the results it is suggested that the −log IC50 value of each isomer (Fig. 2) was significantly ($P < 0.001$) greater than the pD_2' value not only for cumulative acetylcholine- but also for cumulative carbachol-induced contractions (Table 1). How the twitch responses by both isomers might be selectively reduced is still unknown. Further experiments are necessary to clarify the mechanisms involved in the action of Bdph in guinea-pig ileum longitudinal smooth muscle.

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