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Modification of Ca^{2+} Metabolism in the Rabbit Aorta as a Mechanism of Spasmolytic Action of Warifteine, a Bisbenzylisoquinoline Alkaloid Isolated from the Leaves of *Cissampelos sympodialis* Eichl. (Menispermaceae)

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Abstract

The regulation of intracellular Ca^{2+} as a mechanism of spasmolytic activity of a bisbenzylisoquinoline alkaloid, warifteine, isolated from the leaves of *Cissampelos sympodialis*, Eichl (Menispermaceae) was studied in the rabbit aorta.

Warifteine (pD'_2 4·12 ± 0·09) similar to verapamil (pD'_2 6·89 ● 0·05) antagonized, in a noncompetitive and reversible manner, KCl-induced contractions, mediated by Ca^{2+} entry through voltage-operated channels. Noradrenaline-induced sustained contractions mediated by Ca^{2+} entry through receptor-operated channels were also inhibited by warifteine (IC50 $6·03 \times 10^{-5}$ M) and the standard agent sodium nitroprusside (IC50 $1·9 \times 10^{-8}$ M). In Ca^{2+}-free medium, the alkaloid reduced the intracellular Ca^{2+}-dependent transient contraction to noradrenaline by inhibiting the release of Ca^{2+} (IC50 $2·6 \times 10^{-5}$ M) from the stores and the refilling (IC50 $1·9 \times 10^{-5}$ M) of the intracellular stores. The standard agent, procaine, also inhibited the release of Ca^{2+} (IC50 $3·2 \times 10^{-5}$ M) but had no significant effect on Ca^{2+} uptake into the stores. Warifteine failed to affect intracellular Ca^{2+} stores sensitive to caffeine, while procaine inhibited (IC50 $7·9 \times 10^{-4}$ M) the release of Ca^{2+} from these stores.

The results indicate that warifteine may cause muscle relaxation by inhibiting Ca^{2+} channels and by modifying the intracellular Ca^{2+} stores sensitive to noradrenaline.

A hot water infusion of the root bark of *Cissampelos sympodialis* Eichl is used in Brazil for the treatment of asthma, bronchitis, influenza, and rheumatism, among other conditions (Correa 1929). In our laboratory, Araújo et al (1991) detected potent nonspecific relaxant activity of the aqueous fraction of the ethanolic extract of the root in a number of isolated smooth-muscle preparations such as the guinea-pig ileum and trachea, and the rat uterus. A bisbenzylisoquinoline alkaloid, warifteine (Fig. 1), the major component which was isolated from the ethanolic extract, was also found to relax both vascular and nonvascular smooth-muscle tissues (Côrtes 1992). Studies on the mode of action of warifteine showed that it antagonized voltage-dependent Ca^{2+} channels in the guinea-pig ileum, but not in the rat uterus, and that the compound inhibited the release of Ca^{2+} from intracellular stores sensitive to noradrenaline in the rabbit aorta (Côrtes et al 1992). More recent studies on the mechanism of action of warifteine in the rabbit aorta are reported in the present work. The rabbit aorta was chosen, as extensive information on the response of this tissue to standard stimulants and inhibitors of cell calcium is available (Somlyo & Somlyo 1968; Karaki et al 1979, 1986; Reuter 1983). Further, it is also known that the rabbit aorta possesses distinct voltage and receptor-operated Ca^{2+} channels which can be specifically modified by agents (Bolton 1979; Van Breemen et al 1979; Meisheri et al 1981). The alkaloid was tested for its ability to inhibit voltage-dependent and receptor-operated Ca^{2+} channels, and to modify the intracellular Ca^{2+} stores sensitive to both noradrenaline and caffeine. The activity of warifteine was compared with agents that are known to have defined effects on the different parameters under investigation.

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Materials and Methods

General

The aortic strips from albino rabbits, 2·5–3·0 kg, were prepared according to the method of Karaki et al (1979) and Ahn & Karaki (1988). Briefly, the procedure involved cutting the aorta, from which the adventitial layer was previously removed, into helical strips of 2·5–3·0-cm in length. The tissues were suspended in appropriate physiological solution (Krebs-Henseleit (mM): NaCl 118·0, KCl 4·7, KH_2PO_4 1·2, $MgSO_4 \cdot 7H_2O$ 1·2, $NaHCO_3$ 25·0, glucose 11·2, $CaCl_2$ 2·5) at 37°C if not otherwise mentioned, and bubbled with a mixture of 95% O_2 – 5% CO_2. The strips were allowed to equilibrate for 1 h under a resting tension of 1 g. Force generation was monitored with an isometric transducer coupled to a physiograph (Ugo Basile).

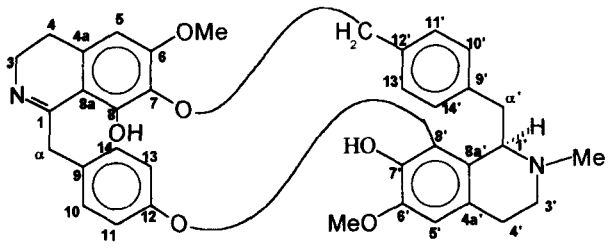

FIG. 1. Structure of warifteine.

Effect on voltage-dependent Ca^{2+} channels in the aorta

Two cumulative concentration–response curves of approximately equal magnitude were obtained with KCl. After washing, the aorta was incubated with warifteine for 15 min and a third concentration–response curve for KCl was constructed in the presence of warifteine. The tissue was washed and when the response to KCl was recovered after repeated washings, the procedure was repeated with different concentrations of the alkaloid. In other tissues verapamil was employed as the standard antagonist. Antagonism was measured by calculating pD_2' values (Van Rossum 1963) defined as the −log molar concentration of the antagonist which produced a 50% reduction of the maximum contraction.

Effect on receptor-operated Ca^{2+} channels in the aorta

In the aorta, two submaximal (65–75% maximal) tonic responses to noradrenaline (10^{-7} M) which stabilized in 15 min were obtained initially. A third response was then obtained and warifteine was added cumulatively at 15-min intervals in an attempt to obtain a concentration–inhibition curve. A 15-min time interval was required to obtain the maximum effect with each dose of warifteine. In other tissues sodium nitroprusside was tested as the inhibitor (Karaki et al 1986) where a time interval of 7 min was used. When possible, the IC50 values were obtained graphically from concentration–inhibition curves.

Effect on intracellular Ca^{2+} stores responsible for noradrenaline-induced transient contractions of the aorta

The inhibitory effects of warifteine and the standard agent procaine on the release of intracellular Ca^{2+} were studied based on the method described by Karaki et al (1979). The tissue was allowed to equilibrate for 2 h in normal Krebs-Henseleit solution under a resting tension of 1 g. Two transient contractions to noradrenaline (10^{-6} M) were obtained as follows. The tissue was washed twice with a Ca^{2+}-free solution (Krebs-Henseleit solution without $CaCl_2$ + 1 mM EGTA) and kept in this solution for 15 min before adding noradrenaline (10^{-6} M) to obtain a transient response to the agonist. The tissue was washed and resuspended in normal Krebs-Henseleit solution (calcium loading) for 15 min. It was found in preliminary studies that noradrenaline was incapable of producing a second contraction in the absence of Ca^{2+} loading. At the end of this period of Ca^{2+} loading, the aorta was washed twice with the Ca^{2+}-free solution and resuspended in this solution for 15 min before obtaining a second noradrenaline response. The above procedure was repeated to obtain a third response to noradrenaline, but in the presence of various concentrations of warifteine ($1{\cdot}0 \times 10^{-5}$–$1{\cdot}0 \times 10^{-4}$ M) or procaine ($1{\cdot}0 \times 10^{-5}$–$3{\cdot}0 \times 10^{-4}$ M) which are added 15 min before the addition of noradrenaline. Inhibition of the noradrenaline response was evaluated by comparing the mean response obtained in the presence or in the absence of the inhibitors. IC50 values were calculated graphically from the concentration–response curves.

The effect of warifteine or procaine on the refilling of intracellular Ca^{2+} stores was studied as described above, except that the test compound was present throughout the 15-min calcium-loading period before the attempt to construct the third noradrenaline response. Thus, at the end of the 15-min incubation period, the tissue was washed twice and resuspended in the Ca^{2+}-free solution for 10 min before adding noradrenaline. Inhibition of the noradrenaline response was evaluated by comparing the mean contractions obtained before and after the exposure of the tissue to the test compound (warifteine or procaine).

Effect on intracellular Ca^{2+} stores responsible for caffeine-induced transient contractions of the aorta

The effects of warifteine and procaine on the release of intracellular Ca^{2+} and refilling of intracellular Ca^{2+} stores responsible for the transient contractions induced by caffeine were studied as described above for the noradrenaline response, except that the experiments were performed at 21°C as recommended by Karaki et al (1987). Similar to noradrenaline, caffeine-induced contractions were also found to depend on replenishing the calcium stores after each addition of caffeine (3×10^{-2} M).

Results

Effect on voltage-dependent Ca^{2+} channels

Warifteine (1×10^{-5} to $1{\cdot}6 \times 10^{-4}$ M) antagonized the cumulative concentration–effect curves to KCl (Fig. 2A). The antagonism was reversible and non-competitive in nature as there was a non-parallel shift to the right and suppression of the maximal response. Further, the maximum inhibition obtained with warifteine was approximately 60% as there was no significant differences between the last two doses of the compound. Verapamil (1×10^{-7} to 8×10^{-7} M) also inhibited the KCl response in a non-competitive manner (Fig. 2B). The pD_2' values for warifteine and verapamil were $4{\cdot}12 \pm 0{\cdot}09$ and $6{\cdot}98 \pm 0{\cdot}05$ respectively.

Effect on receptor-operated Ca^{2+} channels

Warifteine (1×10^{-5}–8×10^{-5} M) and nitroprusside (1×10^{-8}–4×10^{-8} M) added cumulatively to the pre-contracted aorta produced a concentration-dependent relaxation of the tissue and the IC50 values obtained for the two agents were $6{\cdot}0 \times 10^{-5}$ and $1{\cdot}9 \times 10^{-8}$ M, respectively (Table 1). While a 15-min time interval was required to produce maximum effect with warifteine, only a 7-min interval was necessary in the case of nitroprusside.

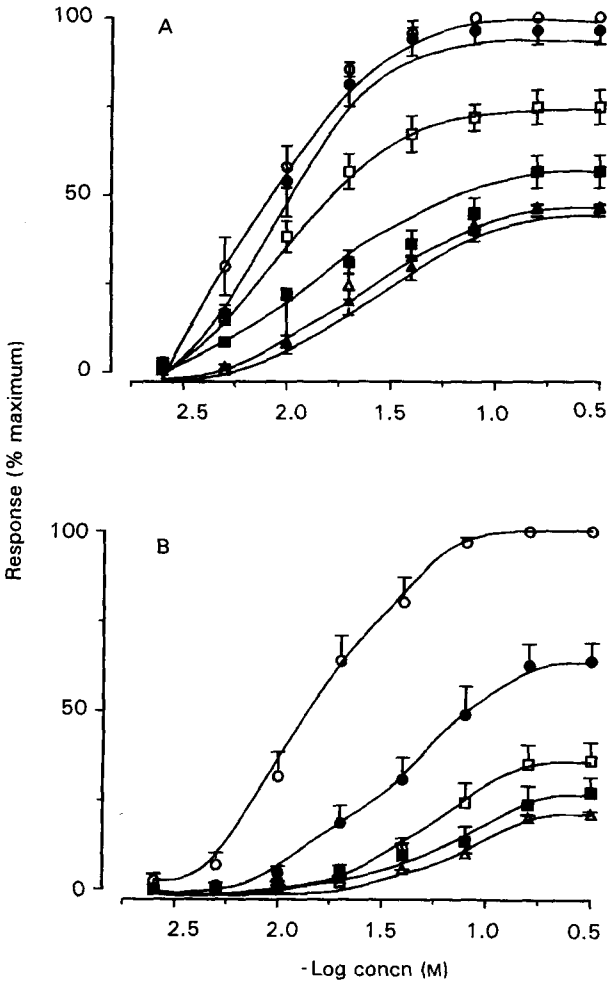

FIG. 2. Effect of warifteine and verapamil on cumulative concentration–response curves to KCl in the rabbit aorta. Values are mean ± s.e.m. (n = 5). A. Warifteine: ○ control, ● 1×10^{-5} M, □ 2×10^{-5} M, ■ 4×10^{-5} M, △ 8×10^{-5} M, ▲ $1{\cdot}6 \times 10^{-4}$ M. B. Verapamil: ○ control, ● 1×10^{-7} M, □ 2×10^{-7} M, ■ 4×10^{-7} M, △ 8×10^{-7} M.

Table 1. Effect of warifteine and nitroprusside on the noradrenaline (10^{-7} M)-induced sustained contraction of the rabbit aorta. Agents were added cumulatively at a time interval of 15 min for warifteine and 7 min for nitroprusside.

Agent	Concn (M)	Inhibition (%)	IC50 (M)
Warifteine	$1{\cdot}0 \times 10^{-5}$	17·0 ± 6·3*	
	$2{\cdot}0 \times 10^{-5}$	28·0 ± 5·3**	$6{\cdot}0 \times 10^{-5}$
	$4{\cdot}0 \times 10^{-5}$	32·3 ± 8·2**	
	$8{\cdot}0 \times 10^{-5}$	74·4 ± 4·9***	
Nitroprusside	$1{\cdot}0 \times 10^{-8}$	34·7 ± 5·2*	
	$2{\cdot}0 \times 10^{-8}$	58·0 ± 11·1**	$1{\cdot}9 \times 10^{-8}$
	$4{\cdot}0 \times 10^{-8}$	64·0 ± 10·4**	

Mean ± s.e.m. (n = 5). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

Table 2. Effect of warifteine and procaine on the release of Ca^{2+} from (experiment A), and refilling of (experiment B) intracellular Ca^{2+} stores responsible for noradrenaline (10^{-6} M)-induced transient contractions. In experiment A, warifteine or procaine was added to the bath 15 min before the addition of noradrenaline. In experiment B, the inhibitors were present for 15 min during the time of refilling of the calcium stores.

Experiment	Agent	Concn (M)	Inhibition (%)	IC50 (M)
A	Warifteine	$1{\cdot}0 \times 10^{-5}$	35·0 ± 3·4*	
		$3{\cdot}0 \times 10^{-5}$	54·2 ± 2·5*	$2{\cdot}6 \times 10^{-5}$
		$1{\cdot}0 \times 10^{-4}$	68·7 ± 0·8**	
	Procaine	$1{\cdot}0 \times 10^{-5}$	34·7 ± 1·5*	
		$3{\cdot}0 \times 10^{-5}$	54·1 ± 3·6*	$3{\cdot}2 \times 10^{-5}$
		$1{\cdot}0 \times 10^{-4}$	65·8 ± 1·6*	
		$3{\cdot}0 \times 10^{-4}$	80·6 ± 2·3**	
B	Warifteine	$1{\cdot}0 \times 10^{-5}$	21·1 ± 3·9	
		$4{\cdot}0 \times 10^{-5}$	36·9 ± 3·4*	$7{\cdot}9 \times 10^{-5}$
		$1{\cdot}6 \times 10^{-4}$	63·6 ± 4·3**	
	Procaine	$1{\cdot}6 \times 10^{-4}$	14·0 ± 8·5	
		$6{\cdot}4 \times 10^{-4}$	0·86 ± 7·8	
		$2{\cdot}56 \times 10^{-3}$	21·3 ± 14·6	

Mean ± s.e.m. (n = 4). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$.

Effect on intracellular Ca^{2+} stores responsible for noradrenaline-induced transient contractions of the aorta

Various concentrations of warifteine (1×10^{-5}–1×10^{-4} M) or procaine (1×10^{-5}–3×10^{-4} M) were added to the aorta suspended in a Ca^{2+}-free medium 15 min before the addition of a previously selected concentration of noradrenaline. Both the agents inhibited the noradrenaline response when compared with the mean of two responses obtained earlier in the absence of the inhibitors (Table 2). Warifteine and procaine were approximately equipotent (IC50 = $2{\cdot}6 \times 10^{-5}$ and $3{\cdot}2 \times 10^{-5}$ M, respectively) in these tests which indicates that both agents are equally capable of inhibiting the release of Ca^{2+} from noradrenaline-sensitive stores.

In the second series of experiments, warifteine (1×10^{-5}–1×10^{-4} M) or procaine ($1{\cdot}6 \times 10^{-4}$–$2{\cdot}56 \times 10^{-3}$ M) was present only during the time of refilling of Ca^{2+} stores of the aorta previously depleted by eliciting a noradrenaline response in a Ca^{2+}-free medium. It was found that the presence of warifteine during the period of calcium loading, significantly inhibited subsequent noradrenaline-induced contraction (Table 2). The effect was reversible and concentration-dependent and the IC50 value calculated graphically was $7{\cdot}9 \times 10^{-5}$ M. Procaine, even at the highest concentration ($2{\cdot}56 \times 10^{-3}$ M) failed to produce a consistent and significant inhibition of noradrenaline-induced contraction of the tissue. The results from these experiments suggest that warifteine may inhibit the refilling of noradrenaline-sensitive intracellular Ca^{2+} stores in the aorta.

Effect on intracellular Ca^{2+} stores responsible for caffeine-induced transient contractions of the aorta

Warifteine in concentrations ranging from 1×10^{-5} to 4×10^{-4} M failed to inhibit caffeine-induced contractions of the aorta suspended in Ca^{2+}-free Krebs-Henseleit solution maintained at 21°C. Otherwise, in concentrations of 4×10^{-5}, $1{\cdot}6 \times 10^{-4}$, $6{\cdot}4 \times 10^{-4}$ and $2{\cdot}56 \times 10^{-3}$ M, procaine inhibited (mean ± s.e.m.) 12·8 ± 3·5, 30·3 ± 5·3, 39·6 ± 3·9 and 60·7 ± 3·3% of the caffeine responses, respectively

(IC50 $7{\cdot}9 \times 10^{-4}$ M), the last three values being statistically significant ($P < 0{\cdot}05$).

Warifteine was also completely ineffective in antagonizing the response to caffeine when the alkaloid was present in a concentration up to 4×10^{-4} M, during the refilling process of Ca^{2+} stores sensitive to caffeine. The refilling of caffeine-sensitive Ca^{2+} stores was also not significantly inhibited by procaine even at the highest concentration ($2{\cdot}56 \times 10^{-3}$ M) where it produced an inhibition of $18{\cdot}2 \pm 6{\cdot}4\%$ which was not significant. These studies show that while warifteine does not modify the caffeine sensitive intracellular Ca^{2+} stores, procaine has significant effect on the release of Ca^{2+} from these stores.

Discussion

The results suggest that warifteine may exert spasmolytic effects by inhibiting a number of calcium regulatory processes in the rabbit aorta.

Experiments with KCl-induced contractions, which occur as a result of Ca^{2+} entry through voltage-dependent Ca^{2+} channels which are sensitive to agents such as verapamil (Rasmussen 1986; Nelson et al 1990), showed that warifteine inhibits these channels. However, the maximum inhibition produced by warifteine was about 60%, which was not altered significantly with further increase in the concentration of warifteine. Thus, inhibition of voltage-dependent Ca^{2+} channels may partially contribute to a reduction in cytoplasmic Ca^{2+} and the consequent relaxation of the muscle.

In the rabbit aorta, agonist-induced Ca^{2+} influx occurs without membrane depolarization (Droogmans et al 1977; Van Breemen et al 1985) and the influx through such receptor-operated channels is resistant to inhibitors of voltage-dependent Ca^{2+} channels (Bolton 1979; Stoytcheva & Venkova 1992). However, these receptor-operated channels are responsible for the sustained phase of the noradrenaline response and are blocked by agents such as nitroprusside which activates protein kinase G through cGMP generation (Karaki 1987; Moncada et al 1987). Warifteine was found to inhibit these channels which indicates another manner in which the compound may inhibit smooth-muscle contraction.

Smooth-muscle relaxants may also act by inhibiting the release of Ca^{2+} from intracellular stores and the process of refilling the depleted stores required for subsequent contractions. The existence of two separate intracellular pools of Ca^{2+} have been proposed, one sensitive to inositol trisphosphate (Berridge & Irvine 1984; Mikoshiba 1993) and the other sensitive to the plant alkaloid ryanodine (Burgoyne & Cheek 1991; Missiaen et al 1992; Sorrentino & Volpe 1993). The ryanodine-sensitive pool is also sensitive to caffeine (Iino 1989; Missiaen et al 1992), and cADP-ribose may be an endogenous ligand of this channel (Galione 1992, 1993; Kim et al 1993).

Warifteine was tested for inhibitory activity on noradrenaline-sensitive and caffeine-sensitive pools of intracellular Ca^{2+}. The initial transient response to noradrenaline is mediated by the release of Ca^{2+} from intracellular pool sensitive to inositol trisphosphate (Exton 1990; Irvine 1992; Berridge 1993). Warifteine was found to inhibit both the release of Ca^{2+} from these pools and also the uptake of Ca^{2+} into these Ca^{2+} stores. However, procaine, the standard agent used in the present studies, only inhibited the release from, but not the refilling of the stores, which confirms the results of an earlier study (Ahn & Karaki 1988). When warifteine was tested for effects on transient contractions induced by caffeine, which depends on a separate intracellular Ca^{2+} store, the alkaloid was inactive even in concentrations higher than those required to produce spasmolytic effects (Côrtes 1992) and effects on other calcium regulatory processes in the present experiment. The lack of effect of warifteine, a bisbenzylisoquinoline alkaloid on the caffeine-sensitive pool is not surprising, as ryanodine is a structurally different neutral alkaloid (Drummond & Hughes 1987). On the other hand, procaine significantly inhibited the caffeine-induced Ca^{2+} release and had a minor insignificant effect on the refilling of caffeine-sensitive intracellular Ca^{2+} stores.

The results of the present studies show that warifteine, in concentrations that produce muscle relaxation, also inhibits several calcium-regulatory processes in the rabbit aorta. Thus, it was found to partially inhibit voltage-dependent Ca^{2+} channels, inhibit receptor-operated Ca^{2+} channels and further was found to interfere with noradrenaline-sensitive intracellular calcium stores. Finally, as the caffeine-induced contractions were unaltered in the presence of warifteine, the alkaloid may have no direct inhibitory effect on the contractile proteins, or modify the calcium sensitivity of these proteins by altering the activity of enzymes like myosin light-chain phosphatase and Ca^{2+}-calmodulin dependent protein kinase II (Allen & Wash 1994; Somlyo & Somlyo 1994).

The structural similarity of warifteine to papaverine which also has a benzylisoquinoline moiety may explain the biochemical basis of muscle relaxation produced by warifteine. Papaverine is known to increase the intracellular concentrations of cyclic nucleotides by inhibiting cyclic nucleotide phosphodiesterase enzymes and thus reduce cytosolic Ca^{2+} which leads to muscle relaxation (Miyamoto et al 1976; Kramer & Wells 1979). A rise in intracellular cyclic adenosine monophosphate levels was also reported to inhibit the levels and the responses of inositol 1,4,5, trisphosphate receptors (Akoi et al 1994). Further, papaverine was also shown to inhibit voltage-dependent Ca^{2+} channels by a cyclic adenosine monophosphate-independent mechanism as well (Iguchi et al 1992).

To conclude, as warifteine is likely to act by modifying cytosolic calcium levels, further investigations along these lines, for example, its effect on ATP-dependent K^+ channels which inhibit voltage-operated Ca^{2+} channels (Nelson et al 1990) are relevant. Direct measurements of changes in the intracellular concentrations of Ca^{2+} cyclic nucleotides and inositol 1,4,5, trisphosphate may also be helpful in clarifying further the mechanism of spasmolytic activity of warifteine.

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