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Ca^{2+}-Channel Blockade in Rat Thoracic Aorta by Crychine Isolated from *Cryptocarya chinensis* Hemsl

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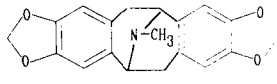
Abstract—The pharmacological effects of crychine, isolated from *Cryptocarya chinensis* Hemsl, on rat thoracic aorta were examined. In the high-K^+ medium (60 mM), Ca^{2+} (0·03–3 mM)-induced vasoconstriction was inhibited by crychine in a concentration-dependent manner (10–100 $\mu g\ mL^{-1}$). Increasing the incubation time from 5 to 30 min did not cause more pronounced inhibitory effect on KCl-induced contraction. The tonic contractions elicited by KCl (60 mM) and Bay K 8644 (0·1 μM) were also relaxed by the addition of crychine and were more marked in the 1·9 mM than in the 5 mM Ca^{2+} medium. The noradrenaline concentration-response curves were antagonized non-competitively by crychine (10–100 $\mu g\ mL^{-1}$). At the plateau of noradrenaline-induced tonic contraction, the addition of crychine also caused relaxation. This relaxing effect of crychine was not antagonized by indomethacin (20 μM) or methylene blue (50 μM), and was still present in denuded aorta or in the presence of nifedipine (2 μM). Caffeine (10 mM)-induced contraction and cAMP or cGMP levels were not affected by crychine. It is concluded that crychine relaxes the rat thoracic aorta mainly by suppressing the Ca^{2+} influx through both voltage- and receptor-operated calcium channels.

Medical plants have been used as traditional remedies or folk medicines in oriental countries over hundreds of years. In our previous works, many biologically active compounds have been isolated from plant sources. Some of them inhibited the contraction of aortic smooth muscles. For example, dicentrine (isolated from *Lindera megaphylla*) is a potent and selective α_1-adrenoceptor antagonist in vascular smooth muscle (Teng et al 1991)) and magnolol (isolated from *Magnolia officinalis*) causes vasorelaxation of rat aorta by releasing endothelium-derived relaxing factor (EDRF) (Teng et al 1990). From Formosan Lauraceous plants, some non-phenolic bases were examined. (+)-*O*-Methylcâryachine is an alkaloid isolated from the tree bark, while crychine is isolated from the wood of *Cryptocarya chinensis* Hemsl (Lu 1966). Recently, we found crychine was an effective vasorelaxant. In the present study, we attempted to elucidate the mechanism of action of this vasorelaxing agent and also compare it with the Ca^{2+}-channel blocker, nifedipine.



Materials and Methods

Mechanical response

Wistar rats of either sex, 250–300 g, were killed by a blow to the head. The thoracic aorta was isolated and excess fat and connective tissue was removed. The vessels were cut into rings of about 5 mm in length and mounted in organ baths containing 5 mL Krebs solution of the following composition (mM): NaCl 118·2, KCl 4·7, $CaCl_2$ 1·9, $MgSO_4$ 1·2, KH_2PO_4 1·2, $NaHCO_3$ 25 and glucose 11·7. The tissue bath solution was maintained at 37°C and bubbled with 95% O_2–5% CO_2. Two stainless steel hooks were inserted into the aortic lumen, one was fixed while the other was connected to a transducer. The aortas were equilibrated in the medium for 90 min with three changes of Krebs solution and maintained under an optimal tension of 1 g before specific experimental protocols were initiated. Contractions were recorded isometrically by a force-displacement transducer connected to a Gould polygraph (Model 2400).

The contractile effects of calcium were studied in rings stabilized in K^+ solution without Ca^{2+}. Calcium was then added from stock dilutions to obtain the desired concentrations, and the effect of each Ca^{2+} concentration was recorded. The maximal tension attained at 3 mM Ca^{2+} was considered as 100%. The high-K^+ solutions were prepared by substituting NaCl with KCl (60 mM) in an equimolar amount.

cAMP assay of rat aorta

The content of cAMP was assayed on aortic rings as previously described (Kauffman et al 1987). After incubation of aortic rings with dimethylsulphoxide, forskolin or crychine for 2 min, the aortic rings were rapidly frozen in liquid nitrogen and stored at −80°C until homogenized in 0·5 mL 10% trichloroacetic acid using a Potter glass/glass homogenizer. The homogenate was centrifuged at 10 000 *g* for 5 min and the supernatant was removed and extracted with 4 × 3 vol ether, and the cAMP content was then assayed using RIA kits. The precipitate was used for protein assay (Lowry et al 1951). cAMP levels were expressed as pmol (mg protein)$^{-1}$.

cGMP assay of rat aorta

Aortic rings prepared as described above, but not put under tension, were placed in 1 mL Krebs solution for 1 h with 95% O_2–5% CO_2 at 37°C. After incubation of aortic rings with

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dimethylsulphoxide (0·1%, control), sodium nitroprusside or crychine for 2 min, the reaction was stopped by immersing the tissue into liquid nitrogen. The tissues were stored at −80°C up to the time of thawing in 10% trichloroacetic acid and 4 mM EDTA. After homogenization with a Potter glass/glass homogenizer for 2 to 3 min, the homogenate was centrifuged at 10000 *g* for 5 min. The cGMP content of the supernatant, after extraction with ether four times, was assayed using RIA kits (Itoh et al 1982), while the precipitate was used for protein assay (Lowry et al 1951). The cGMP levels were expressed as pmol (mg protein)$^{-1}$.

Materials

Crychine was isolated from *Cryptocarya chinensis* Hemsl as previously described (Lu 1966). Noradrenaline, sodium nitroprusside, forskolin, nifedipine, Bay K 8644 and caffeine were obtained from Sigma Chemical Co. cAMP and cGMP RIA kits were purchased from Amersham. If drugs were dissolved in dimethylsulphoxide, the final concentration of which in the bathing solution did not exceed 0·1% and had no affect on the muscle contraction. All experiments with Bay K 8644 and nifedipine were conducted in the dark.

Data analysis

The experimental results are expressed as the mean ± s.e. For each group of preparations, the number of segments reported is also the number of rats. Statistical significance was assessed by Student's *t*-test and $P < 0.05$ was considered significant.

Results

Effects of crychine on high K$^+$-and Bay K 8644-induced contraction

In Ca^{2+}-free Krebs solution containing high K^+ (60 mM), the cumulative addition of Ca^{2+} (0·03–3 mM) to rat aorta caused a stepwise increase of contraction force. The maximum contraction induced by 3 mM Ca^{2+} was 1·4 ± 0·15 g (n = 7). After pretreatment with rat aorta for 5 min, crychine (10–100 μg mL^{-1}) inhibited this contraction in a concentration-dependent manner (Fig. 1A). The IC50 value was calculated to be about 25 μg mL^{-1} for a calcium concentration of 1 mM. Increasing the incubation time from 5 min to 15 or 30 min did not cause more pronounced inhibitory effect on this calcium-dependent contraction (Fig. 1B). A 5-min incubation time was thus used for mechanical response studies. Nifedipine (1 μM) also completely inhibited the high K^+-induced calcium-dependent contraction (Fig. 1A). Exposure of rat aorta to KCl (60 mM) and Bay K 8644 (0·1 μM) caused a tonic contraction maintained for at least 30 min. Addition of crychine (10–100 μg mL^{-1}) or nifedipine (1 μM) during tonic contraction (10 min after the exposure to KCl or Bay K 8644), relaxation could be observed in a concentration-dependent manner (Fig. 2A, B). Elevation of medium Ca^{2+} concentration (from 1·9 to 5 mM) antagonized the relaxation effect of crychine (30 μg mL^{-1}, from 51·8 ± 8·1 to 26·5 ± 3·1% relaxation after addition for 15 min, n = 6).

Effects of crychine on noradrenaline-induced contraction

Cumulative addition of noradrenaline (3 nM–30 μM) to Krebs medium caused a stepwise increase of contractions of rat

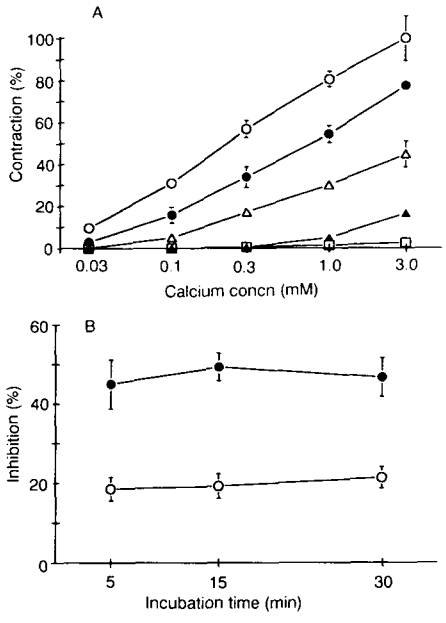

FIG. 1. A. Effects of crychine on the Ca^{2+}-dependent contraction of rat aorta induced by high K^+ (60 mM). The aorta was preincubated with dimethylsulphoxide (○), crychine (10 μg mL^{-1} ●; 30 μg mL^{-1} △; 100 μg mL^{-1} ▲) or nifedipine (1 μM □) at 37°C for 5 min, then cumulative concentrations of Ca^{2+} (0·03–3 mM) were used to trigger the contraction. Each point represents the mean ± s.e. (n = 7). All data points showed a significant inhibition by various concentrations of crychine. B. Effects of incubation effect of crychine on high K^+ (60 mM)-induced contraction. The rat aorta was preincubated with crychine (10 μg mL^{-1} ●; 30 μg mL^{-1} ○) at 37°C for 5, 15 or 30 min, then K^+ was added to trigger the contraction. Each point represents the mean ± s.e. (n = 6–7).

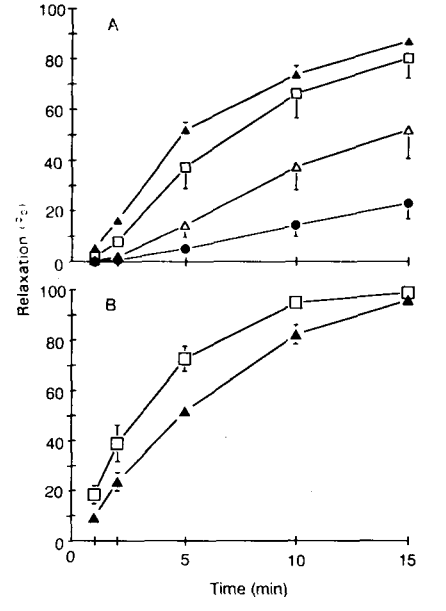

FIG. 2. Relaxation by crychine and nifedipine on high K^+ (A)- and Bay K 8644 (B)-induced tonic contraction in rat aorta. Various concentrations of crychine or nifedipine (1 μM) were added 10 min after the aorta had been exposed to high K^+ (60 mM, A) or Bay K 8644 (0·1 μM, B). The percentage of relaxation was calculated at different time intervals after crychine and nifedipine addition and expressed as the mean ± s.e. (n = 5–7). A. Crychine 10 μg mL^{-1} (●); 30 μg mL^{-1} (△); 100 μg mL^{-1} (▲); nifedipine 1 μM (□); B. Crychine 100 μg mL^{-1} (▲); nifedipine 1 μM (□).

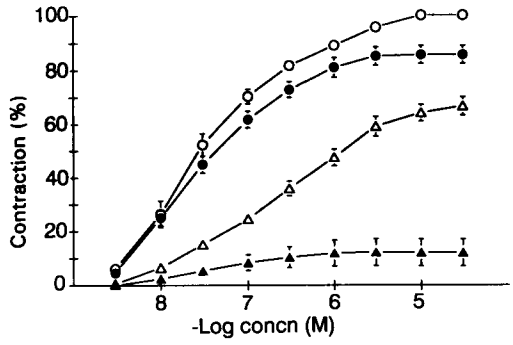

FIG. 3. Antagonism of the concentration-response curves to noradrenaline by 5-min pretreatment of rat aorta with crychine. Control (○), crychine 10 μg mL^{-1} (●), 30 μg mL^{-1} (△), 100 μg mL^{-1} (▲). Each data point represents the mean ± s.e. (n = 7).

aorta. The maximum contraction induced by 10 μM noradrenaline was 2·13 ± 0·13 g (n = 7). Crychine (10–100 μg mL^{-1}) produced a non-competitive blockade of noradrenaline-induced contraction (Fig. 3). Noradrenaline (3 μM) caused a phasic and then a tonic contraction maintained for at least 30 min. If crychine was added at the state of tonic contraction (10 min after the exposure to noradrenaline) a time- and concentration-dependent relaxation was observed (Fig. 4A). This relaxing action of crychine was not blocked by either indomethacin (20 μM) or methylene blue (50 μM) which was added 3 min before noradrenaline (Fig. 4B). In the

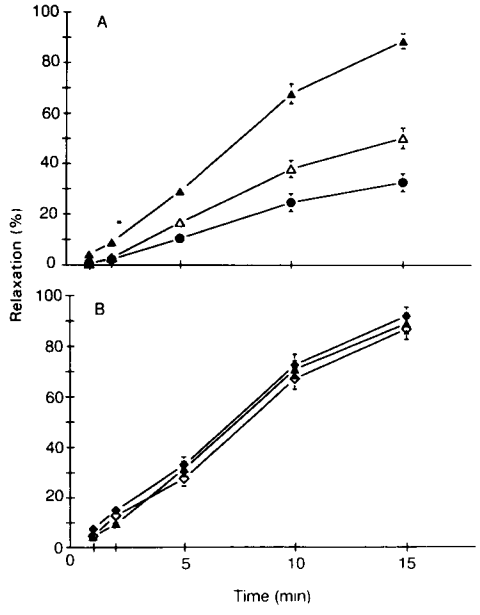

FIG. 4. A. Relaxation by crychine of noradrenaline (3 μM)-induced tonic contraction in rat aorta. Various concentrations of crychine (10 μg mL^{-1} ●; 30 μg mL^{-1} △; 100 μg mL^{-1} ▲) were added 10 min after the aorta had been exposed to noradrenaline. The percentage of relaxation was calculated at different time intervals after crychine addition and expressed as the mean ± s.e. (n = 7). B. Effects of indomethacin and methylene blue and denuded-endothelium on the relaxation effect of crychine. After the endothelium was denuded (▲) or pretreatment of the intact aorta with indomethacin (20 μM ◇) or methylene blue (50 μM ◆) for 3 min, noradrenaline (3 μM) was added to trigger the muscle contraction. Ten minutes later, crychine (100 μg mL^{-1}) was added to induce the relaxation. The percentage of relaxation was calculated at different time intervals after crychine addition and expressed as the mean ± s.e. (n = 7).

Table 1. Effect of crychine on the cAMP and cGMP formation of rat aorta.

	cAMP	cGMP
Treatment	(pmol (mg protein)$^{-1}$)	
Control	2·03 ± 0·30	2·34 ± 0·20
Sodium nitroprusside (10 μM)	—	7·80 ± 0·55*
Forskolin (10 μM)	6·50 ± 0·28*	—
Crychine (100 μg mL^{-1})	1·96 ± 0·21	2·37 ± 0·24

After preincubation of aorta rings with dimethylsulphoxide (0·1%, control), sodium nitroprusside, forskolin or crychine for 2 min, the reaction was stopped by immersing the tissue in liquid nitrogen and the cAMP and cGMP contents in rat aorta were measured. The results are expressed as the mean ± s.e. (n = 5). *$P < 0·001$ compared with the respective control.

denuded rat aorta, the relaxing action of acetylcholine (3 μM) was completely abolished (0% relaxation, n = 7), while crychine still relaxed the aorta (Fig. 4B). After aorta was preincubated with nifedipine (2 μM) for 15 min, KCl (60 mM)-induced contraction was completely blocked while noradrenaline (3 μM)-induced contraction was suppressed by less than 5%. Crychine (100 μg mL^{-1}) relaxed this noradrenaline-induced and nifedipine-resistant tonic contraction (93·4 ± 2·1% relaxation, n = 6). EGTA (10 mM) and Ni^{+} (1 mM) also caused 98·2 ± 1·6 and 96·8 ± 1·8% relaxation of this noradrenaline-induced and nifedipine-resistant tonic contraction, respectively (n = 6).

Effects of crychine on caffeine-induced contraction

After equilibrium in the Ca^{2+}-free Krebs solution for 5 min, caffeine (10 mM) caused a rapid phasic contraction of rat aorta (0·45 ± 0·05 g, n = 7). Crychine (100 μg mL^{-1}) and nifedipine (2 μM) did not affect (0·44 ± 0·05 and 0·46 ± 0·04 g, respectively), while procaine (10 mM) completely abolished this caffeine-induced phasic contraction.

Effects of crychine on cAMP and cGMP formation

The cyclic nucleotide content of aorta measured by radioimmunoassay showed that neither cAMP nor cGMP levels were changed by crychine (Table 1).

Discussion

Pretreatment of rat aorta with crychine inhibited the contractile response to noradrenaline and high K^{+}. It also caused relaxation when crychine was added during the tonic contraction induced by noradrenaline or high K^{+}. All these effects of crychine were concentration-dependent.

Contraction of the vascular smooth muscle required an increase of cytosolic free Ca^{2+}. The high K^{+}-induced contraction of smooth muscle resulted from the increased Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Karaki & Weiss 1979, 1984). Bay K 8644 also promoted Ca^{2+} influx through those in vascular smooth muscle (Franckowiak et al 1985; Stash & Kazda 1989). Crychine inhibited and relaxed high K^{+}- and Bay K 8644-induced contraction. The relaxing effect of crychine was antagonized by increasing the medium Ca^{2+} concentration. Thus, crychine may be a blocker of voltage-dependent Ca^{2+} channels.

However, the antagonism of crychine and nifedipine against Ca^{2+} did not fulfil the requirements of a competitive antagonist, since these two agents shifted the concentration-response curve not only to the right but also suppressed the maximal effects. The reason is probably that much higher concentrations of Ca^{2+} are needed to achieve the maximal contraction. And Ca^{2+} in high concentrations is known to cause auto-inhibitory effects and decreases the permeability of the cell membrane for Ca^{2+} (Fleckenstein 1955).

The tonic tension in response to noradrenaline results from Ca^{2+} entry through receptor-operated Ca^{2+} channels (Bolton 1979). Crychine caused the relaxation of tonic contraction induced by noradrenaline either in intact or in nifedipine-pretreated denuded aorta. The noradrenaline-induced and nifedipine-resistant tonic contraction was also relaxed by EGTA and Ni^{+}, and the noradrenaline concentration-response curve was antagonized by crychine in a non-competitive manner. All these data indicate that crychine also blocks the Ca^{2+} influx through a receptor-operated Ca^{2+} channel. In response to a variety of neurochemical and physical stimuli, endothelial cells release endothelium-dependent vasodilators, such as endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2) (Jaffe 1985; Vanhoutte et al 1986). The relaxing action of crychine persisted in endothelium-denuded or intact aorta in the presence of indomethacin and methylene blue. Thus, the vasorelaxation caused by crychine was independent of endothelium and was not mediated by either EDRF or PGI_2.

It is now generally accepted that caffeine can release intracellular Ca^{2+} in vascular smooth muscle (Itoh et al 1983; Saida & Van Breemen 1984). However, crychine and nifedipine did not affect this process. Other important mediators for relaxing the vascular smooth muscle are cyclic nucleotides. The action of cGMP includes increase in Ca^{2+} extrusion or sequestration (Zsoter et al 1977; Popescu et al 1985) and inhibition of Ca^{2+} uptake (Karaki et al 1984), contractile elements (Pfitzer et al 1986) or receptor-linked phosphoinositide breakdown (Rapoport 1986; Lang & Lewis 1989). cAMP can dilate vascular smooth muscle either by causing phosphorylation of myosin light-chain kinase (Silver & Disalvo 1979; Adelstein & Eisenberg 1980), or by increasing calcium uptake by the sarcoplasmic reticulum (Scheid et al 1979). Neither cAMP nor cGMP content was changed by crychine. This indicates that the inhibitory effects of crychine on the contractile responses caused by high K^{+} or noradrenaline are not due to the increase of cyclic nucleotides.

It is concluded that crychine relaxes the rat aorta by suppressing the Ca^{2+} influx through both voltage-dependent and receptor-operated Ca^{2+} channels.

Acknowledgements

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