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Relaxant Activity of Three Aporphine Alkaloids from *Annona cherimolia* on Isolated Aorta of Rat

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Abstract

In the present study we tested the relaxant effect of three aporphine alkaloids—roemerine, anonaine and dehydroroemerine—isolated from the roots of *Annona cherimolia*, on isolated strips of rat thoracic aorta.

All compounds completely relaxed KCl- and noradrenaline-induced contractions with different potencies depending on their structural characteristics. The experiments, carried out in Ca^{2+}-free medium using two different agonists (noradrenaline and caffeine) which mobilize calcium intracellularly by different mechanisms of action, showed that the alkaloids made no contribution to intracellular calcium processes.

The present study provides evidence that the relaxant effects produced by aporphine alkaloids may be due to the blockade of calcium movements across the cell membrane, mainly through voltage-operated channels, and to the disruption of α_1-adrenoceptors connected to receptor-operated channels.

Annona cherimolia Mill. (Annonaceae) is a tropical South American tree used in traditional medicine as an insecticide and parasiticide (Barriga 1974). Isoquinoline alkaloids from leaves (Villar et al 1985), twigs (Urzua & Cassels 1977) and seeds (Ríos et al 1989) of *A. cherimolia* have previously been described. Bis-tetrahydrofuran γ-lactones with original structures like cherimolin, laherradurin, almunequin, molvizarin, motrilin, otivarin, jeterin and itrabin, have recently been found in seeds of *A. cherimolia* (Cortes et al 1991, 1993a,b).

The presence of aporphine alkaloids in different organs of this plant and the reported smooth muscle activities of other related aporphines (Cortes et al 1990; Chen et al 1991; Speisky et al 1991; Teng et al 1991; Ivorra et al 1992, 1993) prompted us to examine the activity of the aporphine alkaloids isolated from *A. cherimolia* (Fig. 1) in the contractile response of rat thoracic aorta in order to examine their vascular smooth muscle relaxing properties.

Materials and Methods

Plant material

The roots of *A. cherimolia* were collected in July 1990, in Almuñecar (Granada coast, Spain). A voucher specimen (ref. VF 10463) was deposited in the herbarium of the Department of Botany, Faculty of Pharmacy, University of Valencia, Spain.

Extraction and isolation

The dried and powdered roots of *A. cherimolia* (1·5 kg) were macerated with methanol. The methanolic extract (A) was evaporated and concentrated, and the residue (150 g) was re-partitioned between hexane and 90% methanol. After removal of the hexane fraction (B), the hydromethanolic extract was partially evaporated under reduced pressure and CH_2Cl_2 was added. The organic extract (CH_2Cl_2-soluble fraction: (C)) was dried and concentrated to give a residue weighing 20·6 g. The rest of the aqueous extract was alkalinized with 5% NH_4OH and extracted with CH_2Cl_2. This organic fraction (D) was evaporated under reduced pressure to yield a residue weighing 3·3 g. The aporphine alkaloids were contained in the C and D fractions.

The alkaline CH_2Cl_2-soluble fraction D (3·3 g) was applied to a column of silica gel (3·5 × 30 cm) Kiesegel 60H (110 g). The column was eluted with 3 L CH_2Cl_2/MeOH/NH_4OH (97 : 3 : 0·1) at 2 mL min^{-1} to yield two alkaloids, roemerine (**1**: 12 mg, 0·36% of D) and anonaine (**2**: 127 mg, 3·85% of D); these compounds were eluted at 600–610 and 1015–1035 mL, respectively.

The CH_2Cl_2-soluble fraction C (10 g) was fractionated by flash chromatography on a column of silica gel (5·5 × 15 cm) Kiesegel S (145 g). The column was eluted with 3 L CH_2Cl_2/MeOH (97 : 3) at 5 mL min^{-1}, and yielded dehydroroemerine (**3**: 9 mg, 0·1% of C)) between 95 and 105 mL.

Aporphine alkaloids

The structures of the aporphine alkaloids were determined by spectroscopic methods including chemical ionization mass spectroscopy (CIMS), electron-impact mass spectroscopy (EIMS), ^1H NMR and ^{13}C NMR spectra (Guinaudeau et al 1975, 1979; Urzua & Cassels 1977).

Isolated organ-bath studies

Helically cut strips from the thoracic aorta of male Wistar rats, 200–220 g, were prepared and mounted as described by Furchgott & Zawadzki (1980). Each preparation was suspended in a 10-mL organ bath containing Krebs-bicarbonate solution (KBS), maintained at 37°C and gassed with 95% O_2–

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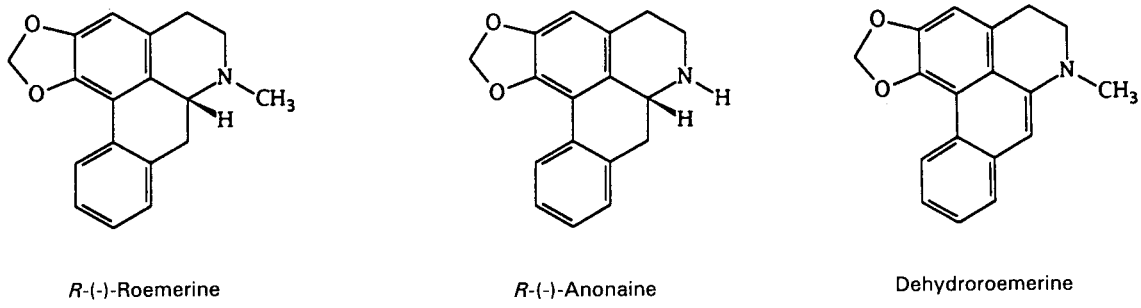

FIG. 1. Chemical structures of *R*-(−)-roemerine, *R*-(−)-anonaine and dehydroroemerine.

5% CO_2. An initial load of 1 g was applied to each preparation and maintained throughout the 75- to 90-min equilibration period before agonist addition. Tension changes were recorded by an isometric force-transducer (Grass RPS 7C8) connected to a Grass polygraph (Model 7E).

The composition of KBS was as follows (mM): NaCl, 118; KCl, 4·75; $CaCl_2$, 1·8; $MgCl_2$, 1·2; KH_2PO_4, 1·2; $NaHCO_3$, 24; and glucose, 11. Ca^{2+}-free solution had the same composition except that $CaCl_2$ was omitted and EDTA (0·1 mM) was added.

Experiments were performed in aorta devoid of endothelium and the absence of a relaxant response after addition of 10^{-4} M acetylcholine to preparations previously contracted with noradrenaline (1 μM) corroborated the absence of a functional endothelium (Furchgott & Zawadzki 1980).

Dose-response curves of relaxation were obtained by addition of cumulative concentrations of prazosin, nifedipine, verapamil and aporphine alkaloids on the sustained contractions induced by noradrenaline (1 μM) or KCl (80 mM). Phentolamine (10^{-5} M) was added in the experiments with depolarizing solution to block the possible action of noradrenaline released by depolarization (Ivorra et al 1993). Contractions in KBS were calculated in mg and relaxations were expressed as a percentage of the maximum tension obtained by the agonist addition. A regression of response against −log concentration of the test compound was performed by the least-squares method for each preparation. The concentration needed to produce 50% inhibition (IC50) was obtained from the linear regression plot of all points between 20 and 80% of the maximal response.

The effects of alkaloids on the contractile responses to noradrenaline or caffeine in Ca^{2+}-free KBS were also studied and experiments were carried out as follows. After incubation for 90 min in Krebs solution to stabilize the preparation, 1 μM noradrenaline or 10 mM caffeine was then administered to contract the muscle. The solution was replaced by Ca^{2+}-free solution and left for 15 min. Noradrenaline (1 μM) or caffeine (10 mM) was added and contractile response was monitored. The solution was replaced by Krebs solution and left for 20 min to refill the depleted stores. Noradrenaline or caffeine was administered after 15 min in Ca^{2+}-free EDTA-containing solution and the contractile response was expressed as a percentage of the response to the second challenge with agonist.

The following drugs were used: L-noradrenaline L-tartrate and acetylcholine (Merck, Darmstadt); anhydrous caffeine and phentolamine methanesulphonate (Sigma, St Louis, MO). Other reagents were of analytical grade. Caffeine was dissolved in Ca^{2+}-free KBS. The other drugs were dissolved in distilled water at pH 7.

The results are expressed as the means ± s.e.m. of five or more preparations (n) obtained from different animals. The statistical significance of differences between the means was assessed using the Student's *t*-test for unpaired data. *P* values of less than 0·05 were considered to represent significant differences.

Results

Fig. 2 shows the smooth muscle relaxing effects of roemerine, anonaine and dehydroroemerine in the rat isolated aorta preparation previously contracted by depolarizing solution (KCl 80 mM) + phentolamine 10^{-5} M. The effects of aporphines on the contraction of the aorta preparation induced

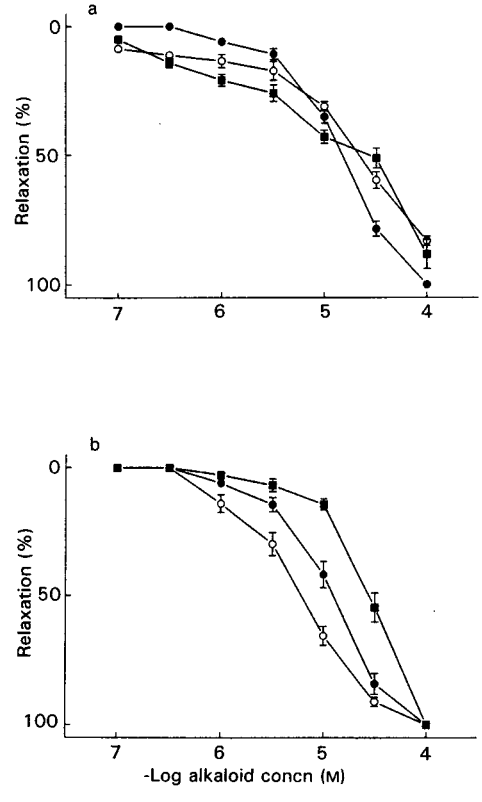

FIG. 2. Relaxation dose–response curves obtained after addition of different alkaloids in aorta previously contracted by 80 mM KCl (a) or 1 μM noradrenaline (b). ○ *R* (−)-roemerine, ● *R* (−)-anonaine, ■ dehydroroemerine. Each point is the mean derived from at least 5 experiments with s.e.m. shown by vertical bars.

Table 1. Inhibitory potencies (IC50, M) of apomorphine alkaloids and different antagonists on contractile responses induced by noradrenaline and depolarizing solution, in rat aorta incubated in KBS.

	Noradrenaline (1 μM)	KCl (60 mM)
Roemerine (10^{-8}–10^{-4} M)	$5{\cdot}8 \pm 0{\cdot}7 \times 10^{-6}$	$1{\cdot}9 \pm 0{\cdot}2 \times 10^{-5}$*
Anonaine (10^{-8}–10^{-4} M)	$1{\cdot}1 \pm 0{\cdot}1 \times 10^{-5}$	$1{\cdot}2 \pm 0{\cdot}1 \times 10^{-5}$
Dehydroroemerine (10^{-8}–10^{-4} M)	$2{\cdot}3 \pm 0{\cdot}1 \times 10^{-5}$	$1{\cdot}7 \pm 0{\cdot}3 \times 10^{-5}$
Prazosin (10^{-12}–10^{-6} M)	$5{\cdot}1 \pm 0{\cdot}4 \times 10^{-10}$	$4{\cdot}8 \pm 1{\cdot}5 \times 10^{-5}$*
Nifedipine (10^{-11}–10^{-6} M)	$4{\cdot}6 \pm 1{\cdot}8 \times 10^{-8}$	$1{\cdot}12 \pm 0{\cdot}05 \times 10^{-9}$*
Diltiazem (10^{-9}–10^{-4} M)	$6{\cdot}2 \pm 2{\cdot}0 \times 10^{-6}$	$2{\cdot}2 \pm 1{\cdot}8 \times 10^{-7}$*

Values are mean ± s.e.m.; number of experiments = 4–7.
*$P < 0{\cdot}001$ compared with the corresponding values for noradrenaline-induced contraction.

by noradrenaline (1 μM) are also shown. Anonaine was the most potent alkaloid inhibiting the KCl-induced contraction with an IC50 value of $1{\cdot}20 \pm 0{\cdot}07 \times 10^{-5}$ M, whereas the contraction elicited by noradrenaline was more potently inhibited by roemerine (IC50 = $5{\cdot}8 \pm 0{\cdot}7 \times 10^{-6}$ M). Dehydroroemerine showed less activity in inhibiting noradrenaline contraction. Similar experiments were performed, adding cumulative concentrations of prazosin, nifedipine and diltiazem and results are summarized in Table 1.

Fig. 3 shows the experimental procedure used to study the action of the compounds tested on noradrenaline-induced contraction in the absence of external Ca^{2+}. The second contraction elicited by noradrenaline in Ca^{2+}-free solution was not obtained in the presence of roemerine and anonaine (100 μM), whereas dehydroroemerine (100 μM) only partially inhibited this response. Standard experiments were performed without adding alkaloids, and after a 20-min resting period in KBS and 15 min in Ca^{2+}-free solution, addition of noradrenaline induced a contraction similar in magnitude to

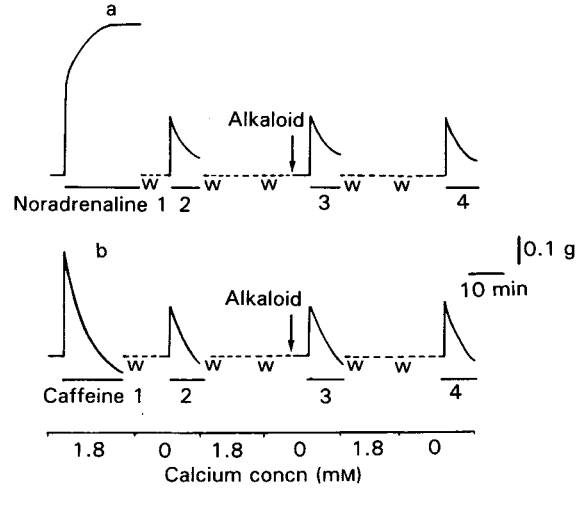

FIG. 3. Schematic representation of the effect of the alkaloids on 1 μM noradrenaline-induced contraction (a) or 10 mM caffeine-induced contraction (b) in the absence of extracellular calcium. w = washing.

Table 2. Effects (% of contraction due to second challenge with agonists) of the alkaloids (100 μM) tested on noradrenaline- and caffeine-induced contraction of the rat aorta in Ca^{2+}-free medium.

	Noradrenaline (1 μM)	Caffeine (10 mM)
Control	$90{\cdot}0 \pm 6{\cdot}1$	$112{\cdot}1 \pm 8{\cdot}0$
Roemerine	0	$114{\cdot}1 \pm 8{\cdot}3$
Anonaine	0	$71{\cdot}6 \pm 13{\cdot}9$
Dehydroroemerine	$53{\cdot}1 \pm 6{\cdot}1$*	$106{\cdot}1 \pm 6{\cdot}0$

Values are mean ± s.e.m.; number of experiments = 4–7.
*$P < 0{\cdot}001$ compared with the effect of the second challenge.

that to the second challenge. Data are expressed in Table 2.

Similar experiments were performed with 10 mM caffeine (Fig. 3) in the presence of roemerine, anonaine and dehydroroemerine (100 μM), but this preincubation did not significantly affect the second response induced by caffeine in Ca^{2+}-free medium. Control experiments were performed without adding alkaloids and results are expressed in Table 2.

We also investigated the contractile response to caffeine after a preincubation with the alkaloids (100 μM) during the Ca^{2+}-loading process (Fig. 4), and the results are summarized in Table 3. Addition of alkaloids (100 μM) during the loading period (20 min) did not produce a modification of the tone of the aorta. After washing and incubating in a Ca^{2+}-free medium for 15 min, the subsequent contraction elicited by caffeine was inhibited (anonaine) or partially decreased (roemerine and dehydroroemerine). Control experiments showed a total recovery of the contractile response by caffeine (Table 3).

Discussion

Three aporphine alkaloids were isolated by column chromatography from the dichloromethane extract of the roots

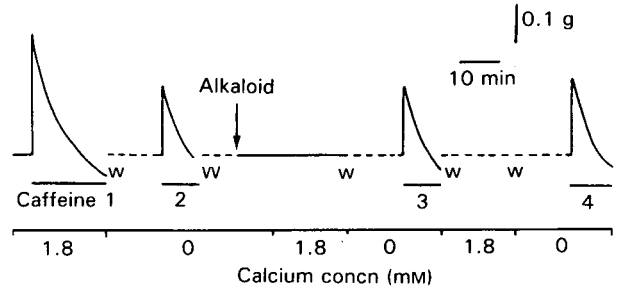

FIG. 4. Experimental procedure to analyse the refilling of internal Ca^{2+}-storage sites in the presence of the testing agents after depletion of caffeine-sensitive intracellular calcium pools. w = washing.

Table 3. Effects (% of contraction due to second challenge with caffeine) of the alkaloids (100 μM) on the refilling process of the Ca^{2+} internal stores.

	Response (%)
Control	$112{\cdot}1 \pm 8{\cdot}1$
Roemerine	$44{\cdot}4 \pm 11{\cdot}2$*
Anonaine	0
Dehydroroemerine	$55{\cdot}2 \pm 5{\cdot}4$*

Values are mean ± s.e.m.; number of experiments = 4–7.
*$P < 0{\cdot}001$.

of *A. cherimolia*, and they were identified on the basis of their spectroscopic data as roemerine (**1**), anonaine (**2**) and dehydroroemerine (**3**) (Fig. 1).

There are two basic mechanisms by which the cell may increase its cytosolic free Ca^{2+}: via release of Ca^{2+} from its intracellular stores after activation of specific receptors and via entry of extracellular Ca^{2+} through voltage-operated calcium channels after membrane depolarization (Karaki et al 1988; Godfraind et al 1989). When alkaloids were tested using KCl + phentolamine-induced contraction (phentolamine 10^{-5} M was added in order to assure the non-activation of the α-adrenoceptor), their participation in the blockade of extracellular Ca^{2+} entry was confirmed. In this way, anonaine was able to inhibit this calcium entry pathway more potently than roemerine or dehydroroemerine. On the other hand, the three alkaloids also inhibited the noradrenaline-induced contraction that was due to the release of intracellular Ca^{2+} stores and extracellular Ca^{2+} entry, and roemerine, with a methyl group at N-6, was the most potent. To confirm the different potencies of action of the alkaloids and to determine their mechanisms of action at the intracellular levels, we designed new experiments in Ca^{2+}-free EDTA-containing solution. The contractile responses in these conditions were induced by noradrenaline or caffeine and were related to the liberation of Ca^{2+} from intracellular stores by different mechanisms (Itoh et al 1983; Sato et al 1988). The fact that anonaine, roemerine and dehydroroemerine behave, as does prazosin, by inhibiting noradrenaline- but not caffeine-induced contractions in Ca^{2+}-free medium may indicate that the alkaloids are able to inhibit the contractile response mediated by α_1-adrenoceptor activation (Ivorra et al 1992, 1993). Nifedipine, a potent calcium-channel blocker, had no effect on noradrenaline- or caffeine-induced contraction in Ca^{2+}-free solution (Ivorra et al 1992). This means that in these experimental conditions, extracellular Ca^{2+} does not participate in the contractile response. In contrast, dehydroroemerine only partially blocked this noradrenaline-induced contraction in Ca^{2+}-free medium, thereby confirming the lower potency of this alkaloid as an adrenergic antagonist.

To study the inhibition of extracellular calcium entry by alkaloids, we carried out some experiments in Ca^{2+}-free medium in which the alkaloids were present during the refilling process of the internal stores via extracellular Ca^{2+} entry. Alkaloids were able to inhibit this refilling process with potencies in a similar range to that obtained against KCl-induced contraction. Anonaine showed a complete inhibition of the response to the third caffeine challenge, whereas roemerine and dehydroroemerine only partially inhibited this response.

The presence of a methyl group at N-6 in roemerine could allow this compound to acquire a greater activity in inhibiting the noradrenaline-induced contraction. On the other hand, dehydroroemerine shows a loss of potency in inhibiting the contractile response induced by noradrenaline. This loss of potency might be due to the less lipophilic character of the molecule and the absence of the chiral carbon atom.

We conclude that aporphines isolated from natural sources exhibit a common feature in relaxing vascular smooth muscle, by altering the function of potential-operated calcium channels and the activity of α_1-adrenoceptors. Modifications in structural properties can modulate both activities, and increase the specificity of the alkaloids for one or another type of mechanism.

Acknowledgements

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