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Inhibition of drug-induced contractions of guinea-pig ileum by *Annona crassiflora* seed extract

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Abstract—A hydro-alcoholic extract of dried *Annona crassiflora* seeds (AR.1) showed a non-specific inhibitory effect on drug-induced contractions of guinea-pig ileum. Maximum responses to histamine, acetylcholine and bradykinin were reduced increasingly with increasing doses of AR.1. Affinities of the gut to the above agonists, expressed as respective pD_2 values were not changed by AR.1. Dose-response curves of calcium-induced contractions in depolarized preparations were shifted to the right but maximum responses were not changed by AR.1. It is suggested that the inhibitory effect of AR.1 is due to a decrease in membrane permeability to calcium. This inhibitory effect may be responsible for the effect of *Annona crassiflora* as a remedy against snake venom.

Accidents involving snake bites are still frequent in Brazil, *Bothrops jararaca* being the most frequent venomous snake involved, followed by *Crotalus durissus terrificus*. Mortality rates of individuals not treated with antiserum is about 25% (do Amaral 1930). The snake venom causes a series of pharmacological effects that include local necrosis, retardation of clotting and lowering of blood pressure. In contrast to many other snake venoms, *Bothrops* venoms develop minimal neurotropic action. All pharmacological effects of snake venom are produced either by the direct effect of their toxic constituents or by release of pharmacologically active substances from tissues (Deutsch & Diniz 1955). Thus rational treatment for snake venom poisoning should include, besides specific antivenin therapy and local treatment of the bite, the use of general measures to combat clotting, haemolysis, cardiac and neurotoxic effects and also enzymic release of other substances (Sarkar & Devi 1968).

However, among some poor and rural Brazilian inland inhabitants, the scientific principles for rational treatment of snake venom poisoning are unknown. Access to medical care being difficult, they rely on plant extracts as remedies for most diseases. Several plant infusions are believed to be remedies against snake venom. *Annona crassiflora* seed infusion is one among these.

Annona crassiflora Mart. (family: Annonaceae) is a species native to open pastures of northeast Brazil. Its fruit (popularly called araticum or nigro-head) has a pleasant taste and smell. The seeds of such fruits are believed in popular medicine to be efficaceous against snake venom, which raised our interest in performing a pharmacological study of semi-purified fractions from the seed extract. We first considered that the seed extract could antagonize the actions of chemical mediators liberated by snake venom. In order to test this hypothesis, an alcoholic extract of *Annona crassiflora* seed was assayed against agonist-induced contractions of guinea-pig isolated ileum.

Materials and methods

Extraction procedures. Dried *Annona crassiflora* seeds were ground and extracted with ethanol: water, 4:1, v/v, in a Soxhlet. The solvent was evaporated, the residue was macerated with n-hexane and the soluble portion was partitioned in n-hexane/water. The aqueous layer from the hexane/water partition was submitted to a second partition with ethyl acetate/water. The ethyl acetate layer was evaporated to dryness under reduced pressure and the residue (AR.1) was tested on the guinea-pig isolated ileum.

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Drugs. Histamine dihydrochloride (Sigma Chemical Co., USA), acetylcholine chloride (Sigma Chemical Co., USA), bradykinin acetate (synthesized in Escola Paulista de Medicina, São Paulo, Brazil) and calcium chloride (Merck, Brazil) were used.

Organ preparation and experimental procedure. Guinea-pigs, ca. 400 g, were killed and the terminal portions of the ilea were immediately removed, washed and mounted in a 10 mL organ chamber, under a resting tension of 1·0 g, containing Tyrode solution of the following composition (mM): NaCl 136·7, KCl 2·7, $MgCl_2$ 1·05, $CaCl_2$ 1·8, NaH_2PO_4 0·4, $NaHCO_3$ 11·9 and glucose 5·56. The solution was adjusted to pH 7·4 and continuously bubbled with air at 37°C. Contractile responses were recorded isotonically on a kymograph using an auxotonic lever (Paton 1957). In some experiments, after an equilibration period of 1 h, AR.1 was added to the organ chamber to examine any contractile effect or any direct influence on the tonus. In other experiments, one agonist (histamine, acetylcholine or bradykinin) was injected into the organ chamber, to obtain complete dose-response curves from the contractions elicited by the different agonists concentrations. Subsequently, a known concentration (2 or 5 $\mu g\ mL^{-1}$) of AR.1 was added to the perfusion fluid and a second, complete, dose-response curve to the same agonist was obtained in the presence of AR.1, to examine any change in the response. In some experiments of this second group, after registering the effect of AR.1, the biological preparation was repeatedly washed out with Tyrode solution, every 5 min, for more than 1 h, and then a third dose-response curve for the same agonist used before was obtained. In a third experimental group, guinea-pig ilea were perfused with Ca^{2+}-free high-K^+ (140 mM) depolarizing Tyrode solution and cumulative dose-response curves for Ca^{2+}-induced contractions were obtained in the absence and later in the presence of AR.1.

Data handling and statistics. pD_2 (co-logarithms of doses producing 50% of maximal responses) were calculated from the adjusted regression line of the experimental points in the dose-response curves (Weinberg 1975). The maximum response of each preparation in the control condition (before adding AR.1) was considered as 100% and all other responses, either in the same dose response-curve or in the second dose-response curve, were expressed as percentages of that value. Results are expressed as means ± s.e.m. Statistical significance was evaluated by paired *t*-tests and differences were considered significant for $P < 0.05$.

Results

Effect of AR.1 on resting tonus. No direct contractile or relaxing effect of AR.1 was observed on the resting ileum, in the absence of an agonist.

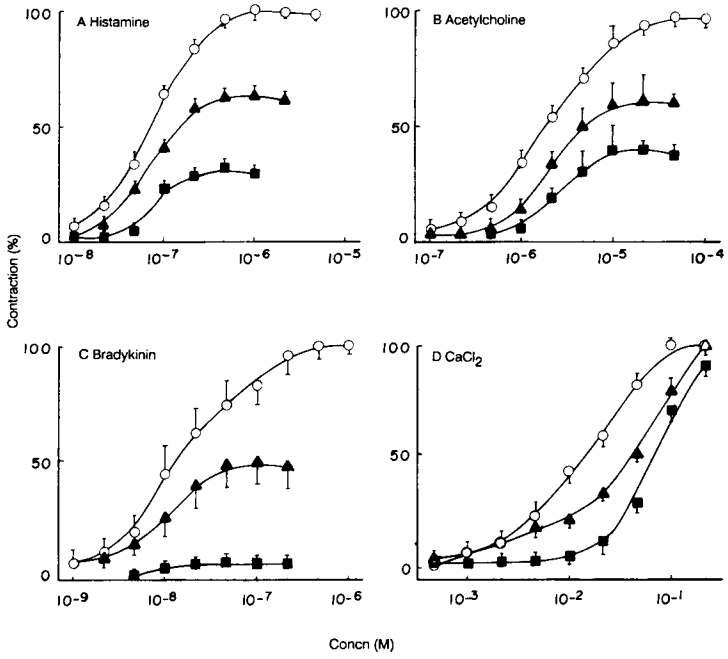

FIG. 1. Inhibitory effect of AR.1 on contractions induced by histamine (A), acetylcholine (B), bradykinin (C) and calcium chloride (D) in guinea-pig ileum. Each point represents the mean of 5–6 experiments; vertical bars show s.e.m. ○ Control, ▲ 2 μg mL^{-1} AR.1, ■ 5 μg mL^{-1} AR.1.

Effect of AR.1 on agonist-induced dose-response curves. AR.1 induced a dose-dependent, nonparallel shift to the right of the dose-response curves to histamine (Fig. 1A), to acetylcholine (Fig. 1B) and to bradykinin (Fig. 1C). Maximum responses to histamine were significantly reduced to 63·7 ± 1·8% of control with 2 μg mL^{-1} of AR.1, and to 32·0 ± 1·9% of control with 5 μg mL^{-1} of AR.1. Maximum responses to acetylcholine were significantly reduced to 59·7 ± 12·0% of control with 2 μg mL^{-1} of AR.1, and to 38·8 ± 1·5% of control with 5 μg mL^{-1} of AR.1. Maximum responses to bradykinin were significantly reduced to 49·3 ± 10·5% of control with 2 μg mL^{-1} of AR.1, and to 6·7 ± 3·0% of control with 5 μg mL^{-1} of AR.1. pD_2 values (7·2 ± 0·1 for histamine, 5·7 ± 0·1 for acetylcholine and 7·9 ± 0·2 for bradykinin) were not significantly different in the presence or absence of AR.1 for any of the agonists.

Irreversibility of inhibitory effect of AR.1. The effects of AR.1 on dose-response curves to histamine, acetylcholine and bradykinin were not reversed upon multiple washouts for more than 60 min.

Effect of AR.1 on Ca^{2+}-induced contractions. Dose-response curves of Ca^{2+}-induced contractions in depolarized ilea were also shifted to the right in the presence of AR.1 (Fig. 1D), but maximum responses were not significantly different in the presence or absence of AR.1.

Discussion

The present study shows that the water-soluble extract of *Annona crassiflora* dried seeds (AR.1) inhibits the contractile responses induced by histamine, acetylcholine or bradykinin in the guinea-pig ileum. Once the maximum responses to these agonists were significantly reduced we conclude that the inhibitory effect is not competitive, at the receptor level (Schild 1954).

Our results on the influence of AR.1 on calcium permeability of depolarized ileum show that this extract decreases the membrane permeability to calcium and that when the external calcium concentration is increased the inhibitory effect is overcome to give the same maximal response obtained in the control condition. Therefore, considering that drug-induced contraction of smooth muscle is dependent on a rise in calcium channel permeability (Hurwitz & Suria 1971), our results suggest that the inhibitory effect of AR.1 on drug-induced contractions might be a consequence of its action in lowering the membrane permeability to calcium. It is unlikely that the inhibitory effect of AR.1 is due to the presence of tannic acid in the extract, since the inhibitory influence caused by tannic acid is easily and promptly reversed by washouts (Calixto & Nicolau 1982), whereas the inhibition due to AR.1 does not return upon multiple washouts for more than 1 h.

The non-specificity of AR.1 inhibition on smooth muscle drug-induced contractions may be the reason for the popular belief that *Annona crassiflora* seed infusion is a remedy against snake venom. Snake venoms are known to liberate chemical mediators in blood (Rocha e Silva et al 1949; Mohamed & Zaki 1957) and tissues (Feldberg & Kellaway 1937, 1938). Some exacerbated physiological effects of these chemical mediators are partially responsible for the clinical picture of snake poisoning (Kellaway 1939; Kaiser & Michl 1968). The non-specific inhibition caused by AR.1 in the smooth muscle, to different chemical mediators, could decrease some of the effects of snake venom due to the liberation of such mediators. This would render a milder clinical picture after a snakebite, allowing some apparent protection against the venom, especially if the amount innoculated is low.

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The disposition of nifurtimox in the rat isolated perfused liver: effect of dose size

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Abstract—The disposition of nifurtimox was studied in the rat isolated perfused liver using a recirculating system. The drug was administered as a bolus (5·0, 15·0 or 30·0 $\mu g\ mL^{-1}$), and its disappearance was monitored by analysing perfusate samples. In all experiments perfusate disappearance was monoexponential, and no significant difference was found between the three doses for the elimination constant (0·016, 0·011 and 0·012 min^{-1}, respectively), half-life (46·6, 65·8 and 66·8 min, respectively), extraction rate (0·128, 0·091 and 0·099, respectively) and distribution volume (41·1, 47·3 and 30·7 mL g^{-1}, respectively). At 30 $\mu g\ mL^{-1}$ the hepatic clearance was lower than the other concentrations of nifurtimox (0·66, 0·51 and 0·34 mL $min^{-1}\ g^{-1}$, respectively). Relatively little parent drug was recovered from the liver at the end of the perfusions. In summary, nifurtimox is cleared slowly from the rat isolated perfused liver, is poorly extracted by hepatocyte cells and is completely metabolized from 2 to 4 h after perfusion.

Nifurtimox is a substituted nitrofuran that has been used successfully for the treatment of *Trypanosoma cruzi* infections. It appears to be effective in amastigote and epimastigote parasite forms, and the reproductive forms are more sensitive to the drug. In-vitro, the minimum inhibiting concentration is 1 μM but over 10 μM is needed to prevent the trypamastigote form from entering the vertebrate cells (Bock et al 1969; Webster 1985).

The mechanism of action of nifurtimox is not completely understood. The trypanocidal action of nifurtimox appears to be related to its ability to form chemically reactive radicals that cause the production of toxic, partially reduced products of oxygen (Docampo & Moreno 1984).

The dose of nifurtimox required to maintain a therapeutic effect, ranges from 5 to 20 mg kg^{-1}. The usual dose is 15 mg kg^{-1}. Adverse effects occur in 40–70% of patients. The central nervous system is mostly affected and effects are dose-related (Brener 1979; Laplume et al 1982).

Little attention has been given to the study of the mechanism of action, the kinetics or the metabolism of this drug, despite the wide morbidity of Chagas' disease and wide nifurtimox use in South America. The dose of this drug has been established by trial and error and the factors which may affect the kinetics and drug disposition remain to be clarified.

Pharmacokinetic studies of nifurtimox in healthy volunteers (Paulos et al 1989) and in patients with chronic renal failure (González-Martin et al 1992) have been made in our laboratories. In these studies we have reported that this drug possesses peculiar pharmacokinetic characteristics. We found low serum concentrations of nifurtimox after an oral dose (15 mg kg^{-1}) suggesting an important presystemic effect with diminished bioavailability. Furthermore, nifurtimox seems to exhibit metabolic features which indicate that the liver could play an important role in its pharmacokinetics.

The main objective of this study was to determine the hepatic disposition of nifurtimox using a rat isolated perfused liver technique.

Materials and methods

Chemicals. Nifurtimox was a gift from Bayer Laboratories (Buenos Aires, Argentina), carbamazepine was a gift from Recalcine Laboratories (Santiago, Chile). All solvents were of HPLC grade and other reagents and chemicals were purchased from Merck Química Chilena, Santiago, Chile.

Animals. Male Sprague-Dawley rats, 140–200 g (Instituto de Salud Pública, Santiago, Chile), were housed in well-ventilated

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