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INVESTIGATION OF THE CARDIOTOXIC PEPTIDES OF THE VENOM OF THE COBRA *Naja oxiana*

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Six populations of basic polypeptides have been found in the venom of the Central Asian cobra Naja oxiana *Eichwald, and four of them – V"c-1, V"c-2, V"c-5, and V"c-6 – have been obtained in the electrophoretically homogeneous state. On a perfused frog heart preparation, all the basic polypeptides proved to be cardiotoxic. However, only some of them (V'c-2, V"c-3, and V"c-4) were characterized by arrhythmic activity as well as by the negative ino- and chronotropic effects, common for all of them. An enhancement of the specific cardiotoxic effect of each of the polypeptides by pure phospholipase A-2 has been shown.*

An investigation of the action of cobra venom on isolated frog heart has revealed a cardiotoxic (CT) effect — systolic cardiac arrest [1].

We have succeeded in separating whole cobra venom into a number of protein–peptide fractions and have obtained in the pure form neurotoxins, phospholipase A-2, cytotoxins, growth factors, and other components: it was shown simultaneously that the CT effect of the venom is due to low-molecular-mass basic peptides [2-6]. It appeared of interest to identify the cardiotoxins and to study their action in more detail.

By an original procedure, combining gel filtration and ion-exchange chromatography [2-4], from the whole venom, together with other components, we have obtained two populations of basic peptides of which one, designated as "F," proved to be homogeneous. The second, designated in the initial separation as "G+H," was characterized, unlike "F," by a powerful phospholipase A-2 activity and consisted of a heterogeneous mixture. This material ("G+H") was subjected to rechromatography on sulfoethyl-Sepharose C-25 (Fig. 1) and, after the separation of the phospholipase A-2, another five fractions of basic polypeptides were obtained which we have designated in the order of issuance from the column as *V"c-2-V"c-6* (fraction F was considered as *V"c-1*).

The electrophoresis of the fractions obtained (Fig. 2) showed that four of them were individual substances: *V"c-1, -2, -5,* and *-6*, while fractions *V"c-3* and *-4* were contaminated with one another. The results of chromatography and electrophoresis showed the basic properties of the peptide fractions isolated. Their cardiotoxic effects were investigated in comparison with the whole venom, the action of which on isolated frog heart has been characterized by ourselves and other researchers previously [1, 7].

On the addition to the perfusate of the whole cobra venom in dilutions of 1:100,000-1:10,000, we observed a disturbance of the cardiac activity expressed in a change in the amplitude and frequency of the cardiac contractions. The nature and efficacy of these changes depended on the concentration of the venom. Thus, in a dilution of 1:100,000, the whole venom caused bradycardia; an increase in the concentration to 1:10,000 led to a gradual decrease in the amplitude of the contractions against a background of bradycardia and to the appearance of Wenckebach–Samoilov periods; a further rise in the concentration of the venom to 5:10,000 led to systolic cardiac arrest only 8 min after its addition to the perfusate (Fig. 3a). Negative ino- and chronotropic effects, and also arrhythmia, were well traced in the case of low concentrations of venom (Fig. 3a), which caused changes of only one, or sometimes two, parameters, with the possibility of their restoration; however, the amplitude of cardiac contraction always remained lowered, and the rate of its lowering depended on the amount

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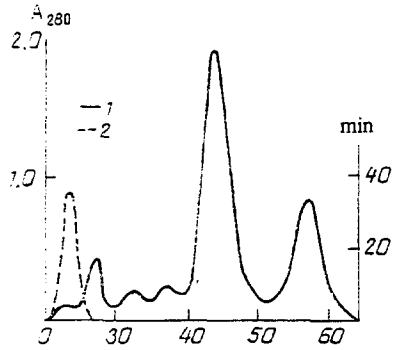

Fig. 1

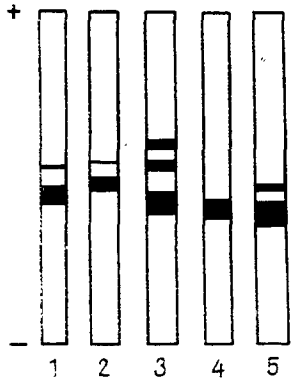

Fig. 2

Fig. 1. Graph of the rechromatography of fractions "G+H" on SE-Sephadex C-25. The column (20 × 400 mm) was equilibrated with 0.05 M ammoniumm acetate buffer, pH 5.0. A single separation was carried out of 150 mg of the material under investigation, dissolved in the equilibrating buffer. Elution (30 ml/h) was carried out with the equilibrating buffer containing a gradientwise increasing concentration of NaCl up to 0.8 M: 1) absorption at 280 nm (protein content); 2) phospholipase A-2 activity (time of coagulating egg yolk, min). *V"c-2* to *V"c-6*) Fractions combined according to the protein peaks.

Fig. 2. Disk electrophoresis of the fraction of membrane-active polypeptides. 15% Polyacrylamide gel, α-alanine buffer, pH 4.3. Electrophoresis for 2.5 h; current strength 6 mA per tube.

of venom in the perfusate. High concentrations of the venom caused a faster development of the events: against a background of a sharp fall in the amplitude of cardiac contraction, the heart stopped immediately and suddenly while the other changes had not yet had time to appear. Therefore, in a study of the dynamics of the cardiotoxic action of the venom and its components the use of medium concentrations (1:10,000-5:10,000) was preferable.

The subsequent analysis of the influence on the isolated frog heart of the fractions and pure substances isolated from cobra venom – neurotoxins, PLA-2, and the basic polypeptides – showed that only the latter produced a cardiotoxic effect. Subsequently, experimentally selected medium concentrations of only these substances were tested.

The perfusion of the isolated frog heart with Ringer solution containing *V"c-1* in a concentration of 5:100,000 (Fig. 3b) caused a twofold decrease in the amplitude of contraction by the 10th minute, but the frequency of contractions had still not changed at this point. By the 25th minute, a threefold decrease in amplitude was recorded together with a 1.4-fold decrease in frequency, and by the 40th minute a further decrease in amplitude – 15-fold – and in frequency. By the 50th minute, the heart was contracting so feebly that the response could not be recorded, and by the 70th minute its complete systolic arrest had set in. Thus, in the concentration used, *V"c-1* causes negative ino- and chronotropic effects followed by cardiac arrest after 50-70 min.

Polypeptide *V"c-5* (5:100,000) caused the changes characteristic for the component *V"c-1* and the whole venom but it was undoubtedly more effective than *V"c-1*. As early as the 3rd minute, a twofold decrease in amplitude was observed, although the frequency had scarcely changed, while at the 6th minute there were decreases in amplitude (5-fold) and frequency (2- to 3-fold). Cardiac arrest was recorded after 10-15 min.

A comparison of the efficacies of the cardiotonic action of the pure polypeptides and that of the whole cobra venom does not appear sufficiently correct, since it is known that the whole venom contains a synergistic factor for the basic polypeptides – phospholipase A-2 [8].

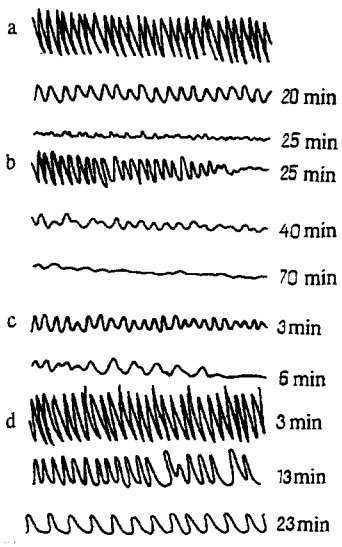

Fig. 3. Cardotoxic effects of the whole venom (a) and of the basic polypeptide components *V"c-1* (b), *V"c-5* (c), and *V"c-2* (d). The substances investigated were added to the perfusate in the following dilutions: a) whole cobra venom – 1:10,000; b) *V"c-1* – 5:10,000; c) *V"c-5* – 5:100,000; d) *V"c-2* – 5:10,000.

A pure preparation of cobra venom phospholipase A-2 did not cause appreciable changes in the cardiac cycle even in a dilution of 1:100. However, the addition of a minute amount of PLA-2 (25:1,000,000) to *V"c-1* and *V"c-5* (5:100,000) sharply potentiated the effect of the CTs under investigation: in the case of *V"c-1* the time of cardiac arrest was shortened by a factor of 2.5 and for *V"c-5* by a factor of 1.2, amounting to 20 and 5 min, respectively.

Thus, the two main populations of polypeptides, in terms of mass, caused dose-dependent negative ino- and chronotropic effects that were intensified in the presence of phospholipase A-2. However, neither a variation in the dilutions used nor the addition of PLA-2 to the perfusate promoted the development of arrhythmia.

The other basic polypeptides differed from the components *V"c-1* and *V"c-5* by a substantially lower level in the venom (Table 1). When *V"c-2* was added to the perfusate in a concentration of 1:10,000, the appearance of arrhythmia was observed after 5-10 min, and a 1.5-fold decrease in amplitude and the failure of alternate contractions by the 15th minute. By the 23rd minute, against the background of a persistent low amplitude, the frequency of contractions decreased by a factor of 4; at the 30th minute a brief stabilization of the cardiac activity was observed in which the amplitude and the frequency were one half that of control specimens. Finally, by the 80th minute the amplitude of contractions had fallen sharply (7-fold) and by the 130th minute systolic ventricular arrest developed on a background of negative ino- and chronotropic effects with arrhythmia. Higher concentrations of the polypeptide *V"c-2* (5:10,000) demonstrated the same effects developing in a shorter interval of time: 25 min after its addition the heart stopped in systole, and the dynamics of the disturbances were not so well-defined.

Fractions *V"c-3* and *V"c-4* were contaminated with one another, and therefore they were added to the perfusate together. In a concentration of 1:10,000 they also caused negative ino- and chronotropic effects and the appearance of arrhythmia, and, against this background, cardiac arrest after 30 min. Lower concentrations (5:100,000) decreased the amplitude by a factor of 1.4 by the 5th minute with the failure of alternate contractions, which led to a fourfold decrease in frequency; the heart stopped by the 40th minute.

TABLE 1. Yields of the Basic Polypeptides after Chromatography of the Whole Venom of the Central Asian Cobra

Name of the material	Stage of chromatography	Yield, %
V"c-1	CM-cellulose	16—18
V"c-2	SE-sephadex	3
V"c-3+V"c-4	SE-sephadex	5
V"c-5	CM-cellulose	18
	SE-sephadex	12—14
V"c-6	SE-sephadex	2

In the same concentration, *V"c-6* demonstrated the changes characteristic for the main, in terms of mass, populations of polypeptides, *V"c-1* and *V"c-5*: at the 14th minute, decreases in the amplitude and frequency of the contractions (3- and 1.2-fold, respectively) were recorded, at the 13th minute brief arrest and, after only a minute, stabilization of the parameters with their subsequent fall again. By the 35th-40th minute the isolated heart was functioning with decreased amplitude and frequency, but no cardiac arrest took place even with a 100-fold increase in the concentration of the polypeptide.

Thus, the cardiotoxic effect of whole cobra venom is caused by the basic polypeptides that had been isolated from it in the individual state and, of these, the most effective in terms of the final effect (systolic arrest) is *V"c-5*. A tendency has been traced to a a rise in cardiotoxicity with an increase in the basicity of the peptides, but it is not absolute, since the most basic of them, *V"c-6*, is weaker than its homologs. It is likely that a more complex relationship exists here that is connected with features of the composition and sequence of the amino acids in the peptides under investigation and, corresponding, with the mechanism of their action. In this connection, the capacity of certain peptides (*V"c-2, V"c-3,* and *V"c-4)* for causing a change in the cardiac contractions of the arrhythmia type attracts attention and requires further study.

Phospholipase A-2 potentiates the peptides isolated, but to a greater degree those which are more cardiotoxically active. Phospholipase A-2 does not change the nature of the cardiotoxic action of the peptides investigated.

EXPERIMENTAL

The venom of the Central Asian cobra, dried in a desiccator over calcium chloride, was purchased from the laboratory of the ecology of snake venoms of the Institute of Zoology and Parasitology, Academy of Sciences of the Republic of Uzbekistan. Batches of venom collected in the early part of the year in the period from 1969 to 1990 were studied.

The other materials used were Sephadexes G-75 and G-10, sulfoethyl(SE)-Sephadex G-25, and Blue Dextran from Pharmacia (Sweden), and carboxymethylcellulose from Reanal (Hungary), and also reagents produced by Reakhim (USSR) of k.h.ch. ["chemically pure"] or "ch.d.a." ["pure for analysis"] grade. A 40-45% (v/v) emulsion in Tris buffer was prepared from egg yolk that had been washed with physiological solution. The activity of the PLA-2 was determined by a semiquantitative method from the time of coagulation of the egg yolk [9].

The fractionation of the whole cobra venom was carried out by gel filtration on Sephadex G-75 and by ion-exchange chromatography on KMTs-32, using a procedure developed specially for the venom of the Central Asian cobra [2-4]. Rechromatography of the "G+H" fraction, possessing a cardiotoxic effect, was performed on a column (16 × 350 mm) of SE-Sephadex C-25 equilibrated with 0.05 M ammonium acetate buffer (pH 5.0). The fractions obtained, combined according to the protein peaks, were concentrated, desalted, and freeze-dried; their purity was checked by disk electrophoresis [10]. The cardiotoxicity of the basic polypeptides was investigated on an isolated frog heart preparation produced by the method generally adopted. The isolated heart was connected to a Jacobi system and was perfused with Ringer solution for cold-blooded animals [7].

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