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RELEASE OF A NEUROSECRETORY HORMONE AS PEPTIDE BY ELECTRICAL STIMULATION OF CRAB PERICARDIAL ORGANS

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INTRODUCTION

The chemical characterization of neurosecretory hormones has been accomplished mainly by analysis of tissue homogenates, and there is little information available on the nature of the material released from axon terminals by stimulation. A cardio-excitor found in extracts of crab pericardial organs (Alexandrowicz, 1953; Alexandrowicz & Carlisle, 1953), neurohaemal structures used in this study, has been identified as a small peptide or peptides (Maynard & Welsh, 1959; Belamarich, 1963; Belamarich & Terwilliger, 1966), as have the active materials found in other neurosecretory systems in animals representing several phyla (e.g. Du Vigneaud, 1956; Edman, Fänge & Östlund, 1958; Davey, 1961; Josefsson & Kleinholz, 1964; Brown, 1965). Only in the case of the vertebrate neurohypophysis have the properties of material released from neurosecretory terminals by appropriate stimuli been compared to the extractable material.

In the neurohypophysis the peptide hormones vasopressin and oxytocin are associated with 'elementary neurosecretory granules' (Lederis, 1962; Barer, Heller & Lederis, 1963), as are proteins (neurophysins) which specifically bind the small active molecules (Van Dyke, Chow, Greep & Rothen, 1942; Ginsburg & Ireland, 1966; Dean & Hope, 1968). Fawcett, Powell & Sachs (1968) have recently been able to detect parallel increase in peptide hormone and neurophysin in the incubation medium when isolated neural lobes are depolarized by raising the external potassium concentration.

In crab pericardial organs the association of the active material with elementary granules has been demonstrated by Terwilliger (1967). The existence of specific binding proteins analogous to the vertebrate neurophysins has not to our knowledge been demonstrated in any other neurosecretory system.

The experiments reported here were undertaken to characterize the cardio-excitor hormone released from an isolated neurohaemal structure when propagated electrical activity is elicited by electrical stimulation (Cooke, 1964; Berlind & Cooke, 1968). We wished to determine (1) whether the cardio-excitor peptides found in extracts represent the form of hormone released by the intact organ; (2) whether we could obtain evidence for a specific binding protein; and (3) whether any protein was released during stimulation.

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METHODS

A pericardial organ of the spider crab *Libinia emarginata* or *L. dubia* was removed to a chamber and prepared for electrical stimulation and recording as previously described (Cooke, 1964; Berlind & Cooke, 1968). The preparation was left in crab saline for two to three hours before tests were begun, the bathing solution being replaced at intervals with fresh crab saline.

The fluid bathing the pericardial organ during each of two experimental periods was analysed. In the first period the pericardial organ was immersed in 0·5 ml of crab saline for 1 h without electrical stimulation, and all fluid from the chamber was collected (sample I). For the second experimental period the pericardial organ was again immersed in 0·5 ml of crab saline for 1 h, during which it was stimulated at a rate of 10/sec. for five periods of 10 min each; each period of stimulation was followed by 2 min without stimulation. During stimulation, propagated electrical activity was monitored at intervals from the dorsal trunk to ensure that the stimulating voltage was optimal, and that the slowly conducted component of the compound action potential, with which release of neurosecretory material has previously been correlated (Cooke, 1964), was still present. The entire volume of fluid was again collected (sample II).

Each of the samples was placed on a 1·1 × 8 cm Sephadex G-25 column (exclusion limits 5000 MW), equilibrated with crab saline, and was eluted with crab saline at a rate of 0·5 ml/min. One ml fractions were collected. Each fraction was halved, 0·5 ml was used for chemical analysis and 0·5 ml for bioassay.

The 0·5 ml aliquots for chemical analysis were hydrolysed in 5 M-NaOH for two hours at 100 °C, neutralized with acetic acid and tested for the presence of free amine groups with ninhydrin. Optical density was measured at 570 mμ on a Coleman spectrophotometer. The amount of material that could be detected as differing significantly from control blanks in these analyses was the equivalent of about 0·02 μM of amino acid in 0·5 ml of fluid.

Bioassays were performed on the isolated perfused heart of the same animal from which the pericardial organ was obtained. The heart exhibits an increase in frequency and/or amplitude of beat when neurohormone is present in the saline being tested.

Distilled-water homogenates of pericardial organs, lysozyme (MW 14,300), 5-hydroxytryptamine (5-HT), and dopamine (DA) were also run through the column. The latter two were tested because they have an effect on the heart similar to that of the pericardial organ hormone. 5-HT is found in low concentrations in pericardial organs (Maynard & Welsh, 1959), as is DA (Cooke, Goldstone, Van Orden and King, in preparation).

RESULTS

I. *Analysis of material released from pericardial organs*

Four pericardial organ preparations provided samples which, after chromatography of sample II (50 min stimulation), showed both heart-exciting activity and ninhydrin-positive material in the column effluent. In these experiments all of the biological activity and all of the ninhydrin-positive material were eluted in the peptide-containing fractions (7–12 ml). The fractions which would be expected to contain proteins of molecular weight greater than 5000 (3–4 ml) were devoid of both biologically active

and ninhydrin-positive material in all four cases. The results of one of these experiments are shown in Fig. 1. In a fifth preparation, heart excitatory activity was found in the fraction emerging at 9–11 ml (peptide) but ninhydrin-positive material was undectectable. Heart-excitatory activity was never detected in the samples collected from unstimulated preparations.

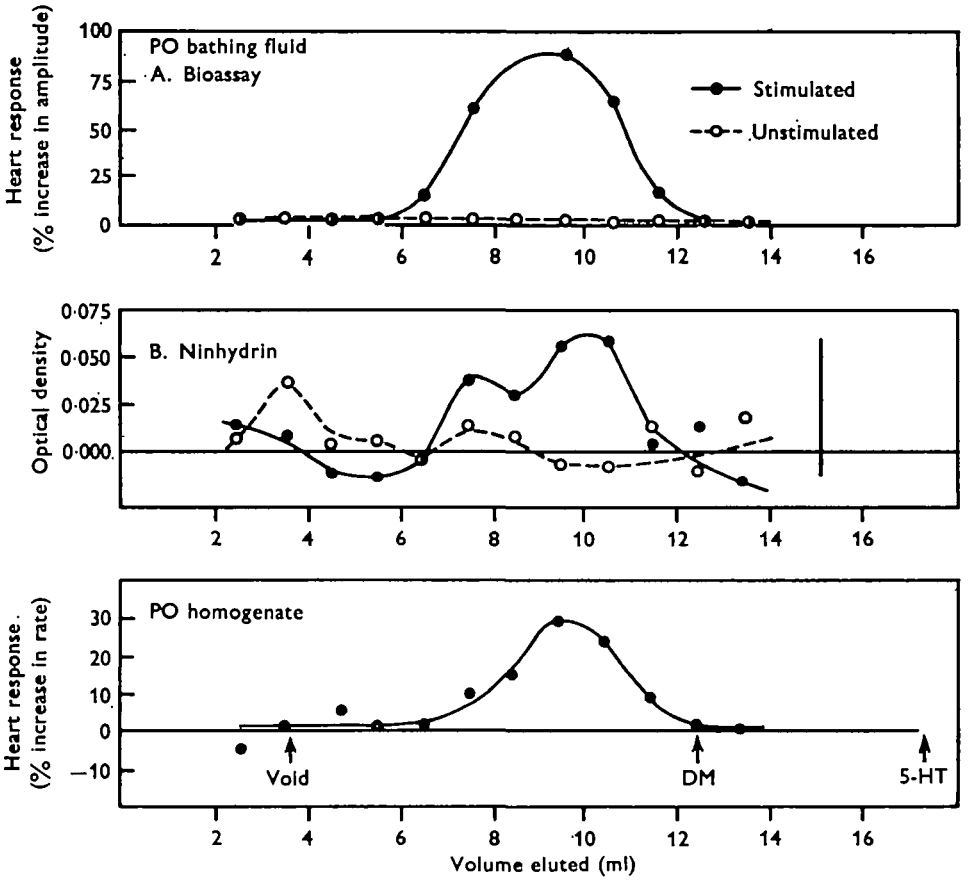

Fig. 1. Results of bioassay (A), and ninhydrin tests (B) on material eluted from a Sephadex G-25 column (1·1 × 8 cm). The samples placed on the column were crab salines in which a pericardial organ had been bathed, with or without electrical stimulation. In A and B the broken lines represent elution of a saline sample in which the pericardial organ remained without stimulation for one hour; the solid lines represent elution of a saline sample in which the same pericardial organ was stimulated for fifty minutes out of 1 h. The period of stimulation began immediately after the period without stimulation had ended. The marker in (B) shows the optical density in the ninhydrin test of 0·05 μM of valine. The bottom record shows the pattern of elution of heart-exciting activity in a distilled-water extract of a pericardial organ. 'Void' marks the elution peak of lysozyme (MW 14,300); DM, the elution peak of dopamine; 5-HT, the elution peak of 5-hydroxytryptamine. The latter two substances are adsorbed on the Sephadex, and emerge long after an entire column has been eluted. Aliphatic amino acids are eluted in a peak which is similar to the peak of cardio-excitor activity. The data given above show that the pericardial organ hormone is present in the external medium as the free peptide.

In trial samples taken from unstimulated preparations which had been rinsed for less than 2 h, ninhydrin-positive material was found in the fractions that would be expected to contain protein. The size (optical density) of this protein peak diminished

with increased duration of washing. A trace of such a peak can be seen in the ninhydrin record of the unstimulated sample in Fig. 1 (broken line), but it has disappeared from the sample taken later, during which the pericardial organ was stimulated. This peak can be attributed to protein leaching from adhering tissue cut during the dissection.

In the five experiments in which cardio-excitor activity was detected in the peptide fractions, the tubes in which dopamine would be eluted were tested; and in one, the very late fraction in which 5-HT should be found was also bioassayed. No heart-exciting activity was detected in any of these fractions.

When a distilled-water homogenate of a pericardial organ is chromatographed on the same Sephadex G-25 column, the only heart-excitatory activity that can be detected is found in the same fractions (7–12 ml) as the active factor released by electrical stimulation. In one case slight activity was detected in the 5-HT fraction. Standardization of the assay heart revealed that it responded well to 0·3 ml of 10^{-9} M of 5-HT perfused through it. This is a sensitivity at least an order of magnitude greater than that usually observed (Cooke, 1966; Berlind & Cooke, unpublished; Cooke & Goldstone, unpublished).

II. *Analysis of extracts for binding of hormone to protein*

To test for protein binding of the pericardial organ hormone in extracts or acetone powders of the neurohaemal organ, several procedures modified from studies of the neurohypophysis were utilized.

(1) To determine whether calcium can disrupt the binding of hormone to protein, two halves of the same extract in calcium-free saline (with 1 mM EGTA) were chromatographed on the same column of Sephadex G-25, one half in calcium-free saline, and the other half after the column had been equilibrated with normal saline. If binding properties of the hormone are similar to those of the neurohypophysial hormones to neurophysin (Ginsburg, Jayasena & Thomas, 1966; Smith & Thorn, 1965; Thorn, 1965), it might be expected that heart-exciting activity would appear with the fast-moving protein peak in the calcium-free column, but with the slower-moving peptide peak in the column with normal saline.

Chromatography of calcium-free, EGTA-treated, extracts gave essentially identical patterns of elution of active material as did chromatography in saline with calcium present; in both cases all activity was found in the peptide peak (identical to that in distilled-water extracts) and none in a protein peak.

(2) To determine whether pH changes can disrupt binding of hormone to protein in the pericardial organ, acetone powders of the neurohaemal structure were taken up in pyridine-acetate buffers isotonic to crab saline, at pH 5·8, 7·4, or 9·0, or pericardial organs were homogenized directly in these buffers. The extracts were chromatographed on G-25 columns equilibrated with the same buffer. Two pooled fractions, one of the peptide, the other of the protein peak, were lyophilized. Material from the protein peak was taken up in 1 M formic acid or 0·1 M acetic acid, and re-chromatographed in acid.

In acetone powders of the neurohypophysis hormone activity migrates with the fast-moving protein peak at pH 5·8; treatment of protein with formic acid or acetic acid results in dissociation of the protein-hormone complex, so that activity from the second chromatography, in acid, appears in the peptide fractions (Lindner, Elmquist & Porath, 1959; Ginsburg, Jayasena & Thomas, 1966).

In extracts chromatographed on Sephadex G-25 in pyridine-acetate buffers at pH 5·8, 7·4, or 9·0, all activity was found in the peptide peak, and no additional activity could be released by treating the protein peak with acid.

In summary, none of the procedures utilized provided any indication that the cardio-excitor factor from the crab pericardial organ is bound to a large non-structural protein molecule intracellularly.

DISCUSSION

Our observations show that neural stimulation of crab pericardial organs results in the appearance in the bathing fluid of a peptide hormone. Both the cardio-excitor activity and ninhydrin-positive material in fluid that had bathed the stimulated neurohaemal organ are found in fractions that would be expected to contain small peptides when chromatographed on Sephadex G-25.

The cardio-excitor activity in fluid collected after neural stimulation and that in distilled-water homogenates of pericardial organs have identical chromatographic behaviour. This provides evidence that work with pericardial organ homogenates is applicable to elucidating the mode of action (Cooke, 1966) and chemical nature of the naturally released hormone. The peptidic nature of material which behaves similarly on Sephadex G-25 in homogenates of *Cancer* has been more completely analysed by Terwilliger (1967).

Biochemical studies have also attributed cardio-excitor activity to peptides (Maynard & Welsh, 1959; Belamarich, 1963). Belamarich & Terwilliger (1966) find two cardio-excitor peptides of molecular weight about 1000 after electrophoresis of homogenates in *Cancer borealis*. The two ninhydrin peaks seen in our figure cannot be assigned any significance, however, and were not duplicated in other experiments. The ninhydrin-positive material observed in our experiments probably includes substances other than cardio-excitor hormone. The column used in this study had a large sample-to-column volume ratio, was short and broad, and was eluted rapidly in order to minimize the deterioration of cardioactive material which has been observed in homogenates (Cooke, 1962). Thus it was not suitable for revealing the presence of different peptides (Flodin, 1961).

Cardio-excitor activity eluted from the column has proven highly stable. Active fractions from one chromatographed extract were frozen, thawed for testing, and re-frozen many times, and retained undiminished activity until they had been used up six months later. By contrast a distilled-water homogenate, heated in boiling water for ½ min, loses its activity within several days.

There is in pericardial organs a system of axons and terminals containing primary catecholamine and one containing 5-HT (Cooke & Goldstone, 1970). When we tested the chromatographic fractions in which dopamine (DA) and 5-HT would be eluted we failed to find cardio-excitor activity. While we cannot rule out the possibility that oxidation of the monoamines rendered them inactive, solutions of the pure drugs in perfusion fluid do not lose their activity within a similar period of time. However, if we consider the total quantities of 5-HT and DA present in a pericardial organ it is not surprising to find no cardio-excitor effect in these fractions. Working from estimates of 5-HT by Maynard & Welsh (1959) and of DA by Cooke, Goldstone, Van Orden & King (in preparation), if all of the monoamine

present were in a 0·5 ml fraction, the 5-HT fraction would approximately correspond to a 10^{-9} M solution and the DA fraction to not more than a 10^{-7} M solution. Since it is unlikely that more than a fraction of the total monoamines present can be released by electrical stimulation, it seems unlikely that the monoamines play even a synergistic role with the peptides in cardio-excitation. It is clear from the observations made in this study that normal cardio-excitor responses are due to the peptides alone.

We sought evidence in this study for the existence of a protein which binds hormone intracellularly and for its release together with hormone during stimulation. We were unable to demonstrate binding of cardio-excitor activity to protein in chromatography of calcium-free, EGTA-chelated, extracts, nor in extracts chromatographed at a range of pH between 5·8 and 9. Terwilliger (1967), however, has found an association of cardio-excitor activity with large granules in density-gradient centrifugation studies. We have not confirmed the appearance of additional activity after acidification of distilled-water homogenates of pericardial organs reported by Terwilliger (1967). Our procedures were not designed to provide information about binding of peptide to structural components. It is clear from our work that if a specific binding protein occurs, its properties are not analogous to those of vertebrate neurophysins.

We have been unable to detect release of protein together with release of peptide hormone during electrical stimulation. Our method for detection required the presence after hydrolysis of approximately 0·02 μM of amino acids in the protein fractions. This is equivalent to 10^{-10} M of a protein having the molecular weight of bovine neurophysin (using Hollenberg & Hope's, 1968, estimate). However, in the absence of any information about a binding protein for the cardioexcitor of pericardial organs or about the proportion of the material in the peptide fractions which is cardio-excitor hormone, it is impossible to know whether our methods have been sufficiently sensitive. We therefore prefer to leave open the question whether a specific protein is released together with the cardio-excitor peptides from crab pericardial organs.

SUMMARY

1. Saline which had bathed an isolated crab pericardial organ was chromatographed on a column of Sephadex G-25. The fractions were divided and assayed for cardio-excitor activity and for ninhydrin colour following hydrolysis.

2. Fluid from pericardial organs which had been stimulated electrically to give a maximum propagated compound action potential showed both cardio-excitor activity and ninhydrin colour. These were always in the same fractions, which corresponded to the volume for elution of small peptides. Fluid from unstimulated preparations gave negative assays.

3. The cardio-excitor activity of fluid from stimulated pericardial organs and of distilled-water homogenates of pericardial organs behaved identically in Sephadex G-25 chromatography.

4. No evidence could be obtained of the cardio-excitor peptide binding to a protein.

5. This work provides evidence that neural stimulation of crab pericardial organs results in release to the bathing fluid of cardio-excitor hormone as free peptide.

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