A. GORĄCA, B. WALKOWIAK *

PARTIAL PURIFICATION AND IDENTIFICATION OF CARDIODEPRESSANT FACTOR FROM THE POSTERIOR PITUITARY LOBE IN RATS

Department of Physiology and *Department of Biophysics, Institute of Physiology and Biochemistry, Medical University of Lodz, Poland

It has been demonstrated previously that the cardiodepressant activity is present in the bovine hypothalamic extract and in the medium incubating the rat’s posterior pituitary lobe “in situ”. In this study medium incubating the posterior pituitary lobe was fractionated by a low pressure gel filtration procedure and the cardiodepressant fractions were pooled and further purified by the HPLC technique on C8 and TSK 3000 SW columns. It was shown, on the basis of mass spectrometry, that cardiodepressant activity is associated with substance(s) with molecular mass of about 500 d. Application of this fraction into the fluid used for incubation of isolated right auricle of the right heart atrium of a two-day-old rat, strongly decreased the frequency of spontaneous discharge of the pacemaker tissue.

Key words: cardiodepressant activity, posterior pituitary lobe, HPLC

INTRODUCTION

The myocardial depressant factor (MDF) was initially identified in the plasma of cats subjected to hemorrhagic shock on the basis of its ability to induce negative inotropic effect (1, 2). MDF occured in the plasma of cats, dogs, rabbits, rats, baboons, and men during the course of shock (3, 4, 5, 6). This factor appears to be a peptide having a molecular weight of 500—1,000 d (7, 8) and originates from the ischemic pancreas (9, 10, 11). A cardiodepressant factor (CDF) has been also isolated by Hallström et al. (12, 13, 14) from the blood plasma of dogs after hypovolemic-traumatic shock. Other authors characterized a filtrable cardiodepressant substance (FCS) which was associated with depressed left ventricular contractility (15). It has been shown that multiple
effects of serum fractions and ultrafiltrates obtained from chronic and acute renal failure patients during dialysis also exhibit the existence of myocardial depressant factor(s) (16). Recently Blake et al. (17) isolated myocardial depressant factor(s) from the ultrafiltrate of plasma heart failure patients and these with acute renal failure.

The presence of the cardiodepressant factor has been demonstrated in the bovine hypothalamus (18) and in the fluid incubating the posterior pituitary lobe “in situ” in rats (19). Experiments with isolated right auricle of the right heart atrium suggest that the cardiodepressant factor(s) acts directly on the heart. Because no exact chemical composition of these factors has been described we have attempted to isolate and partially characterize the cardiodepressant factor from the fluid incubating the posterior pituitary lobe.

MATERIAL AND METHODS

Animals

All the investigations were approved by the ethic committee for animal research. The experiments were performed on male rats, weighing 300—320 g, 5—9 month old, the F1 generation cross-strains of male August and female Wistar from the Institute of Oncology in Warsaw. The animals were anaesthesetized by an i.p. injection of a solution containing 6 mg of chloralose (Roth) and 60 mg of urethane (Flucka Ag, CH-9470 Bucks) per 100 g body weight.

Collection of the medium incubating the posterior pituitary lobe “in situ”

Anaesthetized rats were immobilized on an operating board, ear bars were introduced and the upper jaw was fixed in a clamp. The skin and subcutaneous tissues of the submandibular region were infiltrated with a 2% Polocaini hydrochlorici solution (Polla) and the mandible, the muscles of the floor of the mouth and the soft palate were divided along the midline. A hole was made with a dental drill in the sphenoid bone to expose the dura between the posterior margin of the optic chiasma and the pons. The technique of Worthington (20) of parapharyngeal approach to the pituitary was followed, except that no heparin was injected. Then a longitudinal incision was made in the dura mater with a special fine angular knife and the anterior lobe of the pituitary gland was carefully removed with a stainless steel tube (0.5 mm external diameter) connected to a suction pump as described previously (21). The anterior lobe was removed in the direction from the median eminence to the pons. Care was taken not to touch the surface of the posterior lobe with the suction tube. During the removal of the anterior lobe, the posterior lobe was kept in position with the aid of a fine glass rod. The whole preparation was carried out under a binocular dissecting stereomicroscope P.Z.O., magnification 25×. Special care was also taken in order not to interrupt the vessels between the posterior pituitary lobe and the diaphragm of the hypophysis in order to preserve the blood supply to the posterior lobe. The whole area was then washed several times with 0.95% NaCl solution. With the aid of a micromanipulator two fine polyethylene tubings were placed behind the posterior lobe, a fine polyethylene tube (0.5 mm diameter) was positioned 3 cm above the median eminence. The incubation medium was dropped from the tube so that the pituitary and median eminence were constantly covered with the incubation medium.
The longer tubing with an opening on its side touched the diaphragm of the hypophysis, the shorter one was situated 1.5 mm above the posterior lobe. The medium incubating the pars posterior was constantly aspirated through the shorter tubing. This tubing was connected to a glass tube with methanol (1:2.5 vol/vol of methanol) and dextran (6 mg of dextran MW = 110,000). The glass tube was connected to a suction pump and the incubation fluid was constantly aspirated. The longer outflow tubing was connected with the glass tube at the end of each 30-min interval so that the while volume of the incubating medium could be aspirated (Fig. 1). Sample of the incubation medium collected during the first 30 min was discarded. Then, one sample of the medium was collected during 5 hours of incubation. The incubation medium prepared according to McIlwain and Rodnight (22) had the following composition: NaCl-120, KCl-4.8; KH₂PO₄-1.2; MgSO₄-1.3; CaCl₂-2.8; NaHCO₃-26; glucose- (10.0 mmol/l), pH 7.4, and was dropped at the rate of 1 ml/h. After the methanol precipitation of proteins about 70 ml of the collected incubation medium from 20 rats (one hundred hours) was centrifuged at 10,000 × for 15 minutes. The supernatant was lyophilized and stored at −20°C until bioassay.

**Gel filtration**

The substances after lyophilization of the medium incubating the posterior pituitary lobe was dissolved in 5 ml of 0.1 mol/l acetic acid, and applied onto a Sephadex G-15 column (1.6 × 55 cm) equilibrated with the same buffer. The flow rate was 0.2 ml/min, and 3 ml fractions were collected. The substances were eluted from the column with 0.1 mol/l acetic acid. Presence of peptides in the fractions was determined with spectrophotometry method at 230 nm. All the chromatography steps were carried out at room temperature. Cardiodepressant activity was tested on isolated auricle of the right atrium of two-day-old rats according to the method described previously (23), and active fractions were pooled, lyophilized and subjected to HPLC chromatography.
**HPLC Chromatography**

High performance liquid chromatography was carried out with the CM 4000 system (Milton Roy). The first step of HPLC chromatography involved the C₈ RPC column (SuperPac-Pharmacia Biotech), used in isocratic mode, with the solvent composition: acetonitrile 8%, HPLC-quality water 91.99%, and trifluoroacetic acid (TFA) 0.01%. Both acetonitrile and TFA were delivered by J. T. Baker. The sample, partially purified by low pressure gel filtration, was dissolved in the solvent and 200 µl volume was applied onto the column. The flow rate, fraction volume and run time were set at 1 ml/min, 1 ml and 15 min, respectively. Cardiodepressant activity was assayed for all collected fractions, and usually it was detected in fractions no. 7—10, with maximum activity in fraction no. 8. The procedure was repeated for the remaining material, and fractions containing the activity were collected, pooled and lyophilized.

The next and the last step of peptide purification employed the HPLC gel filtration with TSK G 3000 SW column and TSK precolumn. The system was equilibrated with phosphate — buffered saline (PBS), pH 7.4. The sample — obtained by RPC procedure — was dissolved in PBS, and 50 µl volume was applied into the column. The flow rate, fraction volume and run time were set at 1 ml/min, 1 ml and 40 min, respectively. The cardiodepressant activity was assayed and fractions containing the activity were selected, pooled, lyophilized and stored for further analysis. The purification procedures are summarized in Scheme 1.

### Scheme 1. Summarized procedures for the purification of cardiodepressant factor (s).

<table>
<thead>
<tr>
<th>Step of purification</th>
<th>Peptide (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitation of proteins with methanol.</td>
<td></td>
</tr>
<tr>
<td>↓ Centrifuging 10,000 x g, 15 min</td>
<td>30</td>
</tr>
<tr>
<td>↓ Gel filtration on Sephadex G-15</td>
<td>15</td>
</tr>
<tr>
<td>↓ C₈ HPLC</td>
<td>8</td>
</tr>
<tr>
<td>↓ TSK 3000 SW HPLC</td>
<td>3</td>
</tr>
<tr>
<td>↓ Cardiodepressant factor (s)</td>
<td></td>
</tr>
</tbody>
</table>

The peptide concentration in the investigated sample was measured on the base of the optical density at 230 nm.

**Amino acid analysis**

Approximately 100 pmol of the partially purified substances were subjected to amino acid analysis (AI 420A, analyzer Applied Biosystems, Foster City CA) and quantitative amino acid composition was determined.
Mass Spectrometry

Mass spectrometry (FAB/MS) was performed on a Finnigan Matt Mass Spectrometer equipped with electroscopy interface (Vas Tech, Houston, TX). Samples (1 nmol) were dissolved in acetic acid (2% in water solution) and were introduced into the ion source at a flow rate of 2 μl/min, and electroscopy was emitted at 2500 V.

Determination of Cardiodepressant Activity

Bioactivities of the fractions collected at each purification step were assayed on a pacemaker tissue of the isolated right auricle of the right heart atrium of a two-day-old rat (23). Two-day-old rats (5.5 g) were decapitated and hearts were isolated. The auricle of the right heart atrium was dissected under a stereomicroscope and placed on a platinum wire electrode in a 100 μl chamber in Ringer-Lock solution at 20—22°C. The solution contained (in mmol/l) NaCl-153; KCl-5.6; CaCl₂-3.3; NaHCO₃-1.7; glucose-5.5, was oxygen-saturated and contained additionally atropine sulphate in concentration 5 × 10⁻⁶ mol/l. The solution was continuously exchanged with rate of 0.25 ml/min. After keeping the isolated atrium auricle for 15—30 min in the chamber, it contracted spontaneously at a constant rate. The fractions collected from the columns were dissolved in 0.1 ml redistilled water and injected into the 100 μl chamber in the volume of 20 μl. The contractions of the atrium auricle were observed under a stereomicroscope and the spontaneous discharge of the pacemaker tissue was recorded on the ECG apparatus especially adapted for this purpose. The changes of discharges frequency were analysed by the computer and expressed as a percentage of the greatest decrease in discharge rate in relation to the control, using specially developed software. Discharge rate of the pacemaker tissue was recorded during 20 s before and 100 s after administration of the studied sample. Each sample was tested on six preparations.

The effects of different concentrations of chloride potassium were also tested on the spontaneously discharging isolated right auricle of the right atrium of a two-day-old rat

Statistical analysis

The data reported as mean ± SE were analysed by one-way analysis of variance (ANOVA) followed by Student’s unpaired t-test. P < 0.01 was considered as significant.

RESULTS

Incubation medium was used as a starting material for the purification of CDF, since it contains large amounts of the peptide. After centrifugation of the incubation medium, the supernatant was chromatographed by a low pressure gel filtration. Fig. 2. shows the elution profile of the medium incubating the posterior pituitary lobe from Sephadex G-15, as monitored by absorbance at 230 nm.
Fig. 2. Low pressure gel filtration chromatography of a medium incubating the posterior pituitary lobe in rats. Column dimensions: 1.6 × 55 cm; flow rate, 12 ml/h; eluent 0.1 mol/l acetic acid; fraction size, 3 ml, sample volume, 5 ml. Cardiodepressant fractions (19—25) were pooled.

Fig. 3. Further purification of pooled cardiodepressant fractions from gel filtration (see Fig. 2) with the use of C₈ column (4 × 250 mm) by HPLC. Isocratic elution with 8% acetonitrile, 91.99% water and 0.01% TFA (pH 2.0; 1 ml/min). UV absorbance (A230) is shown by the solid line, and cardiodepressant activity is indicated in the peak.
The pooled active fractions were lyophilized and redissolved and then subjected to HPLC chromatography. Fig. 3 shows a randomly selected chromatogram of a cardiodepressant factor obtained with C₈RPC column. A cardiodepressant factor demonstrated low hydrophobicity, and it was quickly eluted from the column. At this step of isolation procedure several runs were repeated. Fractions 7—10 with the cardiodepressant activity were pooled and lyophilized.

Fig. 4. shows the separation result of the material with a cardiodepressant activity obtained by rechromatography on a TSK 3000 SW column. At this step also several runs were performed. Cardiodepressant activity was found in the 2nd peak. Retention time of the cardiodepressant fraction was about 32 min and it corresponds to the molecular weight about 500, as determined from standard curve.

![Figure 4](image)

*Fig. 4. Separation of cardiodepressant peak from RPC chromatography (see Fig. 3) on column TSK 3000 SW (4 x 250 mm, 1 ml/min). UV absorbance (A230) is shown by the solid line and a cardiodepressant activity is indicated in the peak. Active fractions were pooled.*

Amino acid analysis of the cardiodepressant fraction, eluted from the TSK 3000 SW column, indicated the presence of 7 amino acid residues Asp (1), Glu (2), Gly (2), Ala (1), Lys (1). The composition of amino acids of partially purified cardiodepressant factor is presented in Table 1.
Table 1. Amino acid composition of the cardiodepressant factor (values are expressed as pmol/l)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Quantity</th>
<th>Presumed No of Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>110</td>
<td>1</td>
</tr>
<tr>
<td>Glu</td>
<td>340</td>
<td>2</td>
</tr>
<tr>
<td>Gly</td>
<td>310</td>
<td>2</td>
</tr>
<tr>
<td>Ala</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>Val</td>
<td>135</td>
<td>1</td>
</tr>
<tr>
<td>Lys</td>
<td>120</td>
<td>1</td>
</tr>
</tbody>
</table>

The partially purified factor was also analysed by mass spectrometry. High resolution measurements yielded in an (M+H)+ ion at m/z 558.8 (Fig. 5).

![Graph showing mass spectrometry results](image)

**Fig. 5.** Mass spectrometry of the cardiodepressant fraction purified in Fig. 4. FAB/MS (Finnigan Matt, 13 keV Cs+) of the (M+H)+ ion at m/z 558.8.

Computer modelling performed on the base of results obtained with mass spectroscopy analysis allowed to propose the possible amino acid composition of the peptide: m/z 558.8 amino acids

558.8 Glu, Gly, Asp, Ala, Lys

**Biological action of the cardiodepressant fraction**

Samples from the TSK 3000 SW column, after lyophilization, were dissolved in Ringer-Lock solution and were assayed for cardiodepressant activity using isolated spontaneously discharging pacemaker tissue. One major peak of cardiodepressant activity was observed (Fig. 4). This peak caused a 69% ± 6% (P < 0.01, n = 6) reduction of the discharge rate of the auricle in comparison to Ringer-Lock solution (Fig. 6, 7).
Fig. 6. Change in discharge rate of the isolated auricle of the right atrium after administration of 20 μL of cardiodepressant fraction from the TSK 3000 SW column. (values are means ± SE, P < 0.01).

Fig. 7. Examples of records illustrating changes in spontaneous discharge rate of the pacemaker tissue of the isolated auricle in response to administration: Ringer-Locke solution (A) and cardiodepressant fraction (B). Arrows indicate time of 20 μL sample administration into the chamber with spontaneously discharging isolated auricle of the right atrium.
Chronotropic activity of salts

Experiments showed that chloride potassium in concentration of 179.2 mmol/l caused a decrease in spontaneous discharge rate by 13.4%. Lower concentration had no influence on the cardiodepressant activity (Table 2).

Table 2. Influence of different concentrations of chloride potassium on spontaneous discharge rate of a pacemaker tissue of the isolated right auricle

<table>
<thead>
<tr>
<th>Concentration of chloride potassium (mmol/l)</th>
<th>Decrease of discharge rate (% of control; means ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.6</td>
<td>0</td>
</tr>
<tr>
<td>11.2</td>
<td>0</td>
</tr>
<tr>
<td>22.4</td>
<td>2.1</td>
</tr>
<tr>
<td>44.8</td>
<td>4.4</td>
</tr>
<tr>
<td>89.6</td>
<td>4.2</td>
</tr>
<tr>
<td>179.2</td>
<td>13.4</td>
</tr>
<tr>
<td>358.2</td>
<td>28.1</td>
</tr>
<tr>
<td>716.8</td>
<td>65.12</td>
</tr>
</tbody>
</table>

DISCUSSION

This report describes procedures for the isolation of cardiodepressant factor (s) from the medium incubating the posterior pituitary lobe in rats. Methanol precipitation of plasma proteins, followed by gel filtration on Sephadex G-15 effectively separated the active factor from plasma proteins and salts and thus it was the most significant step in the preparation of purified depressant factor activity. The CDF is clearly distinguishable from free amino acids. Previously, we have tested the effects of free amino acids on the spontaneous discharge rate of a pacemaker tissue. Glutamic acid, leucine and alanine exerted moderate negative inotropic effect by 10—28% when used at the concentration of $10^{-1}$ mol/l (24).

Even if these amino acids were present at a concentration as high as $10^{-1}$ mol/l, their contribution to the cardiodepressant activity would be only modest. However, none of these three amino acids was present at this level in the HPLC isolated fractions having cardiodepressant activity. Lefer and Curtis (8) do not regard single amino acids as cardiodepressant substances.

Lefer and Inge (25) showed previously that the amount of salt required to elicit a negative inotropic effect of MDF, comparable to that of the active factor, is in excess of 450 mequiv/l. In this study we also report that only a high salt concentration (179.2 mmol/l) depresses spontaneous discharge rate of the pacemaker tissue by 13.4%. In our study the fractions containing $K^+$ and
Na\textsuperscript{+} ions were discarded. Goldfarb and Weber (26) also found cardiodepressant activity in preparations free from salts. Previous results, obtained by gel filtration of the medium incubating the posterior pituitary lobe on Sephadex G-15, indicated that all the CDF-like activity substances eluted after the void volume, had molecular weight below 1500 d (19). In the present study we have demonstrated that cardiodepressant activity in the medium incubating the posterior pituitary lobe, is caused by the compound(s) with molecular mass of about 500 d. One group of experiments suggests that MDF isolated from shock plasma has a molecular weight between 500 and 5,000 d (3, 4); whereas other experiments suggest a molecular weight >10,000 d (27, 28). So far, exact chemical composition of myocardial depressant factor (MDF) has not been elucidated, despite intensive investigations. The difficulty in defining the chemical nature of this factor is due to the main fact, that it is present in shock plasma at a very low concentration. A few investigators have tried to isolate, purify, and characterize a specific shock-induced cardiodepressant substance. Greene et al. (29) demonstrated that glycine, serine and alanine were the constituents of a MDF molecule. Only Goldfarb et al. (30) isolated a mixture of L-leucine and L-isoleucine and identified L-leucine as a circulating cardiodepressant factor. They maintain, however, that the myocardial depressant factor appears in the plasma of animals in post-haemorrhagic shock.

Cardiodepressant factor isolated by us from the medium incubating \textit{in situ} contains 7 amino acids: 1 Asp, 2 Glu, 2 Gly, 1 Ala, 1 Lys. This amino acid composition is different from the previously published one, in which it was stated that CDF contained the following amino acids: Gly, Glu, Ser, Ala, Lys, Asp, Leu, Arg and Tre (19). The present composition is also different from that reported earlier for the CDF isolated from the bovine hypothalamus, in which six amino acids were found (Glu, Gly, Ser, Ala and GABA) (31). The discrepancy between our present and previous data, may be due to the fact that at present we have much better methods for peptides purification or that previously obtained were isolated from another source. The previously investigated cardiodepressant fraction could contain amino acids derived from other peptides. These data suggest that the cardiodepressant factor(s) isolated from the medium incubating the posterior pituitary lobe is different from MDF isolated from shock plasma by Brand and Lefer (1). MDF appears to originate, in a large degree, from the ischemic pancreas (10), whereas the cardiodepressant factor originates from the posterior pituitary lobe. Both MDF and cardiodepressant factor circulate in the blood and depress the heart. MDF is accumulated in the blood during the late stage of shock and exerts a direct negative inotropic effect, whereas cardiodepressant factor exerts a direct negative chronotropic effect.

We have also tested the effects of neuropeptides present in the hypothalamus and the posterior pituitary lobe such as: vasopressin, oxytocin,
substance P, leu-enkephalin, met-enkephalin, delta sleep — inducing peptide, atrial natriuretic factor, angiotensin II and substance P on cardiodepressant activity. We have shown previously that the above mentioned peptides at concentrations ranging from $2.1 \times 10^{-7}$ to $10^{-3}$ mol/l had no effect on the contraction frequency of the isolated auricle of the right heart atrium of a two-day-old rat (24). Similarly, Accili et al. (32) have shown no changes in the heart rate in response to neurokinin A, neurokinin B, substance P and vasoactive intestinal peptide. Zhu et al. (33) have shown that in spontaneously beating rabbit atria endothelin-1 had no effect on the beating rate.

In conclusion, we have isolated from the medium incubating the posterior pituitary lobe a cardiodepressant factor containing 7 amino acid residues: 1 Asp, 2 Glu, 2 Gly, 1 Ala, 1 Lys and decreasing in spontaneous discharge rate of a pacemaker tissue. Mass spectrometry analyses of the cardiodepressant fraction resulted in a peak at m/z 558.8 [M + H]+ which corresponded to a peptide Glu, Gly, Asp, Ala, Lys.

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Author's address: A. Gorąca, PhD. Department of Physiology, Institute of Physiology and Biochemistry, Medical University of Lodz, ul. Lindleya 3, 90-131 Lodz, Poland Phone/Fax: (0-42) 678-26-61