Further characterization of cardiodigin, Na^+, K^+-ATPase inhibitor extracted from mammalian tissues

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This study was undertaken to characterize endogenous digitalis-like activity in water extract from mammalian tissues. Prepurified samples obtained from guinea-pig heart were analysed by reverse-phase HPLC using an acetonitrile gradient. The eluent was assayed for its activity as inhibitor of human heart Na^+,K^+-ATPase and digoxin-like immunoreactivity. Both activities were recovered in the same fraction after two successive chromatographic steps. These results provide further evidence for the presence of an endogenous digitalis-like factor, cardiodigin, in mammalian heart.

Cardiodigin *Na^+,K^+-ATPase* *Reverse-phase HPLC* *Endogenous Na^+,K^+-ATPase inhibitor* *Digoxin radioimmunoassay*

1. INTRODUCTION

In recent years, a body of experimental evidence has been accumulated that favors the existence of a physiological regulator of the sodium pump. This factor could play an important role in the regulation of sodium excretion [1–3], in the mechanism of action of cardiac glycosides [4–6] and in the pathogeny of hypertension [7–9].

We have reported the presence in water extracts of mammalian heart of a factor that cross-reacts with digoxin antibodies and inhibits Na^+,K^+-ATPase [10]. This inhibition is specific, reversible, antagonized by potassium and shows species dependency. In addition, the factor displaces specifically bound ouabain.

Here, we report that when a guinea-pig heart extract is further purified using two-step reverse-phase HPLC, the peak for inhibition of Na^+,K^+-ATPase coincides with the peak for specific reactivity with digoxin antibodies. This confirms that the factor so far identified and previously coined cardiodigin has characteristics analogous to digitalis.

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2. MATERIALS AND METHODS

2.1. *Digitalis-like factor preparation*

Guinea-pig hearts were extracted with water by the method in [3], slightly modified.

Shortly, after centrifugation and extraction by methanol, the dry residue obtained from 50 g tissue (from about 60 guinea-pigs) was taken up in 10 ml water and extracted 3 times with 10 ml cyclohexane to remove lipids. After lyophilization of the aqueous phase, dry residue equivalent to 6 g original tissue was dissolved in 0.3 ml water and applied to a Bond Elut C18 extraction column (Analyticem International). The elution was carried out with 3 × 2 ml water and 3 × 1 ml methanol. This last phase was then evaporated at 40°C under low pressure.

2.2. *High performance liquid chromatography*

To purify further the extract, HPLC was performed using a Hewlett-Packard HP-1084B

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chromatograph. The extract was injected into a Lichrosorb® RP-8, 5 μm column (250 × 4 mm) (Merck) combined with a precolumn (30 × 4 mm), filled with Lichroprep RP-8, 25–40 μm, previously equilibrated with 20% acetonitrile in water (Burdick & Jackson, HPLC grade). Elution was carried out at a flow rate of 1 ml/min, with a gradient of 20–50% acetonitrile in water. Fractions were collected every 2 min, during 20 min, lyophilized and tested for immunoreactivity. Corticosterone, chosen as external standard [RT = 16.24 min ± 0.10 (n = 6)] was injected after every fourth injection of the extract. When its retention time differed by more than 0.3 min from the mean retention time of 16.24 min, the system was washed. In addition, the precolumn was replaced.

2.3. *Enzymatic assay*

Preparation of human heart Na^+,K^+-ATPase was performed as in [11]. Usually 1.5–3 μg enzyme protein was incubated for 1 h at 37°C in a medium containing 100 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 3 mM [^{32}P]ATP (Amersham), 1 mM EGTA and 20 mM maleate-Tris, pH 7.4. The final volume was 0.1 ml. The reaction was stopped by the addition of 0.2 ml chilled dextran charcoal suspension in 0.1 M HCl (0.2 g/ml). Total ATPase activity was determined by the method of Bais [12]. Basal ATPase activity was determined in the absence of KCl and in the presence of 1 mM ouabain. Na^+,K^+-ATPase activity was calculated as the difference between total and basal activities.

2.4. *Radioimmunoassay*

Digoxin RIA kit was purchased from Becton Dickinson. Extract and cardiac glycoside dilutions were prepared in a buffer containing 5.5 mM KH_2PO_4, 55 mM Na_2HPO_4, 7.7 mM NaN_3 and 60 g/l bovine serum albumin.

The assay procedure was essentially as described by De Pover et al. [10]. The reaction was stopped by the addition of 0.5 ml of a dextran-charcoal suspension. After centrifugation and decantation, the radioactivity of the pellet was counted.

3. RESULTS

Digoxin antibodies and tracer, ^{125}I-labeled digoxin derivative, were incubated in the presence of various concentrations of digoxin or guinea-pig heart extract, obtained after Bond Elut C18 chromatography. Fig.1 illustrates a typical experiment. Digoxin displaced the tracer bound to the antibodies yielding a sigmoidal displacement curve (fig.1b). The displacement curve obtained with guinea-pig extract was parallel to that of digoxin (fig.1a). As shown in [3], this allowed expression of the concentration of the immunoreactive factor in digoxin RIA equivalents. In 14 successive preparations, the yield of the extraction procedure was 0.22 ± 0.05 pmol/g wet wt tissue (mean ± SE).

The extract was further analyzed by RP-HPLC. As shown in fig.2, digoxin-like immunoreactivity was detected in fractions 5 (8–10 min) to 8 (14–16 min) and peaked in fraction 6 (10–12 min).

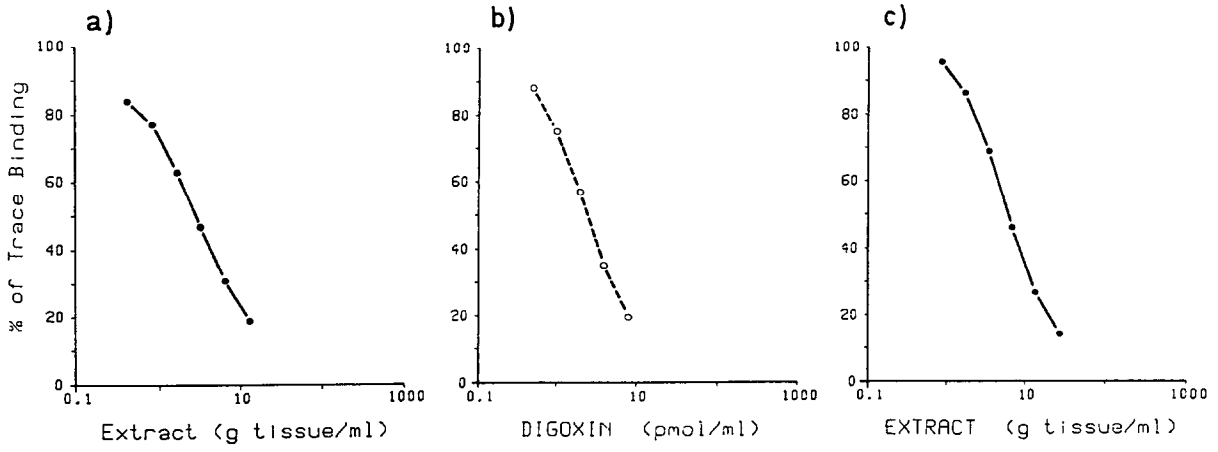

Fig.1. Radioimmunoassay of guinea-pig heart extract obtained after Bond Elut C18 chromatography (a) and fraction 6′ obtained after RP-HPLC (c), compared to digoxin (b).

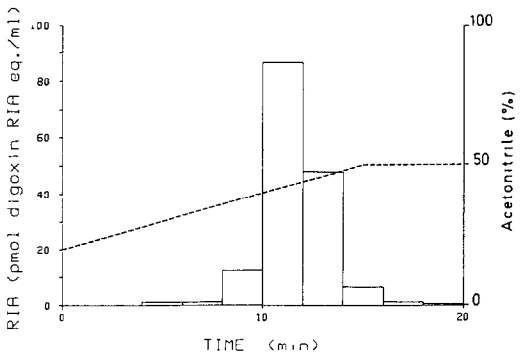

Fig.2. Reverse-phase HPLC of guinea-pig extract obtained after Bond Elut C18 chromatography. The extract was injected in aliquots from about 15 g tissue, into a Lichrosorb® RP-8, 5 μm column (250 × 4 mm). Fractions obtained from 33 successive runs were pooled, lyophilized and assayed for radioimmunological activity. The RIA-response, corresponding to 309 g tissue/ml is expressed in pmol digoxin RIA eq./ml.

The total recovery was about 84%; 46.3% of the initial radioimmunoactivity was recovered in fraction 6 (10–12 min). However, after this first HPLC step, there was no correlation between the immunological and the Na^+,K^+-ATPase inhibitory responses: inhibitory activities were also found in fractions collected between 6 and 20 min (not shown). Therefore, fraction 6 was rechromatographed under the same conditions as the crude extract. As illustrated in fig.3a, fraction 6 led to only one radioimmunological peak with the same retention time as that obtained after the first HPLC analysis (fraction 6′). The recovery was about 94.4% in fraction 6′. As shown in fig.1c, the digoxin-RIA displacement curve obtained with fraction 6′ was parallel to those given by digoxin and the Bond Elut extract.

Fraction 6′ and adjacent fractions were tested for their inhibitory activity on human heart Na^+,K^+-ATPase. The bulk of inhibitory activity was detected in fraction 6′ (fig.3b). This fraction gave a dose-dependent inhibition superposable to that of digoxin when its content in cardiodigin was expressed in digoxin-RIA equivalents (fig.4). The Hill coefficients calculated by linear regression were 0.94 ± 0.02 and 1.01 ± 0.11 for fraction 6′ and digoxin, respectively. Furthermore, Dixon plots (not shown) were straight lines suggesting that inhibition obeyed the mass-action law.

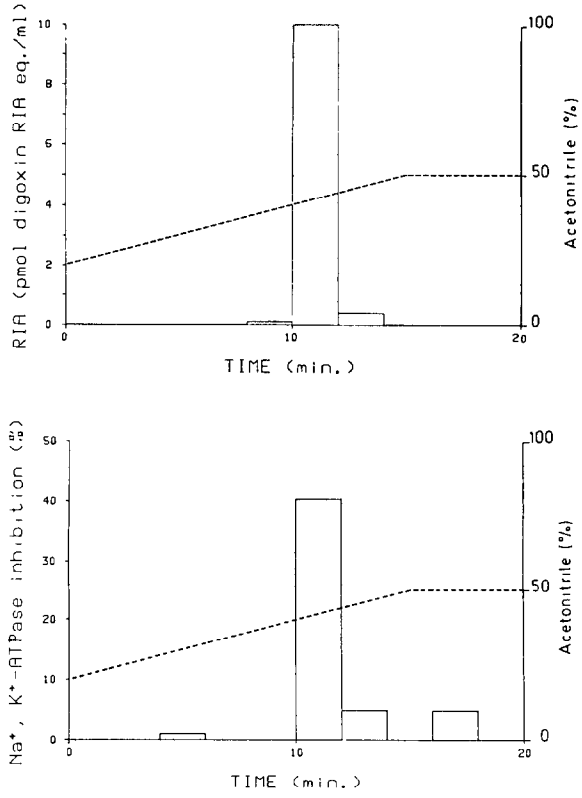

Fig.3. (a) Reverse-phase HPLC of fraction 6 (10–12 min) obtained as described in fig.2. 9 runs of about 15 g tissue were performed, using the same column and gradient as described in fig.2. The RIA response, corresponding to 27.3 g tissue/ml is expressed in pmol digoxin RIA eq./ml. (b) Human heart Na^+,K^+-ATPase inhibition of each 2 min fraction, obtained as in (a). The inhibitory response, expressed in percent, was obtained with 20 μl of each HPLC fraction. This volume of fraction corresponds to 41 g tissue.

4. DISCUSSION

Here, we report the development of a RP-HPLC method applied to guinea-pig heart extract, which yielded a fraction that cross-reacted with digoxin antibodies and evoked a dose-dependent inhibition of human heart Na^+,K^+-ATPase. These results confirm our previous data obtained with a less purified preparation [10].

A digoxin-like immunoreactivity has been reported in mammalian blood [3,13–18], urine [1,19] and tissue extracts [3,4,20]. This im-

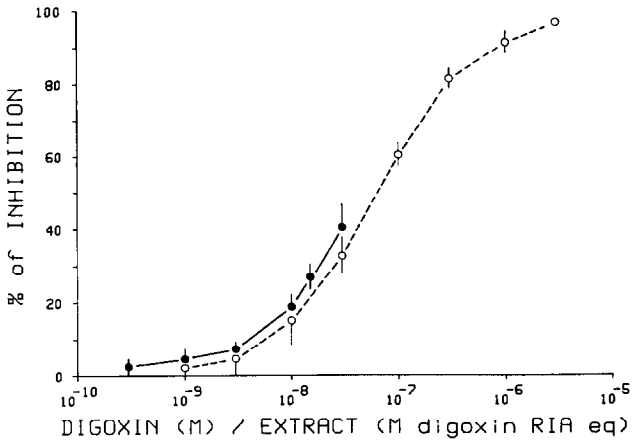

Fig.4. Inhibition of human heart Na^+,K^+-ATPase by fraction 6′ (●) and digoxin (○). Values are means of triplicate determinations and representative of two different experiments.

munoreactivity does not always correlate with Na^+,K^+-ATPase inhibitory activity [16,17,21].

Such discrepancies could be related to a lack of specificity of antibodies as well as to interference with the RIA reaction [22–24]. This is unlikely in view of similar results obtained in the present study and in a previous one [10] with antibodies from two different sources (Diagnostic Products Corp. and Becton Dickinson). Both preparations discriminated digoxin from digitoxin, two molecules differing only in the presence of one OH group in the C 12β position [10]. This indicates that cardiodigin is not an occasional aspecific interference but a chemical entity endowed with structural features critical for its recognition by antibodies.

Some extraction and purification procedures may yield non-specific RIA cross-reacting substances and/or non-specific Na^+,K^+-ATPase inhibitors [10,25,26]. In our experiments, we found that after the first HPLC step, Na^+,K^+-ATPase inhibitory activity was detected in fractions that did not contain digoxin-like immunoreactivity. However, after a second HPLC separation, both activities were recovered in the same fraction. A good correlation has also been found by Gruber et al. [15] after HPLC analysis of plasma of volume expanded dogs. Similarly, in TLC fractions of rabbit adrenal extract, Schreiber et al. [20] found a marked but not complete parallelism between the inhibition of ^{86}Rb uptake and the immunoreactivity.

Further studies are still required for the elucidation of the biological significance and for the determination of the chemical structure of cardiodigin.

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