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Complete purification of two identical Na^+-pump inhibitors isolated from bovine hypothalamus and hypophysis

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We have completely purified, in parallel, a low molecular weight, non-peptidic, non-lipidic, Na^+,K^+-ATPase inhibitory factor from bovine hypothalamic and pituitary tissues. In the final purification step we obtain, from both tissues, a single, homogeneous peak, with a maximal absorbance at 247 nm. This factor, at physiological concentrations of potassium (5–25 mM), inhibits in a dose-response manner Na^+,K^+-ATPase and displaces ouabain from its receptor at the enzyme structure. The factor isolated from both tissues is identical, being the specific activity per weight of tissue higher in hypophysis. No factor was found in cerebral cortex, used as tissue control.

Na^+,K^+-ATPase; Sodium pump; Ouabain

1. INTRODUCTION

The existence of a circulating inhibitor of the ouabain-sensitive Na^+,K^+-ATPase has been implicated in several physiological roles, such as renal sodium excretion or the genesis of certain forms of hypertension [1]. There has been a great deal of effort by several groups to identify and characterize a plasma, urinary or tissue inhibitor of Na^+,K^+-ATPase with controversy about its chemical structure and its mechanism of action [2]. One reason for this disagreement could be due to the use of partially purified preparations that could give conflicting results depending on the grade of purification achieved. Because the midbrain has been implicated in the control of circulating Na^+,K^+-ATPase inhibitors, the hypothalamus was initially selected as a source for study. We have reported the presence, in crude tissue extracts of bovine hypothalamus [3] and hypophysis [4] of a factor that reversibly blocks the active transepithelial sodium transport across anuran membranes and that inhibits Na^+,K^+-ATPase from mammalian kidney, as well as the binding of labelled ouabain to its cellular receptor in frog urinary bladder. We have pursued the purification efforts, using a multiple bioassay strategy, with the final goal of obtaining a completely pure substance in order to determine its chemical structure. In the current communication, we describe the final purification of such factor from bovine hypothalamus and hypophysis. The identity between the factor obtained from both tissues is described. The effect of this pure hypothalamic hypophysary inhibitory factor (HHIF) on Na^+,K^+-ATPase and on displacement of [^{3}H]ouabain from its receptor in the enzyme is also reported. Data are given about some of its physicochemical characteristics.

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Abbreviations: BSA, bovine serum albumin; FFA, free fatty acid; HHIF, hypothalamic, hypophysary, inhibitory factor; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. *Chemicals*

[γ-^{32}P]ATP (3000 Ci/mmol), [^{3}H]ouabain (20.6 Ci/mmol) and [^{125}I]digoxin (833 Ci/mmol) were purchased from the Radiochemical Center (Amersham, England), NADH from Boehringer Mannheim, the enzymes pyruvate kinase and lactate dehydrogenase from Merck (Darmstadt). All the other chemicals were purchased from Sigma. Organic solvents were of high-performance liquid chromatography (HPLC) grade (Merck). Sephadex LH-20 was purchased from Pharmacia and all the HPLC columns from Waters. Deionized distilled water was used for all solutions.

2.2. *Purification*

Tissue extraction: 1 kg of frozen tissue (bovine hypothalamus, hypophysis, cerebral cortex) was extracted according to previously described methods [4,5]. *Chromatographic procedures:* the final extracts were dissolved in methanol and applied to a Sephadex LH-20 column, 3.5 × 85 cm equilibrated and eluted with 100% methanol. Further purification was achieved by 4 steps of HPLC runs; first on a semipreparative C_{18} μBondapak column (7.8 × 300 mm) of 10 μm of particle size, by a step gradient (0–40%, 80 min), (40–80%, 105 min), (80–100%, 110 min) of water/acetonitrile and 0.1% TFA at a flow rate of 2 ml/min; second on an analytical C_{18} Novapak column (3.9 × 150 mm) of 4 μm of particle size by an increasing linear gradient (0–50%) of water/acetonitrile and 0.1% TFA over 260 min at a flow rate of 0.5 ml/min; third in a free fatty acid column (FFA) (3.9 × 300 mm) of 10 μm of particle size by an increasing linear gradient (20–80%) of acetonitrile/water with 0.1% TFA over 180 min at a flow rate of 0.5 ml/min; fourth on an FFA (3.9 × 300 mm) col-

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umn of 10 μm of particle size by a step gradient (20–40%, 60 min), (40–60%, 120 min), (60–80%, 60 min) of water/acetonitrile with 0.1% TFA at a flow rate of 0.5 ml/min. The HPLCs were developed using a Waters 600E system controller with an IBM computer and a Waters 990 photodiode array detector.

2.3. *Bioassays*

The chromatographic fractions were freeze-dried and reconstituted in water. These fractions were assayed as follows. *Na^+,K^+-ATPase activity:* the enzyme was purified from porcine kidney outer medulla by the procedure of Jörgensen [6]. The inhibitory activity was determined by a coupled assay [5,7] and a [γ-^{32}P]ATP assay [8]. One unit (U) of inhibitory activity was defined as the amount of HHIF required to inhibit 8 μg of purified Na^+,K^+-ATPase by 50% in the coupled assay with a concentration of 25 mM KCl. *[^{3}H]Ouabain binding displacement:* we used the method described by Brooker and Jelliffe [9]. *Radioimmunoassay:* a digoxin kit purchased from Immunochem (USA) was used. *Toad bladder assay:* in the initial purification steps, the inhibitory activity of the fractions was confirmed by the toad bladder assay as previously described [3].

2.4. *Characterization*

Physical-chemical treatment: for the acid treatment the conditions for amino acid analysis of peptides were employed. 220 U doses of HHIF were incubated with 5.7 M HCl and 0.005% mercaptoethanol at 115°C for 21 h under vacuum. The remaining HCl was removed by lyophilization and the sample was assayed for inhibition of Na^+,K^+-ATPase. *Alkaline treatment:* 2 U of HHIF were incubated with 0.2 NaOH at 27°C for 2 h, the samples were lyophilized, neutralized with buffer Tris-HCl, pH 5.0, and assayed as described above. Charring was carried out at 250°C for 2 h. In all cases the appropriate reagent controls were also included in the inhibition assay. *Protein incubations:* 2 U of HHIF were incubated for 2 h at 37°C with 6 μg of phospholipase C or 10 μg of BSA in 1 M Tris-HCl, pH 7.4. The incubations were stopped by heating the samples at 80°C for 10 min and centrifugation at 16000 × *g* for 5 min. The supernatants were then assayed for inhibition of Na^+,K^+-ATPase. *Other inhibitors and related compounds:* cholesterol, ouabain, digoxin, dopamine, OH tyrosine, digitoxin, phosphatidylcholinedioleyl, phosphatilaminedioleyl, lysophosphatidylethanolenicleyl, OH-progesterone, androsterone, epiandrosterone, testosterone, chlormanidone, arachidonic, linoleic, glycoquenodeoxycholic, glycocholic, quenodeoxycholic, OH-phenylacetic, OH-phenylpyruvic, OH-benzoic and OH-phenyllactic acids. 100–200 μg of these compounds were run in the final chromatographic system used in the purification (see above) and their retention times and spectra recorded.

3. RESULTS

A crude extract was prepared from 1 kg of bovine hypothalamus, hypophysis or cerebral cortex as described in section 2. The extract equivalent to 250 g of tissue was chromatographed in a Sephadex LH-20 column. The active fractions, eluting just before the salt peak, were concentrated by successive smaller LH-20 columns. At this stage of purification all 3 tissues showed activity in all the assays, including digoxin immunoreactivity (fig.3). The specific inhibitory activity at this purification step was of 1.5 U/g of tissue for hypothalamus and 3.4 U/g of tissue for hypophysis. The material was further purified in a semipreparative C_{18} μBondapak column as described in section 2. The area selected in the hypothalamic and hypophysary profiles from this column was active in all the assays, but did not show digoxin immunoreactivity.

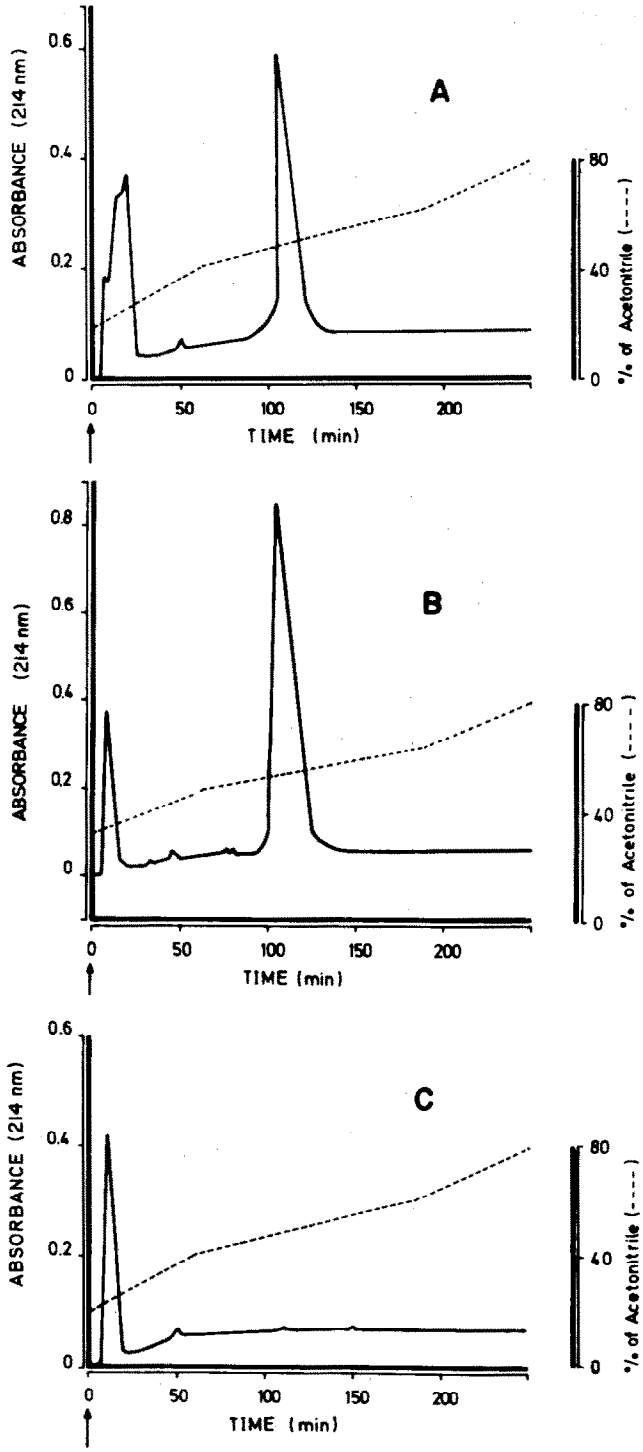

Fig.1. Elution profiles of the final purification by HPLC on an FFA column of 10 μm of particle size. The mobile phase was a step gradient of 20–40% during 60 min, 40–60% during 120 min and 60–80% during 60 min of acetonitrile/water in 0.1% TFA at a flow rate of 0.5 ml/min. The sample injected corresponds to an initial weight of tissue of hypothalamus 350 g (A), hypophysis 42 g (B), brain cortex 350 g (C). The arrow indicates the time of sample injection. Two-minute fractions, except for the peak area which was manually done, were collected and monitored for Na^+,K^+-ATPase inhibitory activity. Along the profiles, inhibitory activity was detected only in the peak that eluted at 108 min in the chromatograms A and B (see fig.2). The chromatogram C can be superimposed on the baseline obtained from injecting the same volume of distilled water (not shown).

Other areas, active only in one assay, were discarded. The profile of brain cortex showed inhibitory activity only in the void volume. From this stage on, the area in the brain cortex profile corresponding to the selected inhibitory area from the other two tissues, was submitted to the same purification steps as the active area in hypothalamus and hypophysis, and used as control.

The next purification steps, in analytical conditions, included a C_{18} Novapak and two FFA runs as described in section 2. In each column the sample injected ranged from 100 to 120 arbitrary units. In the case of brain cortex, where no activity was detected, the amount injected corresponded to the equivalent fraction of hypothalamus injected in each step. Fig.1 represents

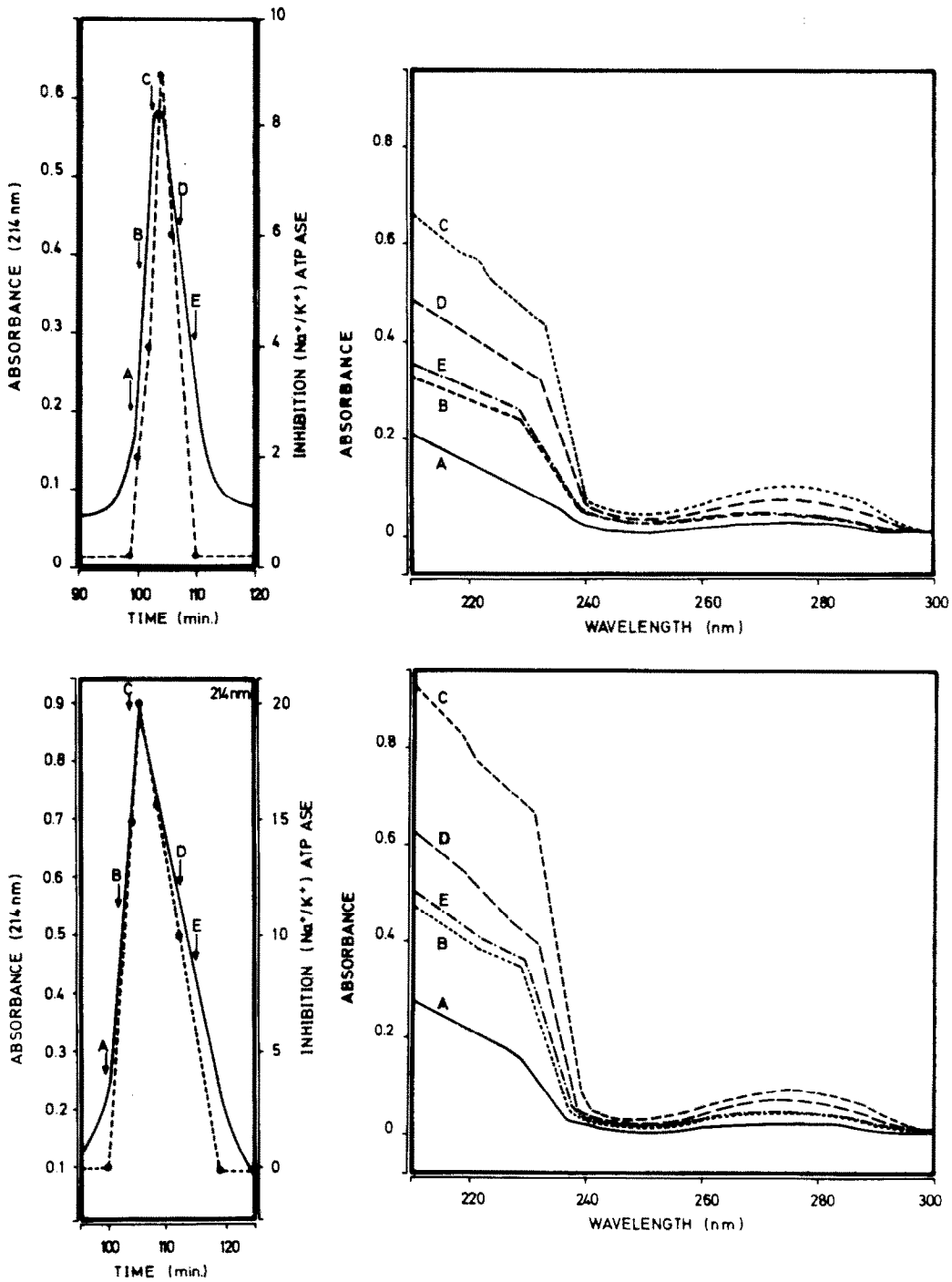

Fig.2. Inhibitory activity and spectral analysis of the peak fractions from the FFA column. The peak profiles were those of fig.1A,B. (Left) Localization of the inhibitory activity (●) in the peaks corresponding to hypothalamus (upper panel) and hypophysis (lower panel). (Right) Automatic overlay spectra obtained from right slope (A,B), peak maxima (C) and left slope (D,E) from 210–310 nm in the peaks corresponding to hypothalamus (upper panel) and hypophysis (lower panel).

the elution profile of the last FFA column in the 3 tissues. The active material eluted as a single peak with a similar retention time in hypothalamus and hypophysis. In the corresponding profile of cortex no peak or inhibitory activity was detected and it can be superimposed on the baseline obtained from injecting distilled water. Fig.2 shows the localization of inhibitory activity in the peaks corresponding to hypothalamus (upper left panel) and hypophysis (lower left panel). The inhibitory activity determined in the standard coupled assay superimposes the absorbance in both peaks. The total inhibitory activity of the peaks is of 50 U for hypothalamus corresponding to 350 g of original tissue and of 105 U for hypophysis corresponding to 42 g of original tissue. The spectral analysis, in both peaks, showed a single component throughout the peak (fig.2, right panels). With an autogain, all the spectra along each peak were superimposed (not shown) with a maximum of absorbance at 274 nm.

The apparent molecular mass from ultrafiltration studies is <1000 (Amicon Diaflo YM2, Amicon Co., Danvers). 500 U of HHIF were not measurable by a microbalance (Cahn 4700, Cerritos, CA: reproducible lower limit, 10 μg; readable lower limit, 1 μg) because of too small amounts. Assuming a molecular mass of 750, we can roughly estimate that the dose necessary to inhibit 50% the standard assay (1 U) is in the range of 10^{-9} M. Charring at 250°C for 2 h destroyed the activity and alkaline hydrolysis for 2 h destroyed 85% of the activity measured in the coupled assay. On the other

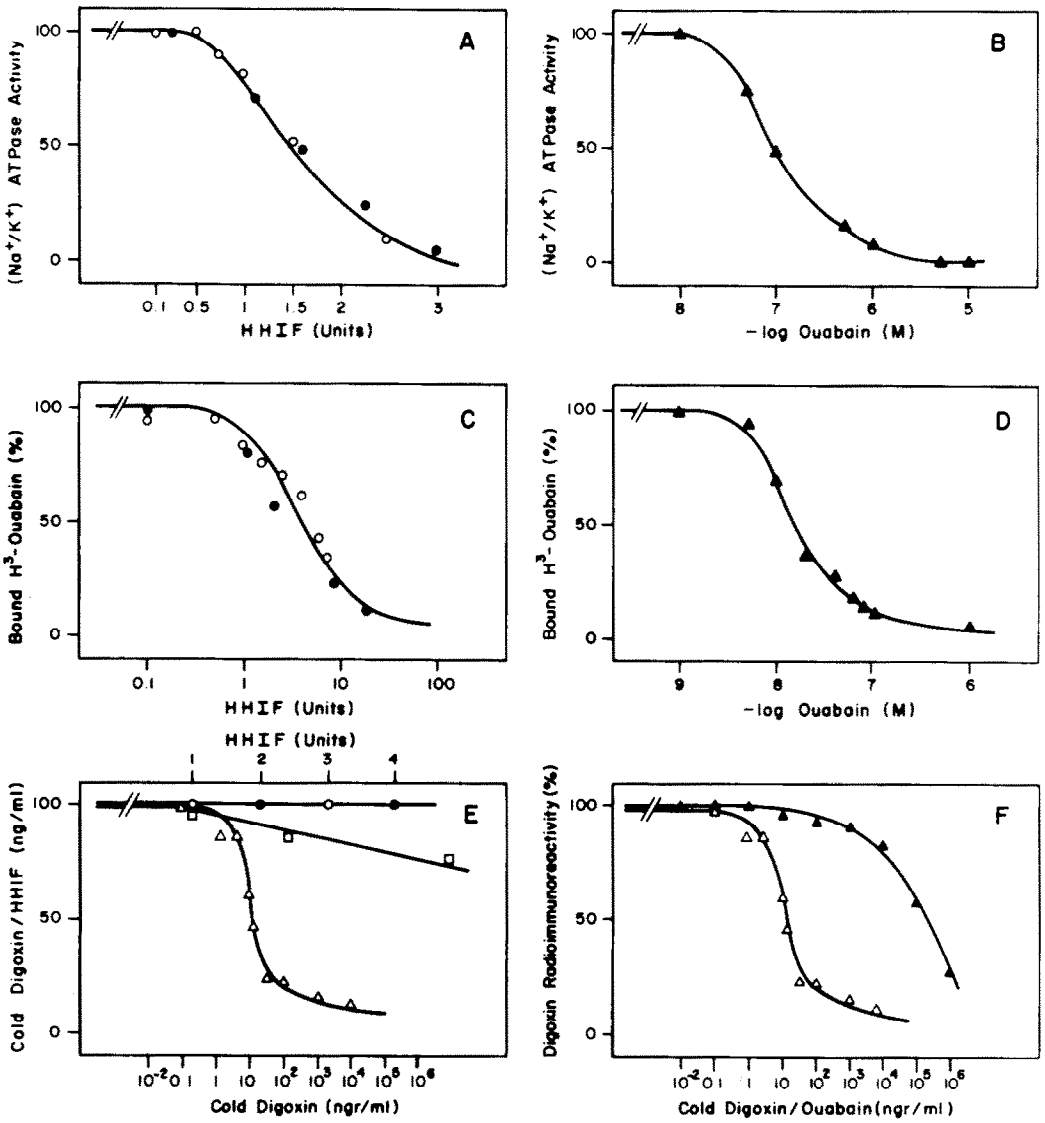

Fig.3. Inhibitory effect of purified hypothalamic (○) and hypophysary (●) factor on Na^+,K^+-ATPase activity (A), on binding of [^{3}H]ouabain to Na^+,K^+-ATPase (C) and cross-reactivity with antidigoxin antibody (E). Dose-response curves of these activities were determined in comparison with ouabain (▲) (B,D,F) and digoxin (△) (E,F) under the same assay conditions. Each point is the mean of duplicate determinations in two separate experiments and in A to D using two different preparations of Na^+,K^+-ATPase. Results are expressed as the percentage of maximal Na^+,K^+-ATPase activity, maximal binding of [^{3}H]ouabain to Na^+,K^+-ATPase, and maximal binding of [^{125}I]digoxin to anti-digoxin antibody. The cross-reactivity of partially purified hypothalamic factor (□) is also represented in E (see text).

hand, the activity was not affected by acid hydrolysis or by phospholipase C digestion (see section 2). The incubation with BSA did not affect the inhibitory activity suggesting that this factor is not lipidic [10]. The lipidic extraction residue (chloroform and petroleum ether residues) [4,5] obtained from 1 kg of hypothalamic tissue was processed through the purification protocol in the same fashion as the tissues, obtaining no inhibitory activity or characteristic peak in the final purification step, suggesting that we are not dealing with a lipidic substance not totally extracted by organic solvents. The inhibition of ATPase by unsaturated fatty acids and lysophosphatidylcholines [8,11] is produced by much higher concentrations than the one calculated for the HHIF. The purified factors obtained from hypothalamus and hypophysis were compared with ouabain for inhibition of Na^+,K^+-ATPase, [^{3}H]ouabain binding to the enzyme and [^{125}I]digoxin binding to anti-digoxin antibody (fig.3). For these studies the same definition of unit was used, that is, the amount necessary to inhibit 50% of Na^+,K^+-ATPase in the coupled assay (25 mM K^+ and 2 h incubation), but the concentration of K^+ used in the coupled assay was 5 mM. The biological activity of the factor purified from both tissues is superimposed in the dose-response curves. The curves of [^{3}H]ouabain displacement obtained with HHIF or ouabain were parallel (fig.3C,D). However, the slopes of the dose-response curves of ATPase inhibition were different for HHIF and ouabain (fig.3A,B). The purified HHIF did not cross-react with the anti-digoxin antibody at the doses tested, while ouabain cross-reacted weakly (fig.3E,F). Partially purified hypothalamic factor, obtained in the lipophilic chromatography did show cross-reactivity with this antibody. The chromatography under the same conditions as in fig.1, of several compounds known to inhibit Na^+,K^+-ATPase or related to them (see section 2) shows that none had a retention time nor spectra similar to the HHIF.

4. DISCUSSION

An endogenous Na^+,K^+-ATPase inhibitor is considered to be a key factor in the pathogenesis of essential hypertension and the midbrain has been postulated as the origin of such a factor [1]. Several groups have found inhibitory activity in crude extracts of brain, plasma and urine [2] but there is disagreement about the chemical structure and mechanism of action due, most probably, to the mixture of compounds in the preparations. We have previously reported the presence of such an inhibitor in extracts of bovine hypothalamic [3] and hypophysary [4] tissues. The present study reports the complete purification of the inhibitor in parallel from both tissues to apparent homogeneity, and the evidence that we are dealing with the same compound. Its absence in brain cortex and the higher concentrations in hypophysis suggest some form of storage in the latter. This compound has a characteristic UV spectrum and is a potent inhibitor of the Na^+-pump at physiological concentrations of K^+. These two characteristics make our compound completely different from the, up until now, only other tissue inhibitor already purified, using bovine adrenal gland as a source [12,13]. The complete purification of this factor is hazardous because the assay systems employed are prone to interference by a number of artifacts [14]. We have overcome these difficulties by the use of a multiassay strategy for the detection of inhibitory activity. In this way we have been able to eliminate false positive areas since each assay is prone to interference by differing substances. The decrease of cross-reactivity with anti-digoxin antibodies during the purification procedure indicates the risk of using such a parameter as a marker in the purification of analogous factors. This non-peptidic, non-lipidic inhibitor has a molecular mass of less than 1000 and it is destroyed by alkaline hydrolysis or ashing. Its chromatographic characteristics and spectral features are different from any of the substances tested. The obtention, by the method described, of sufficient amounts of pure material of this unique compound will permit the study of the chemical nature and the elucidation of its mechanism of action and, therefore, of the role that it may play in the physiopathology of hypertension.

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