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ENDOGENOUS "OUABAIN-LIKE" ACTIVITY IN BOVINE PLASMA

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An endogenous inhibitor of the sodium pump has been detected and concentrated 1000-fold from bovine plasma. The steps of purification included deproteinization and extraction with methanol, removal of lipids by coextractions with a lipopnilic solvent, desalting and further concentration by adsorption on C18-SepPack cartridges and HPLC fractionation on a weak anionic exchange column. The material isolated displaces ^{3}H-ouabain from brain synaptosomes, inhibits red cell membrane Na,K-ATPase without inhibiting Mg-ATPase or Ca,Mg-ATPase. Deproteinization of plasma by boiling may lead to appearance of non-specific inhibitors. The procedures developed should now permit isolation of sufficient amount of material for further purification and structural characterization.

Several studies have implicated circulating endogenous inhibitors of the Na/K pump in the pathogenesis of hypertension in man and experimental animals (1-6). This has lead to attempts to isolate ouabain-like compounds (OLC) from blood plasma and several other sources (for reviews see 5 and 6). Endogenous OLC's have been detected in plasma of normal or hypertensive humans (7-17), in volume expanded dogs (18,19), in acute volume expanded hogs (20) and in rats with corticoid-induced

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Abbreviations: EGTA, Ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'- tetraacetic acid; HPLC, High Performance Liquid Chromatography; NMR, Nuclear Magnetic Resonance; UV, Ultraviolet; P_i, Tris-[tris- (hydroxymethyl)aminomethane] phosphate; PCA, perchloric acid; TRIS, Tris-(hydroxymethyl)aminomethane.

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hypertension (21). At least two reports claim the presence of more than one fraction in human plasma which may be ouabain like (13,17).

The structure of the putative Na/K pump inhibitor is not known. The most serious problem in this respect is the small quantity of inhibitor present in plasma and other tissues. Another problem is that the specificity and mechanism of interaction of inhibitor isolated from plasma with Na,K-ATPase is not well defined. Hence one cannot be sure that isolated compounds are not unspecific inhibitors without biological function.

We wish to report here the partial isolation of an endogenous inhibitor of the Na/K pump from bovine plasma, which behaves like ouabain as judged (22) by its interaction with the Na,K-ATPase in brain synaptosomes, erythrocyte membranes and Na,K-ATPase purified from pig kidney red outer medulla.

Bovine plasma is a convenient, and as it turns out, surprisingly rich source of this material and should be useful for further attempts to purify ouabain-like material in sufficient quantities for both functional and structural characterization.

MATERIALS AND METHODS

1.Ouabain-like compound partial purification.

14 Liters of fresh bovine blood from a slaughter-house were collected in a container with 200 ml of a solution of EGTA 0.24 M and TRIS 1M, pH 7.4. The blood was centrifuged at 7,300 g for 20 min at +6°C. The supernatant was collected by suction (7.5 l) and lyophilized to give a redish cream solid which was extracted with methanol (9.5 l). The insoluble solids were separated by two filtrations over a large Buchner funnel with 30 mm Hg vacuum and a yellow clear solution was obtained and kept at -18°C. An aliquot of 1.5 l (equivalent to 1.2 l plasma) was concentrated by high vacuum distillation using two in series liquid nitrogen traps. The mostly aqueous solution remaining was lyophilized to dryness and later coextracted with methanol (50 ml) and hexane (100 ml). Three phases were obtained, hexane on top, methanol in the middle and a suspension on the bottom. After washing them, the suspension was centrifuged, the supernatant added to the

methanol fraction and the pellet discarded. By evaporation with a high vacuum pump the hexane gave 210 mg of compounds (mostly lipids, according to ^{1}H-NMR) and the methanol 8.6 g of a yellowish mixture, which was redissolved in 50 ml water. This aqueous solution was washed once more with 50 ml hexane. The hexane fraction contained 8 mg of compounds and the water solution was lyophilized to yield 3.6 g of brown powder. It was reconstituted in a smaller volume (8 ml) of water and by cooling and filtration we separated an additional lipid layer.

To further concentrate this fraction and in order to remove salts (especially K^+ which interferes with the [^{3}H]ouabain binding) we passed the aqueous solution three times through three C18-SepPack cartridges (Waters, Milford, MA, USA). The filtrate was discarded and the cartridges were washed each with 2 ml H_2O and twice with 4 ml 50% aqueous acetonitrile. This last solution was evaporated and redissolved in 1 ml H_2O (by atomic absorption, $[K^+]$ = 450 µM). Another SepPack filtration lowered the potassium concentration to negligible levels (13 µM) leaving upon evaporation 3.5 mg of solids.

2.HPLC fractionation

A LKB gradient system (LKB Produkter AB, S-161 26 Bromma, Sweden) including a model 2152 controller, a 2150 pump and a 2210 two-channel recorder, was used. The previous mixture (3.5 mg) was dissolved in 500 µl H_2O and injected in 50 µl fractions on an Alltech amino column (10 µm particle size, 25 x 0.46 cm) equilibrated with 90% aqueous acetonitrile and eluted with a linear gradient from 90% to 70% acetonitrile in water during 40 min and back to 90% in 10 min at a flow rate of 1 ml/min. Peaks were recorded according to their UV absorbance at 300 nm (Knauer variable wavelenght UV detector) and fractions were collected every 60 sec. (ca. 1 ml each). Equal number fractions from different injections were combined, each one was dried by evaporation with vacuum at 40°C and redissolved in 500 µl H_2O.

3.[^{3}H]ouabain binding

The preparation of synaptosomal membranes from male "Sabra" rats and the procedure for the binding assay performed at equilibrium, were as described (23) in a total reaction volume of 500 µl.

50 µl inhibitor were used for each assay and specific binding was calculated by substracting the binding observed in the presence of 100 µM unlabelled ouabain from that in the absence of unlabelled ouabain. The amount of OLC was estimated in terms of "ouabain equivalents" by comparing the [^{3}H]ouabain displacement with that of a known concentration of ouabain (24). The results of this assay are summarized in fig 1 (solid line).

4.Na,K-ATPase Inhibition

Na,K-ATPase was purified from pig kidney red outer medulla by the procedure of Jørgensen (25) and its specific activity was 13 µmoles ATP hydrolized per mg of protein per min; it did not contain ouabain insensitive ATPase.

Inhibition by the HPLC fractions from the plasma extract was tested using a modification of the highly sensitive assay which estimates the release of ^{32}P by hydrolysis of [γ-^{32}P]ATP (22,26) in an incubation buffer containing in the following components in final assay concentrations: histidine, 30 mM; $MgCl_2$, 3 mM; NaCl, 130 mM and KCl, 20 mM; pH 7.5. The total volume of incubation buffer, enzyme and inhibitor was 50 µl. Usually 20 µl of the HPLC fractions, redissolved in water were used for each assay.

Total radioactivity (chemically hydrolyzed ATP),control (tubes containing enzyme but no inhibitor) and blank (no inhibitor and no enzyme) were determined as described (22,26). Blank tubes had less than 4% radioactivity of total counts and in control tubes (considered 100% activity of enzyme) ^{32}P released by enzyme activity was less than 15% of total radioactivity.

5. ATPases Activities in Human Erythrocyte

Calmodulin-stripped membranes from human erythrocytes were prepared as previously described (26). Calmodulin was purchased from Calbiochem (La Jolla, CA, from bovine brain) and stored at -18°C in a solution of 1 mg/ml. Na,K-ATPase, Mg-ATPase and Ca,Mg-ATPase activity in erythrocyte were determined as described in refs. 22 and 26. Tney are expressed in mmoles of ATP per h of assay per 1 cell, and the tested samples are fractions of these totals (table 1).

RESULTS AND DISCUSSION

In all experiments in this report, extractions and manipulations of inhibitory materials have been performed at temperatures lower than 40°C as described in Methods. This precaution has been found important in order to isolate material with specific inhibitory effect (see Table 1). In recent reports in the literature the presence of several factors with ability to inhibit Na,K-ATPase, has been detected in extracts of boiled human plasma (9,12,13,16,17). In preliminary experiments with the bovine plasma we also deproteinized by boiling (at pH 5.5) prior to lyophilization and hot methanol extraction. After application to a reversed phase C_{18} HPLC column we could also

Table 1: Comparison of Effects of OLC and Ouabain on ATPase Activities of Red Cell Membranes

Fraction Number	Na,K-ATPase	Mg-ATPase	Ca,Mg-ATPase
	(Percent of control activities)		
Control	100%	100%	100%
24	0%	136%	104%
24 diluted 1:1	0%	131%	116%
30	26%	128%	86%

Fractions with a retention time of 24 min (neat and diluted 1:1) and 30 min were assayed for specificity of inhibition on different ATPases of human erythrocyte. All samples were performed in duplicates, as described in Materials and Methods. The absolute activities of the control membranes in mmoles ATP hydrolyzed/h assay/1 cells were respectively: Na,K-ATPase 0.27; Mg-ATPase 0.39 and Ca,Mg-ATPase 5.07.

detect several peaks of inhibitory activity (unpublished work) but these materials did not show the specificity properties expected of ouabain-like compounds (see Table 1 below). Other groups, using extraction procedures which avoid boiling the plasma report the detection of one inhibitory factor from bovine hypothalamus (27) or guinea pig hearts (28,29).

Figure 1 shows that in fractions collected from the amino column, we could detect a single large peak of material having ability to displace [^{3}H]ouabain from rat brain synaptosomes (fractions 17 to 27) and inhibit Na,K-ATPase activity of the purified pig kidney enzyme. This same HPLC separating system has been found previously to be effective for separating ouabain-like material extracted from toad skin (22). Calculation of the absolute amount of ouabain displacing activity showed tnat

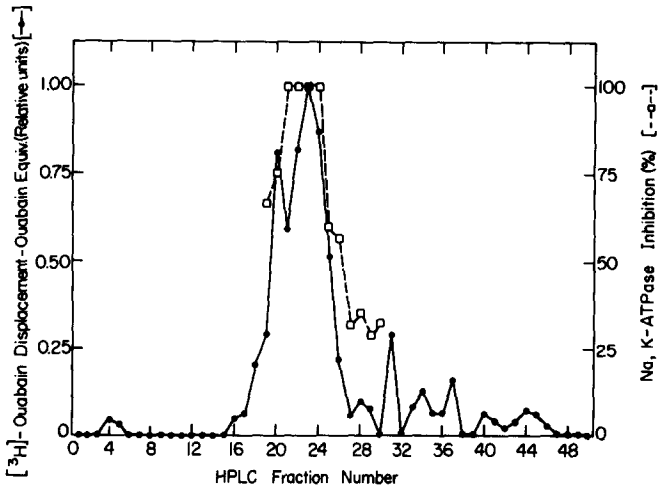

Figure 1: Fractionation of plasma extract by an amino HPLC column using a step-gradient elution program. A 50 µl aliquot of extract equivalent to 120 ml of original plasma was injected in water onto a weak anionic exchange HPLC column (Alltech NH_2) and then eluted with a linear gradient from 90% to 70% acetonitrile in water, during 40 min, and back to 90% in 10 min at a flow rate of 1 ml min^{-1}. This separation was repeated ten times and equal number fractions were pooled. [^{3}H]ouabain displacement activity (—●—) and Na,K-ATPase inhibitory activity (--□--) were performed as described in the Methods.

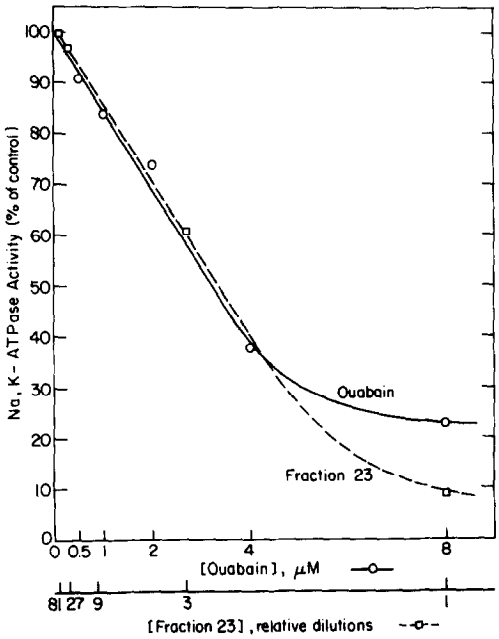

Figure 2: Effect of ouabain and serial dilutions of HPLC fraction 23 on Na,K-ATPase from pig kidney outer medulla. Fraction 23 (--□--), ouabain (—o—). HPLC fraction 23, redissolved in water, was diluted serially by a factor of 3, and 20 ul aliquots were used in the Na,K-ATPase assay.

approximately 10 nmoles of ouabain equivalent were extracted per liter of original plasma.

Fig. 2 shows curves for the degree of inhibition of Na,K-ATPase of pig enzyme using different concentrations of ouabain, or serial dilutions of fraction 23 from the HPLC separation. A dilution of 27 fold in fraction 23 reduced inhibition from 91% to 3% i.e. showing saturation of the inhibitory effect over a small range of concentrations. This shows that we are not dealing with a mixture of inhibitors having widely different affinities for the enzyme. Estimation of the dilution of inhibitor required to produce the same degree of inhibition as produced by ouabain, allowed rough calculation of the inhibitor concentration in ouabain equivalents. About 10 μM ouabain equivalent is present in fractions 21 or 23, and show that the extraction procedures developed concentrate the

inhibitor by a factor of about 1000 fold compared to the calculated plasma concentration of 10 nM.

A critical requirement for identification of true ouabain-like compounds is that inhibition is highly specific for Na,K-ATPase activity. Table 1 shows an experiment to look at effects of fractions 24 or 30 on Na,K-ATPase, Mg-ATPase or calmodulin activated Ca,Mg-ATPase activities of human red cell membranes. At concentrations of fraction 24 which inhibit completely Na,K-ATPase, a slight stimulation of Mg-ATPase was observed, while Ca,Mg-ATPase was quite unaffected. This therefore demonstrates a very high degree of specificity for the inhibitory material in this fraction. Fraction 30 (tail of the peak) appeared to be slightly less specific in that a concentration inhibiting Na,K-ATPase 74%, gives a small (14%) inhibition of Ca,Mg-ATPase.

In conclusion the preliminary work described in this report suggests:

(a) that an effective series of procedures has been developed to concentrate and partially purify ouabain-like compound(s) from bovine plasma.

(b) that deproteinization of plasma by boiling may lead to the appearence of non-specific Na,K-ATPase inhibitors, and

(c) the calculated quantities of inhibitor present in bovine plasma (~10 nM) suggest that it will be feasible to isolate and purify a ouabain-like compound from this source in amounts sufficient for determination both of inhibitory properties and of chemical structure. Such experiments are now in progress.

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