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A SEARCH FOR AN 'OUABAIN-LIKE' SUBSTANCE FROM THE ELECTRIC ORGAN OF *ELECTROPHORUS ELECTRICUS* WHICH LED TO ARACHIDONIC ACID AND RELATED FATTY ACIDS

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The electric organ of *Electrophorus electricus* contains substances which inhibit ($Na^+ + K^+$)-ATPase activity, the specific binding of [^{3}H]ouabain to purified ($Na^+ + K^+$)-ATPase and $^{86}Rb^+$ uptake by chick cardiac cells in culture. The active organic material was extracted from microsomal membranes. Its purification was carried out by chromatography on Sep-Pak C-18 and thin-layer chromatography. Reverse-phase liquid chromatography and mass spectrometry identified the active material as a mixture of unsaturated fatty acids. Linoleic (18:2), arachidonic (20:4), linolenic (18:3) and docosahexaenoic acids (22:6) contributed to about 60% of the total activity of the active material. The other active substances could be arachidonic analogs, since they have both a lipophilic and carboxylic character. Pure unsaturated fatty acids have been shown to be active in the different biological assays used to analyze the endogenous 'ouabain-like' activity. Linolenic, arachidonic and docosahexaenoic acids were the most active, whereas saturated fatty acids and glyceryl esters or methyl esters of unsaturated fatty acids were inactive. It is possible that in pathological situations in which the level of unsaturated fatty acids increases, these molecules may then act as physiological inhibitors of the sodium pump.

Introduction

Several recent reports have described the existence of endogenous factors having digitalis-like activity in the skin [1,2] and plasma [3] of the toad *Bufo marinus* as well as in mammalian plasma [4], brain [5–8], hypothalamus [9] and heart [10,11] tissues. However, none of these factors has yet been obtained in a pure form and their structure remains therefore unknown. The search for such a compound is rendered difficult by the fact that small amounts of K^+ or vanadate or of divalent cations might be, in certain cases the factors responsible for inhibition of [^{3}H]ouabain binding and/or ($Na^+ + K^+$)-ATPase activity.

This paper describes a search for an 'endogenous' ouabain-like molecule in the electric organ of *Electrophorus electricus*. Since the electric organ is composed of an homogeneous population of cells containing large amounts of ($Na^+ + K^+$)-ATPase and because this ($Na^+ + K^+$)-ATPase has a high affinity for ouabain (K_d = 30 nM) [12], this preparation was chosen as a likely source of an endogenous digitalis-like molecule, assuming such a molecule exists.

Materials and Methods

Chemicals. [^{3}H]Ouabain and $^{86}Rb^+$ were purchased from New England Nuclear Corporation.

Natural phospholipids were purchased from Serdary Research Laboratories. They included lysophosphatidylcholine, cerebrosides, ganglio-

sides and sphingomyelin from beef brain; lysophosphatidylethanolamine and phosphatidylinositol from pig liver; cardiolipin from beef heart; phosphatidylethanolamine, L-phosphatidylcholine and phosphatidylglycerol from egg yolk.

ATP, ouabain, fatty acids, biliary acids, steroids and bovine (Fraction V) albumin were purchased from Sigma; prostaglandins from Upjohn.

$(Na^+ + K^+)$-ATPase preparation. The $(Na^+ + K^+)$-ATPase from the electric organ of *Electrophorus electricus* (stored at $-70°C$) was purified according to Dixon and Hokin [13].

Determination of protein concentration. Protein concentration was measured according to Hartree [14] using bovine serum albumin as standard.

Ouabain-binding assay. Ouabain binding was carried out by the Millipore filtration method [12] with slight modifications; [^{3}H]ouabain was 3.5 Ci/mmol, the incubation medium was supplemented with 0.1 mM EGTA, and the incubation time was 90 min at 25°C. The [^{3}H]ouabain-binding capacity of the $(Na^+ + K^+)$-ATPase preparation was 2.28 ± 0.11 nmol of [^{3}H]ouabain per mg protein. Inhibition of [^{3}H]ouabain binding was measured by addition of increasing concentrations of unlabeled ouabain, endogenous fractions or fatty acids to the incubation medium. Fatty acids were solubilized by addition of up to 3% methanol. These concentrations of methanol had no effect on control and inhibition values.

Determination of $(Na^+ + K^+)$-ATPase activity. $(Na^+ + K^+)$-ATPase in the presence or absence of inhibitors was incubated for 90 min at 25°C in the medium described for the ouabain-binding assay. $(Na^+ + K^+)$-ATPase activity was then assayed by the sensitive pyruvate kinase/lactate dehydrogenase linked system, as described by Gache et al. [15]. The activity of the control $(Na^+ + K^+)$-ATPase activity was 10 ± 0.5 μmol P_i/min per mg protein (at 25°C). The level of the ouabain-insensitive ATPase activity was less than 1% of the total. Neither ouabain nor endogenous active fractions at the concentration used had any significant effect on the efficacy of the ATP regenerating system (pyruvate kinase + lactate dehydrogenase) as determined by inspection of rates of NADH oxidation in response to addition of 20 μM ADP. The methanol concentration used to solubilize fatty acids had no effect on control and inhibition values.

$^{86}Rb^+$ flux experiments. Rates of $^{86}Rb^+$ uptake by cardiac cells from 11-day-old chick embryos [16] were determined as follows. Cells were incubated for 1 h at 37°C in a K^+-free medium containing 140 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 25 mM Tris-Hepes at pH 7.4 ('Hepes' buffer) and the desired concentrations of inhibitors. Under these preincubation conditions and because of the K^+-free medium $(Na^+ + K^+)$-ATPase activity was inhibited, Na^+ efflux was prevented, and the intracellular concentration of Na^+ increased [17]. The long time of preincubation was found to be sufficient to reach equilibrium for the association of ouabain and endogenous fractions with ouabain activity in cardiac membranes. 2 μCi/ml of $^{86}Rb^+$ in 5 mM RbCl was added to start Rb^+ flux measurements. After 3 min of $^{86}Rb^+$ influx, cardiac cells were washed three times with 1 ml of the Hepes buffer and the radioactivity incorporated in the cells was counted. The ouabain-sensitive ^{86}Rb uptake corresponded to the difference between the uptakes in the presence and absence of 0.1 mM ouabain. The methanol concentration used to solubilize fatty acids had no effect on control and inhibition values.

Determination of Ca^{2+}-ATPase and alkaline phosphatase activities. Ca^{2+}-ATPase (100 μg/ml) purified according to Dupont [18], was incubated with endogenous fractions under similar conditions as those described above, except that EGTA was removed from the incubation medium. Ca^{2+}-ATPase activity was assayed by the sensitive pyruvate kinase, lactate dehydrogenase linked system, as described by Rossi et al. [19] in the presence of 0.1 mM $CaCl_2$. The Ca^{2+}-ATPase activity was 3.7 ± 0.1 μmol P_i/min per mg protein. Ouabain-sensitive ATPase activity in the Ca^{2+}-ATPase preparation was less than 1% of the total. The Ca^{2+}-insensitive ATPase activity, determined by addition of 0.5 mM EGTA in the incubation medium, represented 25% of the total activity.

Alkaline phosphatase (Grade I, Boehringer) (0.6 IU/ml) was incubated at 25°C for 30 min with a 0.1 M ethanolamine-HCl buffer (pH 10)/0.4 M NaCl and various concentrations of endogenous fractions active on the $(Na^+ + K^+)$-ATPase. The activity was measured at 25°C by the hydrolysis of 10 mM *p*-nitrophenylphosphate [20].

Radioimmunoassay. Endogenous fractions were incubated overnight at 4°C with [^{3}H]ouabain and rabbit specific anti-ouabain antibodies in a 0.1 M citrate buffer at pH 6.2. The antibodies were precipitated by addition of plasma and poly(ethylene glycol). After centrifugation and removal of the supernatant, the radioactivity contained in the pellet was counted.

Endogenous fractions were also assayed with the ^{125}I-labelled digoxin clinical radioimmunoassay kit (New England Nuclear).

Purification of an 'ouabain-like' fraction. 120 g of electric organ of *E. electricus* was homogenized in 0.3 M cold sucrose (500 ml) and centrifuged at $1000 \times g$ for 10 min. the supernatant was then centrifuged at $100\,000 \times g$ for 1 h and the pellet washed with 500 ml of sucrose. The pellet was resuspended in 1.2 l of water, frozen overnight at -70°C, thawed and centrifuged at $100\,000 \times g$ for 1 h. The supernatant was lyophilized and the dry matter suspended in methanol (60 ml) and centrifuged at $30\,000 \times g$ for 10 min. After removal of the pellet, the methanol extract was evaporated under low pressure and the dry residue taken up in 5 ml of water, centrifuged at $100\,000 \times g$ for 1 h and the supernatant retained. A similar type of extraction was previously described by De Pover et al. [11] for the purification of a potential digitalis-like substance from guinea-pig heart. The active supernatant was applied to a column of Sep-Pak C-18 (Waters) previously activated with methanol and water. The separation was carried out by eluting with 4-ml aliquots of solvents of increasing hydrophobicity in the following order: water, acetonitrile/water (1 : 9, v/v), acetonitrile/water (1 : 4, v/v), acetonitrile/water (1 : 1, v/v) and acetonitrile. The ouabain-like fraction was eluted by the 1 : 1 (v/v) acetonitrile/water mixture and this eluate was then lyophilized.

The lyophilized fraction was taken up in 50 μl of methanol and subjected to a two-dimensional thin-layer chromatography (TLC) on plates coated with silica gel 60 F-254 (Merck, Darmstadt, F.R.G.). Precoated plates (20 × 20 cm, 0.25 mm) were cleaned before use by elution in system I: $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v). After the first development in system I, a second migration was performed with system II: $CHCl_3/CH_3COOC_2H_5/CH_3COOH$ (75 : 25 : 5, v/v). Silica gel was scraped from each area of 1 cm^2, extracted with methanol and, after concentration by evaporation of methanol, tested for ouabain-like activity. An active fraction could be stored for a few days at -30°C.

The TLC step was carried out under nitrogen atmosphere and all solutions were bubbled with nitrogen before use.

Identification of active substances in the 'ouabain-like' fraction. The active fraction was submitted to HPLC (Waters Ass.) on a reverse phase column (Lichrosorb RP-18, 25 × 0.4 cm, Merck) using a solvent programmer (Model 660), a variable wavelength detector (Model 450) and two pumps (Model 6000A and M45). The detailed pattern of elution is described under Results.

Methylation of active HPLC peaks and of various fatty acids from commercial sources was carried out with diazomethane [21]. Native or methylated substances were analyzed on a Ribermag (Rueil-Malmaison, France) mass spectrometer (Quadrupole Model R-10-10B) using a direct probe method under ammoniac positive ionic desorption (CID). Mass analysis showed a main peak at $M + 18$ ($M + NH_4^+$).

Determination of inorganic ions. In the various active endogenous fractions K^+, Na^+, Ca^{2+}, Mg^{2+}, NH_4^+ was routinely analyzed by emission flame photometry, vanadium and Hg^{2+} by atomic absorption, and Zn^{2+}, Cd^{2+}, Pb^{2+} and Cu^{2+} by anodic stripping voltametry.

Results

Active endogenous substances have been extracted from a membrane fraction from the electric organ of *Electrophorus electricus* with the idea that we might extract a ligand that may be normally bound to the membrane ($Na^+ + K^+$)-ATPase of which is in large amounts in this preparation. The activity of the various fractions extracted from the membrane was routinely assayed following both the inhibitory effect on [^{3}H]ouabain binding and on ($Na^+ + K^+$)-ATPase activity. In order to avoid the possibility that the extracts were contaminated with divalent cations which are inhibitory by themselves, the two chelators EGTA and dithiothreitol were both present in all the assays. ($Na^+ + K^+$)-ATPase activity contained in the

membrane was first destroyed by freezing and thawing and exposure to a hypotonic shock. An inhibitory substance was obtained and then purified with 73% of the initial activity recovered after Sep-Pak extraction and 30% recovered after TLC extraction. The recovery of 'ouabain-like' activity at each stage was measured as the quantity of active substance which produced 50% inhibition (ED 50) of $(Na^+ + K^+)$-ATPase activity and [^{3}H]ouabain binding. The values of both these tests were not significantly different. TLC analysis of the Sep-Pak extract indicated the presence of one active spot with a R_F of 0.90 in system I and a R_F of 0.63 in system II. These R_F values correspond to the migration of long-chain free fatty acids (Fig. 1). The activity of the endogenous fraction disappeared after methylation by transesterification or also when the activity assay was carried out in the presence of serum albumin (0.1%). The active fraction was then submitted to a HPLC step on a reverse phase Lichrosorb RP-18 and eluted with an isocratic acetonitrile/water mixture. The chromatogram is shown in Fig. 2. Several active peaks migrated in the same way as unsaturated fatty acids.

Identification of each active peak was performed by mass spectral analysis. The following unsaturated fatty acids were identified in quantities expressed as pmol per g of fresh tissue (uncorrected recovery): palmitoleic acid (16 : 1) 900, linoleic acid (18 : 2) 389, oleic acid (18 : 1) 373, arachidonic acid (20 : 4) 208, linolenic acid (18 : 3) 144 and docosahexaenoic acid (22 : 6) 133. The total 'ouabain-like' activity due to these unsaturated fatty acids corresponded to about 60%

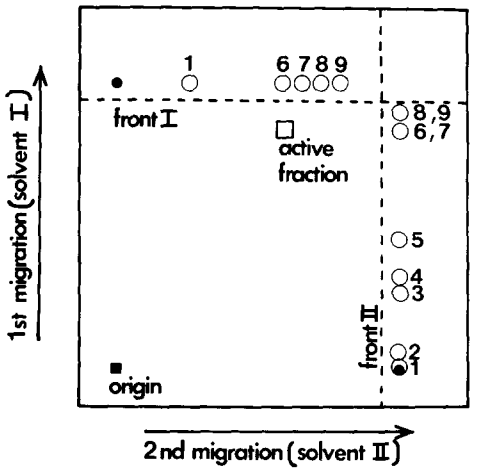

Fig. 1. Two-dimensional thin-layer chromatography (TLC). The active fraction from Sep-Pak chromatography was solubilized in 50 μl of methanol and applied (■) to a silica gel 60F-254 (0.25 mm, 20×20 cm). The plate was developed in the first dimension in solvent system I: $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) and then in a second dimension with solvent system II: $CHCl_3/CH_3COOC_2H_5/CH_3COOH$ (75 : 25 : 5, v/v). The whole chromatoplate was divided into equal areas (1 cm^2), the silica-gel was scraped off, extracted with methanol and tested. The active fraction migrated as indicated by □. Various known molecules were applied (●) to the silica gel systems and their migrations were compared to that of the active fraction. Their migration was visualized under ultraviolet light and iodine vapor. These molecules are: (1) monoolein, (2) lysophosphatidylcholine, (3) ouabain, (4) cardiolipin, (5) cholic acid, (6) long-chain free fatty acid, (7) diolein, (8) long-chain fatty methyl ester, (9) triolein, progesterone. The active fraction migrates like long-chain fatty acids.

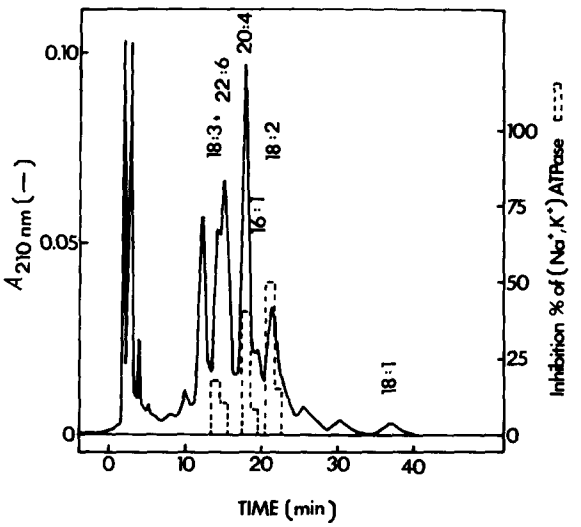

Fig. 2. HPLC chromatogram of the endogenous fraction active on $(Na^+ + K^+)$-ATPase activity. The active fraction from TLC (37 equiv. g of fresh tissue in 20 μl of methanol) was applied to a Lichrosorb RP-18 7 μm column (Merck). The elution was carried out with acetonitrile/water (79/21 v/v) at a flow rate of 1 ml/min. The absorbance at 210 nm AUFS 0.2 was monitored. Fractions of 1 ml were collected, dried under nitrogen and taken up in 20 μl methanol. Inhibition of $(Na^+ + K^+)$-ATPase activity was assayed with 5 μl of each fraction (117 equiv. g tissue per ml of incubate). The chromatogram demonstrates the presence of long-chain fatty acids. Shorthand notation is used for the fatty acids indicating chain length : number of double bonds. A calibration of the chromatographic system was also carried out with the different pure long-chain fatty acids (not shown).

of that of the total activity of the material loaded on the HPLC. The different unsaturated fatty acids contributed to the endogenous activity in the following order: linoleic acid (18:2) 7.5% of the initial activity, arachidonic acid (20:4) 6%, linolenic acid (18:3) 2.5%, docosahexaenoic acid (22:6) 1.5%, and palmitoleic acid (16:1) 0.5%. Peaks near the void volume (retention time 2–8 min) were inactive. Their retention times corresponded to those of oxidized unsaturated fatty acids.

The properties of the active TLC extract and of pure arachidonic and linoleic acids were studied comparatively (Fig. 3) by looking at their inhibitory activity on $(Na^+ + K^+)$-ATPase hydrolyzing activity, their activity in inhibiting [^{3}H]ouabain binding and ATPase catalyzed $^{86}Rb^+$ fluxes in chick cardiac cells in culture. Fig. 3 also shows that the half-maximum concentrations of arachidonic or linoleic acid required for a 50% inhibition (IC_{50}) of $(Na^+ + K^+)$-ATPase activity and [^{3}H]ouabain binding (*E. electricus* ATPase) is near 20–30 μM. These concentrations are of course much larger than those corresponding to the half-maximum effect of ouabain in the same conditions: 0.07–0.1 μM. The half-maximum inhibition of $^{86}Rb^+$ fluxes catalyzed by the $(Na^+ + K^+)$-ATPase in chick cardiac cells was observed at 60 μM for arachidonic acid and at 6 μM for ouabain. The active endogenous fraction did not inhibit other ATP hydrolyzing enzymes such as Ca^{2+}-ATPase and alkaline phosphatase, even when it was used at concentrations higher than those having an effect in the different assays presented in Fig. 3. In addition, this fraction was not recognized by specific antibodies against ouabain or digoxin (data not shown).

Long-chain fatty acids like myristic acid have long been known to inhibit the ATP hydrolyzing activity of the $(Na^+ + K^+)$-ATPase [22] and [^{3}H]ouabain binding was found to be inhibited by lipid fractions from liver [10]. A variety of lipidic substances were assayed for their inhibition of $(Na^+ + K^+)$-ATPase activity and [^{3}H]ouabain binding and the properties of their inhibition are summarized in Table I. The most active compounds were arachidonic, linolenic, docosahexaenoic and linoleic acids. Results, in Table I, suggest that there exists a correlation between the

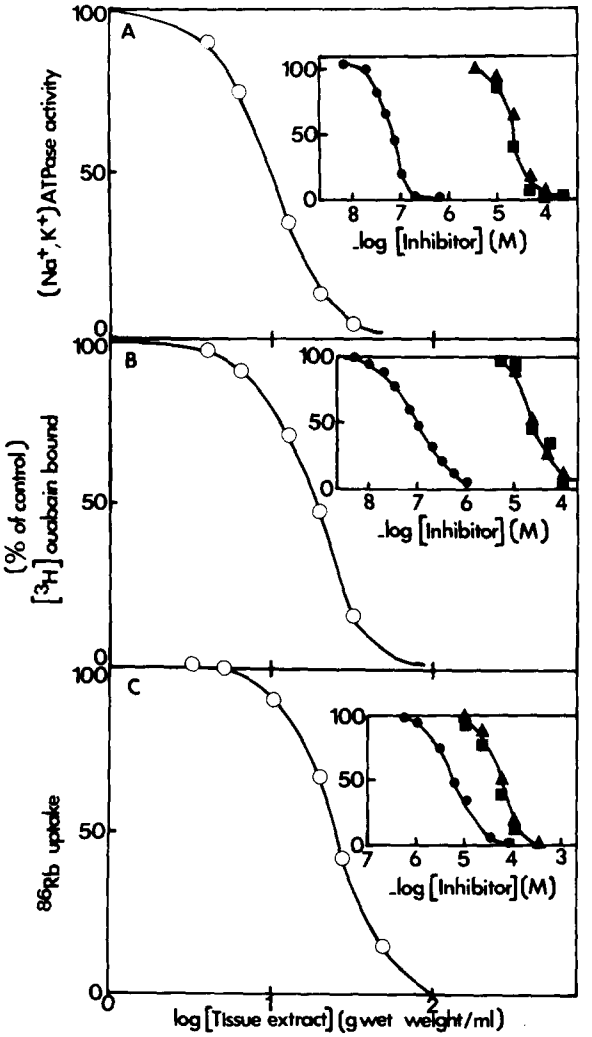

Fig. 3. Biological activity of the active endogenous fraction extracted from electroplax membranes. Dose-response curves of $(Na^+ + K^+)$-ATPase activity (A), [^{3}H]ouabain binding (B) and ouabain-sensitive ^{86}Rb uptake by cardiac cells in culture (C) were determined in relation to the concentration of the endogenous active fraction purified by thin-layer chromatography (Fig. 1). $(Na^+ + K^+)$-ATPase and [^{3}H]ouabain binding activities were completely inhibited by the active fraction. The maximal inhibition of total ^{86}Rb uptake by the active fraction was 65%. It was identical to the maximum inhibition found with ouabain itself. Insets: comparative inhibitions produced by unlabeled ouabain (●), arachidonic acid (■) and linolenic acid (▲).

degree of unsaturation of the free fatty acids and their inhibitory action, the most highly unsaturated fatty acids being the most active. Cholic and deoxycholic acid, phospholipids except

TABLE I

THE INHIBITORY POTENCIES OF UNSATURATED LONG-CHAIN FATTY ACIDS AND OTHER LIPIDIC SUBSTANCES ON $(Na^+ + K^+)$-ATPase ACTIVITY AND [^{3}H]OUABAIN BINDING

No inhibition was obtained with 200 μM of the following substances: docosahexaenoyl methyl ester, phosphatidylglycerol, lysophosphatidylethanolamine, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, phosphatidylinositol, dioleoyl phosphatidic acid, cerebroside, ganglioside, cholic acid, sodium deoxycholate, diolein, prostaglandin $F_{1\alpha}$, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ (6-keto prostaglandins).

		ED_{50} (μM)	
		$(Na^+ + K^+)$-ATPase activity	[^{3}H]ouabain binding
Arachidonic acid	(20:4)	20	30
Linolenic acid	(18:3)	20	30
Docosahexaenoic acid	(22:6)	20	30
Linoleic acid	(18:2)	30	40
Palmitoleic acid	(16:1)	60	80
Retinoic acid	(20:5)	80	120
Erucic acid	(22:1)	100	150
Stearic acid	(18:0)	> 200	> 200
Oleic acid	(18:1)	> 200	> 200
Elaidic acid	(18:1)	> 200	> 200
Petroselinic acid	(18:1)	> 200	> 200
Nervonic acid	(24:1)	> 200	> 200
Cardiolipin		10	20
Lysophosphatidylcholine		70	90

cardiolipin and lysophosphatidylcholine, fatty methyl ester, glyceryl ester and some of the prostaglandins were inactive in inhibiting $(Na^+ + K^+)$-ATPase activity or [^{3}H]ouabain binding.

Discussion

It is tempting to speculate that there exist endogenous ouabain-like molecules in excitable tissues which could regulate the activity of the pharmacological receptor of the drug. Several groups have claimed to have identified a substance of this kind. However, no one has yet obtained the molecule in the pure form and hence the structure of this hypothetical compound remains unknown.

The purification of such a substance is complicated, since both the enzymatic activity of the $(Na^+ + K^+)$-ATPase and [^{3}H]ouabain binding are sensitive to the presence of low concentrations of cations, the most classical inhibitor of [^{3}H]ouabain binding being K^+. The difficulties of this research are illustrated when one uses acid-acetone extraction, a technique frequently used to isolate endogenous substances from mammalian brain [5–9]. Our own experience has shown that this technique applied to the electric organ of *E. electricus* does provide an endogenous substance with properties similar to those of ouabain or its analogs. It inhibits $(Na^+ + K^+)$-ATPase activity, [^{3}H]ouabain binding and $^{86}Rb^+$ influx into cardiac cells. However the activity disappears when assays are carried out in the presence of chelators (dithiothreitol and EGTA) although the following ions are undetectable: K^+, Na^+, Ca^{2+}, Mg^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+} and vanadium. For this *Electrophorus* enzyme, small concentrations of cations like Hg^{2+} (ED_{50}, 0.3 μM) or Cu^{2+} (ED_{50}, 2 μM) or Zn^{2+} (ED_{50}, 10 μM), for example, inhibit both the ATP hydrolyzing activity and [^{3}H]ouabain binding.

This paper describes a purification, directly from *E. electricus* electroplax membranes, of endogenous molecules which are able to inhibit simultaneously $(Na^+ + K^+)$-ATPase activity, [^{3}H]ouabain binding to purified ATPase and ouabain-sensitive flux of ^{86}Rb in intact chick embryo cardiac cells.

The strategy of the procedure is based on the assumption that if there are endogenous ouabain-like molecules at least a proportion will be bound to their receptor on the membranes.

Long chain unsaturated fatty acids, i.e., arachidonic, docosahexaenoic, linolenic and linoleic acids, were shown to be present in the 'ouabain-like' fraction. These molecules represented at least 60% of the total purified endogenous activity. Some of the active components have not been chemically identified; they could be analogs of free fatty acids or metabolites of arachidonic acids as some prostaglandin-like compounds. Free arachidonic acid is probably not present in membranes in the relatively high concentrations required to see inhibition of $(Na^+ + K^+)$-ATPase activity, [^{3}H]ouabain binding or $^{86}Rb^+$ influx. Arachidonic acid is released from cellular membrane phospholipids through the action of Ca^{2+}-dependent phospholipase A_2 [23] after which it becomes precursor of prostaglandins and leukotrienes [24].

In order to investigate the contribution of free fatty acid to the observed endogenous inhibitory activity, a series of authentic fatty acids and other lipidic substances were examined for their inhibitory potencies on $(Na^+ + K^+)$-ATPase activity and [^{3}H]ouabain binding. Among the fatty acids tested, the more potent substances correspond reasonably to the active endogenous fatty acids found. The inhibitory potency of fatty acids has already been determined on membrane $(Na^+ + K^+)$-stimulated ATPase activity [22,25] and [^{3}H]ouabain binding [26]. These authors showed that inhibition is dependent on concentration, methyl esters are inactive, and unsaturated fatty acids are more inhibitory than their corresponding saturated congeners. Thus, among C_{18} unsaturated acids linolenic and linoleic acids were much more potent than oleic acid [26]. Similar conclusions can be drawn for our own study.

The membrane-bound Ca^{2+}-ATPase was not inhibited by the concentrations of the endogenous fraction which caused a complete loss of $(Na^+ + K^+)$-ATPase activity. However, in a recent work, Wetzker et al. [27] show that the Ca^{2+}-dependent enzyme was activated by a variety of saturated fatty acids but also by oleic acid or arachidonic acid. We have also found (data not shown) an activation of the Ca^{2+}-ATPase by a factor which can be as high as 2 with concentrations of the endogenous fraction which are 10-times higher than those which inhibit the $(Na^+ + K^+)$-ATPase.

Free arachidonic acid has been implicated in many pathological conditions including brain edema, brain ischemia hypoxia and convulsive seizures [28–31]. All these effects, as well as its inhibition of neurotransmitter uptake by brain slices and synaptosomes [32], could be due at least in part to $(Na^+ + K^+)$-ATPase inhibition. In experiments performed on cultured beating heart cells, the unsaturated fatty acid erucate (22 : 1) has been shown to increase the action potential duration and to decrease the beating frequency [33]. The heart $(Na^+ + K^+)$-ATPase was found to be inhibited when free fatty acid levels in plasma increased after heparin [34] or adrenalin treatment [35]. All these observations indicate that arachidonic acid and other unsaturated fatty acids might even be possible candidates as the non-proteic circulating inhibitor with an unknown structure which was recently found at raised levels in plasma of hypertensed patients [36].

One could even envisage at this stage, although it is very hypothetical, that there exists a cascade in which increased levels of internal Ca^{2+} activate a phospholipase which releases arachidonic and other polyunsaturated fatty acids which then serve to inhibit the $(Na^+ + K^+)$-ATPase. Under a number of pathological conditions in which phospholipases are activated at a high level, more fatty acids are released and extensive pump inhibitions are observed. It is still possible that the unsaturated fatty acids mimic the action of more potent effectors of the $(Na^+ + K^+)$-ATPase which have not been identified here.

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