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Identification of Na^+/K^+-ATPase inhibitors in bovine plasma as fatty acids and hydrocarbons

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A preparative purification of endogenous inhibitors of the Na^+/K^+-ATPase has been carried out from bovine blood. Dried plasma was deproteinized, hexane-extracted and desalted, followed by further purification through a series of reverse-phase HPLC fractionations. Fractions active in inhibiting Na^+/K^+-ATPase activity and displacing ouabain were collected and purified further. By comparison with ouabain, the final extract was found to have a steeper concentration-effect curve in the inhibition of Na^+/K^+-ATPase. In displacement of [^{3}H]ouabain, the extract had again a steeper concentration-effect curve than does ouabain, and in addition it enhanced ouabain binding at high dilutions. These properties are indicative of nonspecific interactions with the Na^+/K^+-ATPase. The active fraction was identified by TLC, HPLC, NMR, GLC and GC-MS, to be a mixture of three unesterified fatty acids, mainly oleic acid (72% of the total) and three saturated hydrocarbons. The assignment of structures was corroborated by comparison with authentic samples.

Introduction

A large body of experimental and clinical evidence (reviewed in Ref. 13), has lead to the proposal of the existence of an endogenous Na^+/K^+-ATPase inhibitor(s), implicated in the pathogenesis of essential hypertension and regulation of extracellular fluid volume. This might be similar in structure or effect, to the cardiac glycosides – leading to the term 'endogenous ouabain-like compound' (OLC). Many laboratories have attempted to isolate such a factor from a wide variety of tissues and species, but its structure remains unknown. In an earlier publication [3] we described a procedure to detect and concentrate 1000-fold a putative Na^+-pump inhibitor from bovine plasma. We report in this paper results of the purification of inhibitory compound(s) from bovine plasma, in sufficient quantity for structural and enzymological characterization. This search lead only to isolation of fatty acids and hydrocarbons, i.e. molecules which are unlikely to play the physiological role of an endogenous Na^+-pump inhibitor.

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Materials and Methods

General. Thin-layer chromatography was performed on Merck silica gel 60 F_{254} plates (5 × 7.5 cm, 0.2 mm thickness) and spots were visualized with iodine vapour.

Proton magnetic resonance spectra were obtained on a Varian FT-80A, 80 MHz instrument operating in the FT mode with the solvent deuterium used as internal lock. Spectral data are reported in δ (ppm) downfield from internal tetramethylsilane, for $CDCl_3$ solutions.

Gas-chromatography analyses were performed on a Spectra Physics SP7100GC with a flame-ionization detector on a 10% SE-30 on Chromosorb DMCS column at 220 °C. For mass spectral analyses of the methylated fatty acid samples we used a GC-MS Finnigan gas chromatograph-mass spectrometer model 4500 with a SBP5 capillary column (30 m) and an oven-heating program from 150 °C to 220 °C at 15 C°/min and then holding at the final temperature. The electron source of the spectrometer was set at 70 eV.

The separation of lipid classes was achieved with a Vac-Elut processing station and 3 ml-capacity NH_2 aminopropyl Bond-Elut columns (20 μm porosity, upper and lower stainless steel frits, from Analytichem International, CA, U.S.A.).

Chemicals. [21,22-^{3}H]Ouabain and [γ-^{32}P]ATP were purchased from Amersham International (U.K.), ATP (disodium salt, by phosphorylation of adenosine, vanadium-free), ouabain and other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.) or local sources as reagent grade. Solvents were of analytical purity or 'all-glass grease-free apparatus' redistilled. Eluents for HPLC were purchased from Bio-Lab Laboratories (Jerusalem, Israel) and were of HPLC grade filtered through 0.2 μm.

Preparative purification of inhibitors from bovine plasma. 250 liters of fresh bovine blood were collected in four batches from a slaughter-house, mixed immediately with an EDTA solution (pH 7.4, final concentration 8 mM) and centrifuged on the same day at $3500 \times g$ for 10 min (yielding 124 liters of plasma). The plasma was dried in a bulk lyophilizer in two batches of 62 liters each (using the facilities of Bio-Yeda, Rehovot, Israel) to give 13 kg of a tan light powder which was kept at $-18°C$ until use. All the remaining steps were preceded by a semi-industrial scale procedure in order to become acquainted with the problems arising from the increased scale of starting material.

12 kg of dried plasma were deproteinated by mixing for 4 h with 95 liters of redistilled methanol in an industrial mixer equipped with a mechanical propeller (Bio-Yeda). The suspension obtained was filtered through cheese-cloth and then Whatman's filter paper No. 1. The homogeneous solution thus obtained was evaporated to yield 1150 g of a dry paste. At this stage aliquots of the residue were treated with different solvents or combinations of them to extract lipids; hexane was found to be the most effective. Thus, the deproteinated paste was coextracted with 5 l of hexane and 5 l of water. The aqueous layer, rich in salts, was applied to a large flash-chromatography column with 250 g of LiChroprep RP-18 (40–63 μm from Merck, F.R.G.) and eluted with a step-gradient of increasing concentrations of acetonitrile in water at a constant pressure of $1.5 \cdot 10^5$ Pa.

The majority of the salts were eluted at the beginning and these fractions were discarded. The fractions eluting with acetonitrile/water (1 : 4, v/v) were evaporated and stored, at $-18°C$. The 40% acetonitrile/water fractions were collected and evaporated to dryness, yielding 5.78 g of material, which represent a 0.05% of the dried plasma. By flame photometry this powder was found to contain a high Na^+ content. Therefore the mixture was loaded onto a preparative HPLC reverse-phase column for further desalting. The conditions were as follows: column, PrepPAK-500/C_{18} cartridge with a radial pneumatic-drive pressure of $42.5 \cdot 10^4$ Pa; solvent flow, 150 ml/min; fractions collected, 50×250 ml each; eluent, wash with 5 l of water, 4 l of 20% acetonitrile/water, 4 l of 35% and 5 l of 50%. The first water wash lowered the content of salts by a factor of 300; subsequent fractions were collected. Results of an ouabain displacement assay of these samples depict a peak of activity, which coincides with the inhibitory activity found on the Na^+/K^+-ATPase inhibition assay. These active samples were combined, an aliquot equivalent to 5 l plasma was put aside for further studies (see Results and Discussion) and the rest was separated on HPLC on a semipreparative scale. The instrument was a Waters Liquid Chromatograph equipped with two model 6000A pumps, a model 660 solvent programmer and a model 440 absorbance detector set at 254 nm. The column used was LiChrosorb RP-18, 10 μm particles in a stainless-steel 9×250 mm column and the elution was done at a flow of 4 ml/min with a linear gradient from 30 to 60% methanol/water in 30 min, holding at 60% for 23 min and then increasing linearly from 60% to 100% methanol in 10 min.

Based on Na^+/K^+-ATPase inhibition one single large peak was detected and it was collected in three separated groups: I, fractions 32–49 (7.3 mg); II, fractions 50–60 (24.1 mg); III, fractions 61–72 (72.9 mg).

Aliquots of these fractions were further fractionated and studied, as detailed in Results.

Fractionation into lipid classes. This fractionation was done according to the procedure of Kaluzny et al. [5]. Basically, the method utilizes aminopropyl bonded phase columns under pressure, eluted with various solvents, to separate, isolate and purify individual polar and neutral lipid classes, rapidly and in high yields (> 96%) and purity (> 98%).

HPLC separation of lipids. Fraction II and fraction III were HPLC-separated by a common method used for free fatty acids on octadecyl bonded phase columns. The conditions of separation were: column, Radial-PAK C_{18}, 10 μm particle size, 8 mm $\times$ 10 cm; elution, isocratic, acetonitrile/water (79 : 21, v/v) at a flow rate of 1 ml/min, on a Waters 600 pump system; detection, absorbance at 210 nm, 0.080 AUFS measured with a Waters 490 programmable multi-wavelength detector and recorded on a Omniscribe recorder. Five main peaks were detected and collected.

Identification by GLC and GC-MS. Prior to analyzing the samples by gas chromatography they were methyl esterified by dissolving them in methanol and adding a catalytic amount of concentrated HCl. After two hours, the esters were extracted with hexane. By GLC six peaks were detected, which were then analyzed by GC-MS. The mass-spectra of these compounds revealed three fatty acids and three hydrocarbons: saturated fatty acids palmitic (5% of the material) and stearic (3%) and mono-unsaturated oleic acid (72%); the hydrocarbons being $C_{22}H_{46}$ (7%), $C_{23}H_{48}$ (8%) and $C_{24}H_{50}$ (5%).

Na^+/K^+-ATPase preparation. Na^+/K^+-ATPase was purified from pig kidney red outer medulla, according to the simpler of the two procedures of Jørgensen [1]

and stored at −80 °C until use. Its specific activity varied between 12 and 18 μmol of hydrolyzed ATP per min per mg protein, depending on the batch. Ouabain-insensitive ATPase activity was not detected.

Na^+/K^+-ATPase inhibition assay. Inhibition of the Na^+/K^+-ATPase activity was tested using a modification of the sensitive assay which estimates the release of $[^{32}P]P_i$ by hydrolysis of $[\gamma\text{-}^{32}P]ATP$ [2,3].

[^{3}H]Ouabain binding assay. This assay was modified during the course of the present work. Initially, ouabain binding was measured on microsomes from pig kidneys [1] but subsequently partially purified renal Na^+/K^+-ATPase was used.

A typical procedure measures the displacement of ouabain from its binding site at equilibrium, in 100 μl final volume. The assay mixture contains 40 μl of cocktail (50 mM Tris-HCl buffer (pH 7.4), 80 mM NaCl, 4 mM $MgCl_2$, 2 mM ATP (Tris, vanadium-free) and 11.2 nM [^{3}H]ouabain ($5 \cdot 10^4$ cpm/tube), 40 μl Na^+/K^+-ATPase suspension from one of the sources mentioned above in 25 mM histidine and 1 mM EDTA and 20 μl of inhibitor solution. The tubes were incubated at 37 °C for 60 min with 100 shakes per min, the reaction stopped with 3 ml ice-cold 50 mM Tris-HCl and tubes transferred to an ice bath. The suspensions were filtered through Whatman GF/B filters, washed twice with 3 ml of the same buffer and dried at 100 °C. The filters were counted in minivials with 3 ml scintillation fluid, after 24 h equilibrium extraction. The specific binding was calculated by subtracting the binding observed in the presence of 1 mM unlabelled ouabain (nonspecific) from the total binding. Nonspecific binding represented up to 5% of the total binding under control conditions. The total radioactivity counts were measured by adding 40 μl cocktail to a dry filter, drying it at 100 °C and counting as the samples.

Results and Discussion

Much experimental evidence has lead to suggestions that an endogenous ouabain-like compound(s) exists in plasma of healthy subjects, and is present in higher amounts in patients or animals with essential hypertension [6,7]. In an attempt to isolate this compound(s) we have sought to develop effective purification schemes to obtain a pure 'ouabain-like' substance from bovine plasma.

The initial steps in the purification procedure involved the deproteinization of the bovine plasma by methanol extractions of the dried plasma, followed by the removal of neutral lipids by hexane extractions and desalting by successive water washings on C_{18} reverse-phase flash- and high-performance liquid-chromatographies. The desalted residue was fractionated by elution from the HPLC column, with increasing concentrations of acetonitrile as described in the Methods. Inhibition

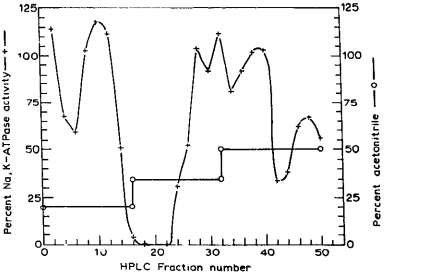

Fig. 1. Inhibition of Na^+/K^+-ATPase activity by HPLC fractions. The deprotecinized, delipidized and desalted dried bovine plasma was fractionated by a preparative Prep-PAK-500/C_{18} cartridge using a water-acetonitrile step gradient, as shown by the straight lines at a flow rate of 150 ml/min. 50 fractions of 250 ml were collected and monitored for inhibition of Na^+/K^+-ATPase activity (+).

of Na^+/K^+-ATPase activity by 1:21 diluted samples of these fractions is illustrated in Fig. 1. The diluted sample of fraction 42 displaces ouabain only partially and the diluted sample of fraction 6 actually enhanced binding (250% of control), while the fractions from the main peak both inhibit the ATPase activity and the binding of [^{3}H]ouabain. Therefore we concentrated our efforts on this band (C). An aliquot of these combined fractions (14–27), equivalent to 5 l plasma, was studied separately. It was concentrated, applied to a Sep-PAK C_{18} cartridge, washed with water and eluted with 40% and 50% acetonitrile/water (v/v). The 40% and 50% acetonitrile samples were applied separately to analytical reverse phase HPLC columns and the resulting fractions were assayed for 'ouabain-like' activity. While the fractions from the 40% eluant samples revealed a region of inhibitory activity in the Na^+/K^+-ATPase assay no ouabain-displacement activity was observed. The analytical samples from the 50% acetonitrile eluant revealed somewhat different behaviour; they inhibited the ATPase activity but actually enhanced the binding of [^{3}H]ouabain. The active fractions from this latter separation were combined and concentrated 50-fold. This concentrated sample now did displace [^{3}H]ouabain but a concentration curve revealed a relatively steep shape with respect to the ouabain titration curve (Fig. 2).

The latter result, as well as the enhancement of displacement by the diluted samples, suggested that the extract is interacting nonspecifically with the Na^+/K^+-ATPase. In the light of publications stating that 'ouabain-like' material in other sources are in fact mixtures of lipidic compounds [8–11], the original ac-

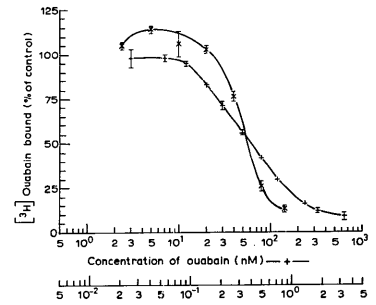

Fig. 2. [^{3}H]Ouabain displacement by ouabain and a 'ouabain-like' fraction. A dose-response curve of this activity was determined for a 50-fold concentrated active combined fractions from an analytical chromatography (×) in comparison to ouabain (+). The values shown are the means ± S.E. of duplicate determinations and are expressed as the percentage of [^{3}H]ouabain bound to the Na^+/K^+-ATPase.

tive fraction C was investigated for the presence of lipids. In order to concentrate and reduce the amount of material, a semipreparative HPLC separation was performed first, using octadecylsilane bonded phase columns and elution with a methanol/water gradient. One single peak inhibited the Na^+/K^+-ATPase activity and it was collected in three fractions: I, II and III. Dilution of 1 : 20 of the samples showed no inhibition in I, while II and III fully inhibited the enzyme's activity. Thin-layer chromatography with hexane/ether/acetic acid (70 : 30 : 1, v/v/v) revealed an identical R_f value (0.35) for these fractions and for a standard fatty acid (oleic). ^{1}H-NMR spectrum of the fractions was very similar to those of unsaturated fatty acids (not shown). An aliquot of fraction III was then fractionated into lipid classes according to the procedure of Kaluzny et al. [5]. The eluates were checked by thin-layer chromatography as before, and one major spot was revealed in the fatty acid fraction ($R_f = 0.37$) and minute traces in the phospholipid fraction ($R_f = 0.0$). By gravimetry, the free fatty acid accounted for nearly 100% of the material, and inhibited the enzyme. A proton NMR of this fatty acid fraction showed clearly an unsaturated free fatty acid profile (or mixture of them): δ 0.88 ppm (triplet, $-CH_3$), 1.25 (singlet, $-CH_2-$), 2.02 (multiplet, $-CH_2-C=O$), 2.34 (triplet, $J = 7$ Hz, $-CH_2-C=C$), 5.34 (triplet, $J = 3$–4 Hz, -CH=CH-) and 11.00 (broad singlet, -COOH). Fractions II and III were separately divided into individual compounds by a HPLC free-fatty acid method in isocratic conditions. Five main peaks were detected and collected from both fractions, each inhibiting the ATPase activity and having a similar concentration-effect curve. Only the most concentrated compound (fraction 5, about 70% of the total) could displace [^{3}H]ouabain from its binding site. This major component was compared at different dilutions with ouabain, both for the ATPase inhibition and the displacement of bound [^{3}H]ouabain. The curve for the Na^+/K^+-ATPase inhibition by ouabain has a hyperbolic shape, but HPLC fraction 5 showed a sigmoidal and steeper curve (Figs. 3 and 4). Between 80% to 20% inhibition of Na^+/K^+-ATPase it requires a 26-fold dilution of ouabain, but only a 5-fold dilution of fraction 5.

Another fact that does not fit the assumption that the isolated compound is 'ouabain-like' is that Na^+/K^+-ATPase inhibition requires 100-fold lower concentration than for [^{3}H]ouabain binding.

These results clearly indicate that the isolated compound is interacting nonspecifically with the Na^+/K^+-ATPase to induce a positive cooperative inhibition effect, rather than acting at a specific site on the enzyme and acting in a competitive fashion with the [^{3}H]ouabain.

A final identification of the isolated material was obtained by a GC-MS fragmentation study. First, all the samples were methyl esterified and analyzed on TLC and GLC to verify that all the compounds reacted quantitatively and that a satisfactory separation was achieved. Then they were injected on the GC-MS ap-

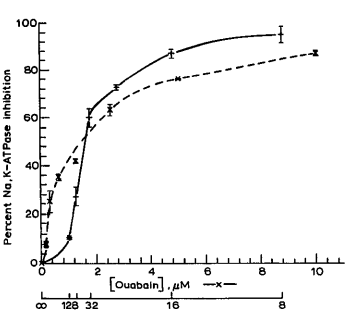

Fig. 3. Inhibition of Na^+/K^+-ATPase activity by ouabain and a 'ouabain-like' fraction. A dose-response curve of this activity determined for ouabain (×) and for fraction 5 (+) from HPLC-separation of lipids on a C_{18} reverse phase column eluted isocratically with acetonitrile/water (79 : 21, v/v) at a flow rate of 1 ml/min. The values expressed as percentage of inhibition of Na^+/K^+-ATPase are the means ± S.E. of duplicate determinations.

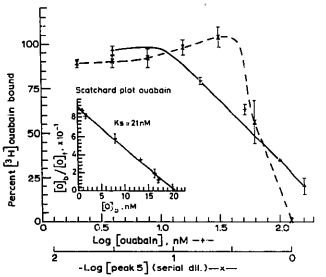

Fig. 4. [^{3}H]Ouabain displacement by ouabain and a purified inhibitor fraction. A dose-response curve of the [^{3}H]ouabain displacing activity for ouabain (+) and peak 5 (×) from HPLC separation of lipids on a octadecyl bonded phase column, with isocratic elution, acetonitrile/water (79:21, v/v) at 1 ml/min. The values presented are the means ± S.E. of duplicate determinations. Inset: a Scatchard plot for the titration of ouabain.

paratus and from the mass-spectra it was concluded that the compounds are: palmitic acid (5% of the total isolated fraction), stearic acid (3%), oleic acid (72%) and three saturated hydrocarbons C_{22}, C_{23} and C_{24} in 7%, 8% and 5%, respectively. The formula assignments were confirmed by comparison with spectra of original standards.

Possible explanations of the results of our search are: (1) there is no endogenous 'ouabain-like' compound, (2) there is a potent inhibitor of the Na^+/K^+-ATPase with 'ouabain-like' properties, but because of its minute concentration it is 'masked' by other inhibitors, such as fatty acids, or (3) the current approaches for searching and characterizing such a compound are missing the target. It is important to bear in mind that Na^+/K^+-ATPase is readily inhibited by a large number of compounds, differing greatly in chemical structure [15]. The spectrum covers steroidal substances such as progesterone derivatives [15], bile salts [16], bufotoxins [17], or cortisone [18], up to lignans [19], ascorbic acid [20], or dopamine [21], just to mention a few. Our search detected none of these compounds in the isolated active fractions.

In considering our results and those of previous studies reporting that unsaturated nonesterified fatty acids have Na^+/K^+-ATPase inhibitory activity as well as ouabain displacing activity [8,12] we should ask ourselves whether the nonesterified fatty acids could be the long sought physiologically relevant regulators of cellular sodium transport? The most likely answer is 'no', despite two reports of a 10-fold concentration increase in humans [11] and animals [9] with hypervolemia. Indeed inhibitory effects of oleic acid on Na^+/K^+-ATPase have been known for many years [14].

Thus, it is evident that thirty years of effort to isolate and identify such a compound, have not yet thrown light on the question whether this compound exists or not, and if it does, its chemical structure remains obscure.

Acknowledgments

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