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Isolation and Characterization of a Specific Endogenous Na^+,K^+-ATPase Inhibitor from Bovine Adrenal†

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Received September 16, 1987; Revised Manuscript Received February 8, 1988

ABSTRACT: In order to identify a specific endogenous Na^+,K^+-ATPase inhibitor which could possibly be related to salt-dependent hypertension, we looked for substances in the methanol extract of bovine whole adrenal which show all of the following properties: (i) inhibitory activity for Na^+,K^+-ATPase; (ii) competitive displacing activity against [^{3}H]ouabain binding to the enzyme; (iii) inhibitory activity for ^{86}Rb uptake into intact human erythrocytes; and (iv) cross-reactivity with sheep anti-digoxin-specific antibody. After stepwise fractionation of the methanol extract of bovine adrenal glands by chromatography on a C_{18} open column, a 0-15% acetonitrile fraction was fractionated by high-performance liquid chromatography on a Zorbax octadecylsilane column. One of the most active fractions in 0-15% acetonitrile was found to exhibit all of the four types of the activities. It was soluble in water and was distinct from various substances which have been known to inhibit Na^+,K^+-ATPase such as unsaturated free fatty acids, lysophosphatidylcholines, vanadate, dihydroxyeicosatrienoic acid, dehydroepiandrosterone sulfate, dopamine, lignan, ascorbic acid, etc. This substance was further purified by using an additional five steps of high-performance liquid chromatography with five different types of columns. Molecular mass was estimated as below 350 by fast atom bombardment mass spectroscopy and ultrafiltration. Heat treatment at 250 °C for 2 h and acid treatment with 6 N HCl at 115 °C for 21 h almost completely destroyed the inhibitory activity of the purified substance for Na^+ pump activity. Additionally, alkaline treatment with 0.2 N NaOH at 23 °C for 2 h destroyed approximately 70% of the inhibitory activity, whereas boiling for 10 min and various enzyme digestion did not destroy the activity. The dose dependency for the four types of the activities for this substance paralleled those of ouabain, spanning 2 orders of magnitude in concentration range. The inhibitory potencies of the purified substance for Na^+,K^+-ATPase, Na^+ pump, and ouabain binding activities were diminished with increasing K^+ concentration, exhibiting a characteristic typical of cardiac glycosides. This substance had no effect on the Ca^{2+}-ATPase activity or the Ca^{2+} loading rate into the vesicle prepared from skeletal muscle sarcoplasmic reticulum. These results strongly suggest that this water-soluble nonpeptidic Na^+,K^+-ATPase inhibitor may be a specific endogenous regulator for the ATPase.

The elevated levels of a humoral factor or factors that inhibit ouabain-sensitive Na^+,K^+-ATPase and Na^+ pump and specific binding of ouabain to the enzyme have been shown in both experimentally hypertensive animals (Buckalew & Nelson, 1974; Huot et al., 1983; Pamnani et al., 1983; Castaneda-Hernandez & Godfraind, 1984; Kojima, 1984; Tamura et al., 1985) and clinically hypertensive subjects (Hamlyn et al., 1982, 1985; Devynck et al., 1983; Grault et al., 1983; Crabos et al., 1984; Graves & Williams, 1984; Vassallo, 1985; Deray et al., 1986). In addition, a similar factor that cross-reacts with anti-digoxin antibodies has also been reported to increase in plasma from various patients with essential hypertension (Deray et al., 1986) chronic renal insufficiency (Kramer et al., 1985; Valdes, 1985) and hypertensive pregnant women (Graves & Williams, 1984; Valdes, 1985), as well as experimental animmals whose extracellular fluid volume was expanded by saline infusion (Gruber et al., 1980) or chronic excessive Na^+ loading (Castaneda-Hernandez & Godfraind, 1984; Kojima, 1984). These observations suggested that there is an endogenous hormone, presumably a specific Na^+,K^+-ATPase inhibitor, that modulates the Na^+ ion transport across

† This work was supported by National Institutes of Health Grants HL35323, HL14192, and HL07323.
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0006-2960/88/0427-4244$01.50/0

cell membranes, is involved in the regulation of extracellular fluid volume, and is hypothetically implicated in the regulation of vascular smooth muscle tone. Accordingly, we had attempted to isolate this humoral factor from volume-expanded hog plasma using a method generally used for the purification of steroids or their conjugates (Tamura et al., 1985, 1987a,b). While some lipidlike Na^+,K^+-ATPase inhibitors were isolated, a specific ouabain-like ATPase inhibitor was not identified. On the other hand, the existence of a humoral and transmissive hypertensive factor has been inferred from cross-circulation experiments (Zidek et al., 1986) and various other experiments (Iwai et al., 1969; Knudsen et al., 1969; Hirata et al., 1984). It has been demonstrated that this hypertensive factor may be produced or activated in the adrenal or kidney since either adrenalectomy or nephrectomy suppressed the appearance of the factor in plasma (Iwai et al., 1969; Knudsen et al., 1969; Zidek et al., 1986). In addition, high levels of immunocross-reactivity with anti-digoxin antibody have been reported in the adrenal (Castaneda-Hernandez & Godfraind, 1984). Since humoral Na^+,K^+-ATPase inhibitor has been postulated in the etiology of Na^+ (or salt)-dependent hypertension and since the adrenal is known to be related to volume-dependent hypertension (Buckalew & Gruber, 1984; deWardener & Clarkson, 1985), it is of interest to determine whether the adrenal contains a specific endogenous Na^+,K^+-ATPase inhibitor. In this paper, we report the isolation and characterization of a specific endogenous Na^+,K^+-ATPase inhibitor which is functionally similar to ouabain.

Materials and Methods

Chemicals. [12α-^{3}H]Digoxin (19.0 Ci/mmol), [G-^{3}H]-ouabain (20.0 Ci/mmol), $^{45}CaCl_2$ (21.92 mCi/mg), and ^{86}-RbCl (2.72–4.82 mCi/mg) were purchased from New England Nuclear. Antisera against digoxin conjugated to bovine serum albumin raised in sheep were the generous gift of Dr. Edgar Haber of Massachusetts General Hospital. Human red blood cells, type Rh(−) O, were supplied by the blood bank of Vanderbilt Hospital. Adenosine 5′-triphosphate (vanadium free), digoxin, ouabain, protease (type XIV), trypsin (type III), deoxyribonuclease I (type II), ribonuclease A (type III-A), and phospholipase C (type XIV) were obtained from Sigma Chemical Co. Deuterium oxide (99.96 atom % D) was purchased from Aldrich Chemical Co. Organic solvents were of high-performance liquid chromatography (HPLC)[1] grade. Other chemicals were of analytical grade.

Na^+,K^+-ATPase Activity. Membrane-bound Na^+,K^+-ATPase was prepared from canine kidney outer medulla by a modification of Jorgensen's rapid method (Jorgensen, 1974a). Unless otherwise stated, the capacity to inhibit Na^+,K^+-ATPase activity was estimated at 37 °C by the rate of P_i release from 3.1 mM Na_2ATP at pH 7.4 in a 1.3-mL reaction mixture containing 77 mM NaCl, 17 mM $NaHCO_3$, 0.5 mM KCl, 2.7 mM $MgCl_2$, 0.77 mM Tris–EGTA, 23 mM imidazole, 11.5 mM Hepes, 77 μg of fat-free bovine serum albumin (BSA), and 6.8 μg of the purified enzyme protein. After incubation for 30 min, the reaction was terminated by addition of 0.5 mL of 5% SDS. The P_i released was measured according to the method of Hegyvary et al. (1979). The ouabain-sensitive Na^+,K^+-ATPase activity was calculated as the difference between P_i released in the absence and presence of 0.24 mM ouabain.

In view of the previous finding (Tamura et al., 1985, 1987a,b) that free fatty acids and lysophosphatidylcholines are less specific inhibitors and that their effect on the ATPase can be eliminated by the addition of BSA, fat-free BSA was used for this assay. Although fat-free BSA tended to increase the ATPase activity slightly, this effect was also advantageous for increasing the sensitivity of the assay since lesser amounts of the enzyme protein can be employed for the assay. Additionally, fat-free BSA did not affect the inhibitory effect of ouabain.

Ouabain Displacement Assay. The ouabain displacing activity was determined by measuring the binding of [^{3}H]-ouabain to Na^+,K^+-ATPase in competition with an inhibitor as described previously with partially purified rat brain Na^+,K^+-ATPase (Tamura et al., 1985). A 0.5-mL reaction mixture contained 50 mM Tris-HCl buffer, pH 7.4, 0.5 mM EDTA, 80 mM NaCl, 4 mM $MgSO_4$, 2 mM Na_2ATP, 62.5 nCi of [^{3}H]ouabain, 80 μg of Na^+,K^+-ATPase preparation, and various concentrations of samples or ouabain standards (2 nM–2 μM). After incubation at 37 °C for 60 min, the reaction was terminated by adding 3 mL of ice-cold 50 mM Tris-HCl buffer, pH 7.4. Bound [^{3}H]ouabain was trapped by filtering on GF/B glass fiber filters (Whatman Inc., Clifton, NJ), and the radioactivity on the filter as quantified by liquid scintillation spectrometry.

Na^+ Pump Activity. Inhibition of Na^+,K^+ pump activity in intact human erythrocytes was determined by measuring ^{86}Rb uptake activity according to the method of Diamandis et al. (1985). Red blood cells were washed 3 times with potassium-free Ringer's solution containing 162.1 mM Na^+, 1.34 mM Ca^{2+}, 1.26 mM Mg^{2+}, 127.4 mM Cl^-, and 4.5 mM glucose by centrifugation for 5 min at 2000g. For assay, 0.2 mL of packed red cells was mixed with 0.4 mL of potassium-free Ringer's solution and 0.1 mL of samples or ouabain standards (10 nM–0.1 μM). After preincubation for 2 h at 37 °C, 20 μL of 1.5 μCi of ^{86}RbCl in water was added to each tube, and the reaction mixture was incubated for an additional 1 h in a water bath with periodic shaking. The reaction was terminated by the addition of 4 mL of ice-cold potassium-free Ringer's solution, and the red cells were washed 3 times with the Ringer's solution by centrifugation for 5 min at 2000g. Radioactivity taken up into the erythrocytes was counted in a γ counter (Micromedic System 2/200, Atlanta, GA).

One arbitrary unit was defined operationally as the activity that shows a 50% inhibition of the *total* ^{86}Rb uptake by intact human erythrocytes when the purified substance was added to the present assay system. The standard variation for this specification averaged 3.5% ± 0.8% (n = 6).

Radioimmunoassay. The cross-reactivity with anti-digoxin antibody was measured by radioimmunoassay according to the method of Gruber et al. (1980) using sheep anti-digoxin antibodies. Free digoxin was separated from antibody-bound digoxin by adsorption to dextran-coated charcoal. The sensitivity of this radioimmunoassay system was 130 pg/tube.

Ca^{2+} Pump and ATPase Activities. The Ca^{2+} pump (Ca^{2+},Mg^{2+}-dependent ATPase) of longitudinal sarcoplasmic reticulum, prepared from rabbit fast twitch skeletal muscle (Saito et al., 1984), was generously supplied by Dr. Sidney Fleischer of Vanderbilt University. The Ca^{2+} pumping rate and Ca^{2+},Mg^{2+}-dependent ATPase activities were determined according to the method of Chamberlain et al. (1983). In the

[1] Abbreviations: HPLC, high-performance liquid chromatography; ODS, octadecylsilane; C-8, octylsilane; NH_2 column, aminopropyl column; CN column, cyanopropyl column; FAB, fast atom bombardment; ^{1}H NMR, proton nuclear magnetic resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; GC–MS, gas chromatography–mass spectroscopy.

assay for Ca^{2+} pump activity, the Ca^{2+} loading rate was determined at 23 °C by measuring ^{45}Ca uptake into the vesicles. A 0.1-mL reaction mixture contained the following constituents 100 mM KCl, 4 mM $MgCl_2$, 60 μM $CaCl_2$, 20 nCi of $^{45}CaCl_2$, 30 μM EGTA, 3.5 mM Na_2ATP, 5 mM potassium oxalate, 100 mM sucrose, and 20 mM imidazole hydrochloride, pH 6.9. Aliquots of the purified inhibitor were preincubated with 2.5 μg of protein of vesicles in the reaction mixture for 10 min. The Ca^{2+} uptake reaction was initiated by the addition of ATP. After incubation for 30 s, the reaction was stopped by adding 1.0 mL of ice-cold Ca^{2+}-free reaction mixture containing 1 mM EGTA. ^{45}Ca taken up by the vesicles was separated from free ^{45}Ca by filtration through a 0.2-μm pore size disk filter (Gelman Sciences, Inc., Ann Arbor, MI). Radioactivity in aliquots of the total reaction mixture and filtrate was counted in a liquid scintillation counter. Ca^{2+} loading rate was calculated from the difference between radioactivity in the total mixture and the filtrate.

Ca^{2+}-ATPase activity was estimated at 23 °C by the difference between the two rates of P_i formation from ATP in the presence of Ca^{2+} (60 μM) and in the absence of Ca^{2+} with 1 mM EGTA. These rates were determined under the conditions employed for the measurement of Ca^{2+} uptake as mentioned above. The reaction mixture was incubated for 45 s at 23 °C, and P_i released was determined by the method of Baginsky et al. (1967) and Ottolenghi et al. (1975). Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Extraction of Na^+,K^+-ATPase Inhibitor. Bovine adrenals (1.5 kg) obtained from a local slaughterhouse were trimmed of fat and connective tissue 1 h after collection and kept at −60 °C until use. Approximately 400 g of the tissue was homogenized in 10 volumes (v/w) of ice-cold methanol using a Waring blender at low speed for 30 s. The homogenate was filtered through Whatman 4 filter paper. The residue on the filter paper was rehomogenized and filtered again under the same condition as the first extraction. The methanol in the combined filtrate was evaporated by a rotary evaporator, and the resultant water phase was lyophilized.

Reverse-Phase Flash Chromatography. Two hundred grams of octadecylsilane (ODS) bonded to silica gel (40-μm average particle diameter) was packed in a glass column (5.0 × 60 cm) and washed with 1 L of 100% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and then with distilled water containing 0.1% TFA. In all subsequent column chromatography, eluting and washing solvents including distilled water were acidified with 0.1% TFA. The extract obtained from approximately 150 g of bovine adrenal was suspended in 1 L of acidified distilled water, and the suspension was filtered through Whatman 4 filter paper. The yellowish turbid suspension was applied to the column preequilibrated with acidified distilled water. After the column was washed with 1 L of acidified distilled water, the inhibitory activities were fractionated by stepwise elution first with 1 L of 15% acetonitrile and then with 85% acetonitrile at room temperature. The flow rate of approximately 0.8 L/h was controlled by nitrogen pressure. Acetonitrile in the eluates was evaporated by the rotary evaporator, and the remaining aqueous solutions were individually lyophilized. The column was regenerated by sequential washes with 100% acetonitrile, 2-propanol, hexane, 2-propanol, acetonitrile, and distilled water, and the chromatography was repeated 4 times with fresh extract. The dry materials obtained from the 15% acetonitrile fraction were dissolved in 10 mL of water. The dry materials obtained from the 85% acetonitrile fractions were dissolved in 10 mL of methanol for the subsequent purification by HPLC.

High-Performance Liquid Chromatography. Reversed-phase HPLC on a Zorbax ODS column (0.46 × 25 cm; Du Pont, Wilmington, DE), on a SynChropak ODS column (1.0 × 25 cm), and on an octylsilane (C-8) column (0.41 × 25 cm; SynChrom, Linden, IN) was run with linear gradients of acetontrile in 0.1% TFA at a flow rate of 1 mL/min for analytical columns and at 3 mL/min for the semipreparative column. HPLC on an aminopropyl (NH_2) column (0.46 × 25 cm; Alltech, Deerfield, IL) and on a cyanopropyl (CN) column (0.46 × 25 cm; Du Pont) was carried out with a decreasing linear concentration gradient of acetonitrile at a flow rate of 1 mL/min. The absorbance of the eluate was monitored at 214 nm.

Structure Analysis. Fast atom bombardment (FAB) mass spectra of the purified substances were obtained on a VG70/250 GC–MS instrument equipped with a high-field magnet and a VG 11/250 data system. The fast atom bombardment gun was of the saddle-field type (Ion-Tech) and was operated at 8 kV with xenon gas as the source of fast atoms. The mass spectrometer was adjusted at an accelerating voltage of 6 kV. Scans were obtained at 10 s/decade by switching on alternative scans between the positive ion and negative ion modes. The purified samples (approximately 250 units of AP10Cα and 750 units of AP10Cβ) in distilled water (1–1.5 μL) were introduced on a stainless-steel target with a thin film of neat glycerol as a matrix.

^1H NMR spectra were recorded on Bruker AM-400 and IBM NR-300 spectrometers operated at 400 and 300 MHz, respectively. Entire purified samples from 1.5 kg bovine adrenals which correspond to 431 units of AP10Cα and 1702 units of AP10Cβ were dissolved individually in 0.5 ml deuterium oxide and were separately transfered into an NMR tube (0.5 × 17.78 cm) and then subjected to the NMR studies.

UV spectrum was recorded on Hewlett Packard HP 8451A diode array spectrophotometer using water as a solvent.

Enzymatic Digestions. The purified inhibitor AP10Cβ (1 unit) exhibiting 50% inhibition of the *total* ^{86}Rb uptake activity was incubated with 0.5 mg of hydrolytic enzymes except for phospholipase C (0.55 μg) at 37 °C for 2 h in 0.5 ml of potassium-free Ringer's solution (pH 7.4). The reactions for the proteolytic enzymes and nucleases were terminated by heating in boiling water for 10 min. The reaction for the phospholipase C was terminated by adding EDTA (final 10 mM) and then Ca^{2+} and Mg^{2+} concentrations in the reaction mixtures were adjusted to those of potassium-free Ringer's solution by the addition of $CaCl_2$. After centrifugation for 5 min at 13,000 × g, 0.2 ml aliquots were assayed for inhibitory activity on ^{86}Rb uptake into red blood cells. As a control, the heat-denatured enzymes and EDTA-inactivated phospholipase C were incubated with inhibitor and were assayed identically.

Physical–Chemical Treatment. For the acid treatment, the conditions for amino acid analysis of common peptides were employed. The purified inhibitor AP10Cβ (1 unit) was incubated with 6 N HCl at 115 °C for 21 h under vacuum. After the remaining HCl was removed by lyophilization, the sample was assayed for its inhibitory activity on ^{86}Rb uptake into red blood cells. Alkaline treatment was performed in 0.02 and 0.2 N NaOH at 23 °C for 2 h. After neutralization with HCl, the sample was lyophilized and assayed for its inhibitory activity as described above. As a control, neutralized NaOH solutions were lyophilized identically and used for the preparation of the standard curves only for the alkaline treatment. Charring was carried out at 250 °C for 2 h.

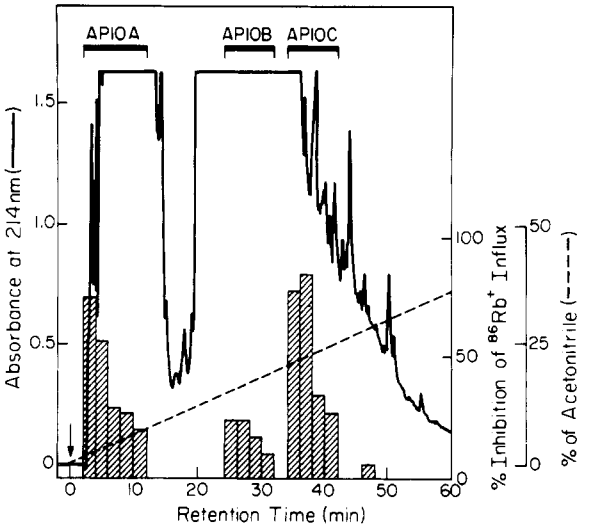

FIGURE 1: Elution profile of inhibitory activities of ^{86}Rb uptake by erythrocytes of HPLC on a Zorbax ODS column. An aliquot of the eluate (0–15% acetonitrile fraction) from reverse-phase C_{18} open column chromatography, which was derived from 22 g (wet tissue) of bovine adrenal, was fractionated by HPLC on a Zorbax ODS column with a linear gradient of acetonitrile (0–36%) containing 0.1% trifluoroacetic acid over 60 min at a flow rate of 1 mL/min. The arrow indicates the time of sample injection. Fractions were collected every 2 min and lyophilized, and then the effect on ^{86}Rb uptake activities of each fraction was assayed.

RESULTS

Purification of Na^+,K^+-ATPase Inhibitor. A crude extract was prepared from 1.5 kg of bovine adrenals as described under Materials and Methods. A reverse-phase ODS open column was used for flash chromatography as the second step of purification for preliminary fractionation of the activities into water-soluble (0–15% acetonitrile) and insoluble (15–85% acetonitrile) fractions. The water-soluble activity in the 0–15% acetontrile fraction obtained from the flash chromatography was separated into three distinct activity peaks by HPLC on a Zorbax ODS column, and each activity peak was designated AP10A, AP10B, and AP10C according to the eluting order (Figure 1). The third peak with highest Na^+ pump inhibitory activity which eluted at a retention time of 34–42 min on an ODS HPLC was further purified by an Alltech NH_2 column (Figure 2A). Although two minor activities were separated from the major activity peak in this second step of HPLC, these two activities accounted for only less than 5% of the total activity applied to the column. Strong gold fluorescent material was also separated by this normal-phase HPLC. The major activity peak, which eluted from the NH_2 column at a retention time of 22–32 min, was subsequently purified by HPLC on a SynChropak ODS semipreparative column (Figure 2B) and on a Zorbax CN column (Figure 2C). As shown in Figure 2C, the activity from the ODS semipreparative column was resolved into two activity peaks, which were designated AP10Cα (retention time, 2–6 min on CN column) and AP10Cβ (retention time of 16–26 min). Most of the small UV absorbance peaks under these activity peaks on CN HPLC were found to be the solvent peaks and the background peaks. The materials under two activity peaks were further chromatographed individually by an additional two steps of reverse-phase HPLC on a C-8 column and on a Zorbax ODS column (Figure 3). Figure 3 represents the elution profile only of AP10Cβ. The active materials in AP10Cα and AP10Cβ were eluted as a single peak. Approximately 431 units of AP10Cα and 1702 units of AP10Cβ were obtained. The ratio of Na^+ pump inhibitory activities due to AP10Cα and AP10Cβ was approximately 1:4, and this ratio corresponded to that of the peak heights as well as the peak areas. The quantities of the AP10Cα and AP10Cβ recovered from the present experiment were not measurable by a microbalance (type M5; Mettler Instrument Corp., Hightstown, NJ; reproducible lower limit, 10 μg; readable lower limit, 1 μg)

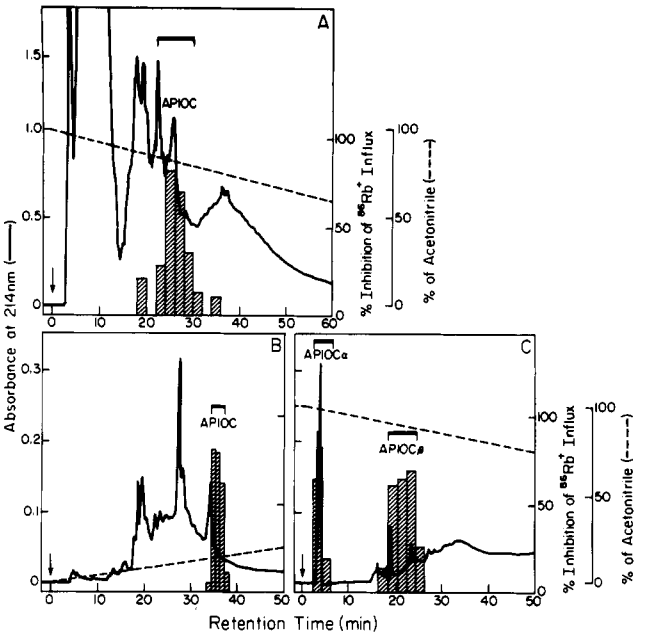

FIGURE 2: Elution profiles of inhibitory activities of ^{86}Rb uptake by erythrocytes on HPLC in purification steps. The fraction AP10C (retention time of 34–42 min), indicated by the brackets in Figure 1, was purified by HPLC on an NH_2 column with a decreasing linear gradient of acetonitrile (100–60%) in 0.1% TFA over 60 min at a flow rate of 1 mL/min (A). The fraction indicated by the bracket, corresponding to a retention time of 22–30 min, was further purified by HPLC on an ODS semipreparative column with a linear gradient of acetonitrile (0–20%) in 0.1% TFA over 50 min at a flow rate of 3 mL/min (B). The fraction (retention time of 34–37 min), indicated by the bracket in Figure 2B, was purified by normal-phase HPLC on a CN column with a decreasing linear gradient of acetonitrile (100–75%) in 0.1% TFA over 50 min (C) at a flow rate of 1 mL/min. The arrows indicate the time of sample injection. Two-minute fractions for the normal-phase HPLC and 1-min fractions for the reverse-phase HPLC were collected, and the activities were monitored according to the method described in the legend for Figure 1 and under Materials and Methods.

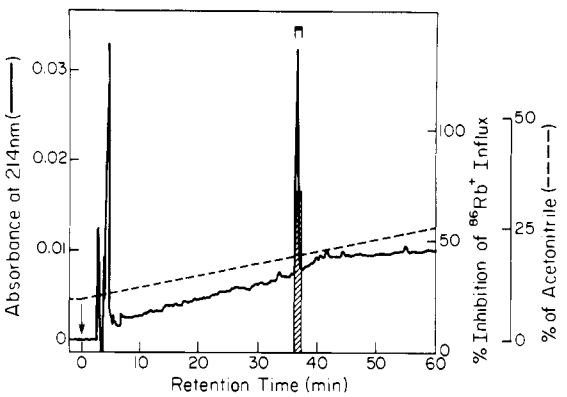

FIGURE 3: Elution profile of the inhibitory activity of ^{86}Rb uptake by erythrocytes obtained by HPLC on an ODS column. The fraction AP10Cβ (retention time of 16–24 min), indicated by the bracket in Figure 2C, was purified by HPLC on an ODS column with a linear gradient of acetonitrile (9–25%) in 0.1% TFA over 60 min at a flow rate of 1 mL/min. The arrow indicates the time of sample injection. Two-minute fractions, except for the active peak area which was manually collected, were collected and monitored for inhibitory activity of ^{86}Rb uptake by erythrocytes.

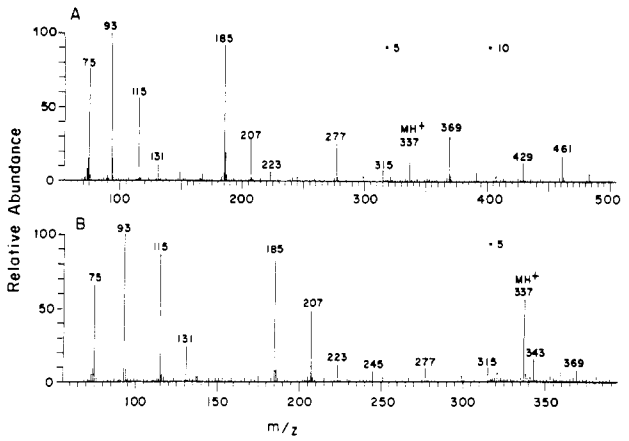

FIGURE 4: Positive ion spectrum from FAB mass spectrometry of purified AP10Cα (A) and AP10Cβ (B). The ions at m/z 93, 185, 277, 369, and 461 were respectively protonated mono-, di-, tri-, tetra-, and pentaglycerol ions from the matrix employed in FAB mass spectrometry.

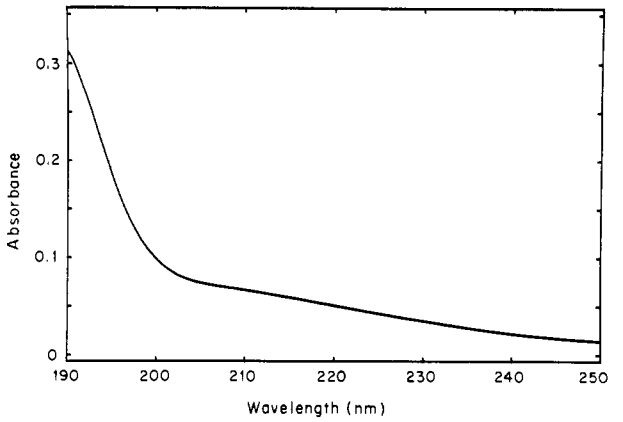

FIGURE 5: UV absorption of purified AP10Cβ. Approximately half the amount (800 units) of the purified AP10Cβ was dissolved in 1 mL of distilled water and measured UV absorption in a quartz cuvette with a 1-cm optical path length.

because of too small an amount. In each step of the entire purification, recovery and elution profiles of the inhibitory activity of ^{86}Rb uptake into erythrocytes were easily reproducible. The entire purification was repeated 3 times for the additional characterizations.

Attempts toward Identification and Structure Analysis of Na^+,K^+-ATPase Inhibitors. The two substances obtained by HPLC on an ODS column (approximately half of the total purified substance) were subjected to FAB mass spectrometry. The nominal masses obtained from positive-ion FAB mass spectra provided the molecular mass of both compounds. Both AP10Cα and AP10Cβ produced only single protonated dominant ion peaks at m/z 337 (Figure 4). Intense protonated peaks attributable to the various lengths of polymeric glycerol (m/z at 93, 185, 277, 369, and 461) and Na^+ + glycerol (m/z at 115, 207, 299, and 391) or K^+ + glycerol (m/z at 131, 223, and 315) were also detected (glycerol being used as matrix). Negative-ion FAB mass spectra obtained from both substances showed more intense ion peaks attributable to the glycerol matrix (data not shown). In order to ensure the results obtained from FAB mass studies of both purified substances, HPLC fractions (on an ODS column) adjoining the active peak were also analyzed for background information. The only signals derived from the matrix employed were observed. ^{1}H NMR spectra obtained from both entire purified substances by prolonged accumulation (8 h) at 400 MHz did not produce signals related to the inhibitor due to insufficient quantity of the substances.

The two purified substances with the inhibitory activities against ^{86}Rb uptake into erythrocytes were ultrafilterable through an Amicon YM-2 membrane (Amicon Co., Danvers, MA) that has a 1000 molecular weight exclusion limit. Both of the purified compounds lacked a characteristic spectral peak between 190 and 820 nm. The UV absorption spectrum between 190 and 250 nm of 800 units of AP10Cβ is given in Figure 5. No absorbance was observed at a higher wavelength region. The spectra from both compounds are not characteristic for peptides or common steroids. The purified compounds, AP10Cα and AP10Cβ, were found to elute from the ODS column at the same retention time of 37 min. Furthermore, AP10Cα and AP10Cβ were resolved into two activity peaks with retention times of 2–6 and 16–26 min by individual rechromatography on a CN column. From the results obtained from FAB mass spectrometric study, UV absorption spectroscopy, and HPLC on reverse-phase columns of the purified compounds, it is most likely that Na^+,K^+-ATPase inhibitory substances in AP10Cα and AP10Cβ are structurally very similar.

Table I: Effect of Various Physical–Chemical Treatments and Enzyme Digestions on the Inhibitory Activity of the Purified Na^+,K^+-ATPase Inhibitor AP10Cβ on ^{86}Rb Uptake Activity[a]

treatment	% inhibition of ^{86}Rb uptake act.
control	47.7 ± 2.0
heat (10 min in boiling water)	45.0 ± 3.1
charring (250 °C, 2 h)	4.7 ± 0.8
acid (6 N HCl, 115 °C, 21 h)	0.3 ± 1.5
alkaline (0.02 N NaOH, 23 °C, 2 h)	41.3 ± 2.5
alkaline (0.2 N NaOH, 23 °C, 2 h)	14.1 ± 5.5
protease (Pronase E)	50.9 ± 0.7
trypsin	48.7 ± 3.1
deoxyribonuclease I	55.0 ± 3.2
ribonuclease A	51.1 ± 1.6
phospholipase C	44.3 ± 3.1

[a] Values are the mean ± SE of quadruplicate determinations.

The chemical nature of the inhibitor to Na^+,K^+-ATPase was further investigated by inactivation of its inhibitory activity for Na^+ pump activity (Table I). Charring at 250 °C for 2 h and acid treatment with 6 N HCl at 115 °C for 21 h almost completely destroyed the inhibitory activity of the purified substance AP10Cβ for Na^+ pump activity. Alkaline treatment with NaOH at 23 °C for 2 h also destroyed the inhibitory activity in a dose-dependent manner. On the other hand, heat treatment at 100 °C for 10 min and various enzyme digestions did not affect the inhibitory activity. Although a little variation of the inhibitory activity was observed in various enzymatic digestion, the difference between the inhibitory activities from enzyme-treated and denatured enzyme treated samples (data not shown) was not significant.

Characterization of the Inhibitor. The purified compound AP10Cβ and authentic ouabain were compared for their inhibitory potencies against Na^+,K^+-ATPase activity, [^{3}H]-ouabain binding to the enzyme, Na^+,K^+ pump activity, and digoxin binding to anti-digoxin antibody (Figure 6). For these studies, a concentration required for the 50% inhibition of *total* ^{86}Rb uptake by intact human erythrocytes was arbitrarily designated as 1 unit concentration of AP10Cβ. In the study of Na^+,K^+-ATPase inhibitory activity, the slope of the dose–response curve from AP10Cβ paralleled that of ouabain (Figure 6A). The Na^+,K^+-ATPase activity was completely inhibited by both AP10Cβ and ouabain at 126 units and 0.75 μM, respectively. Under the present condition, a 50% inhibition of the ATPase activity was obtained at 7.6 units of

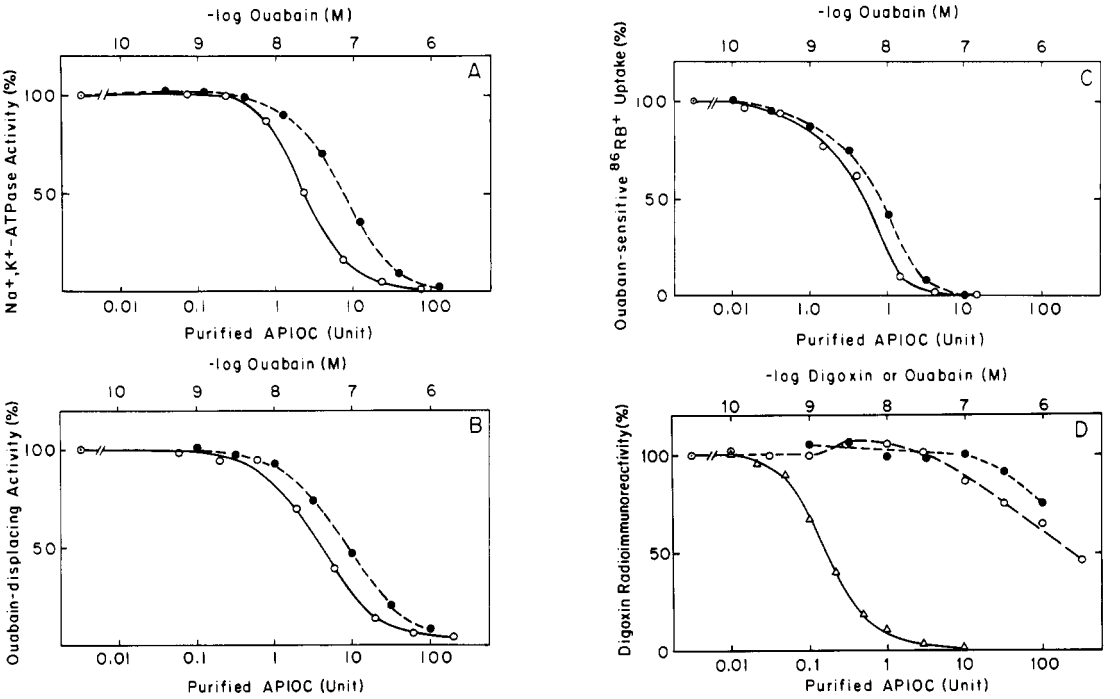

FIGURE 6: Inhibitory effect of purified AP10Cβ (●) on Na^+,K^+-ATPase activity (A), on binding of [^{3}H]ouabain to Na^+,K^+-ATPase (B), and on ^{86}Rb uptake by human erythrocytes (C) and cross-reactivity of AP10Cβ with anti-digoxin antibody (D). Dose-response curves of these activities were determined in comparison with ouabain (○) and digoxin (△) under the same assay conditions. Each point is the mean of duplicate determinations in two separate experiments and expressed as the percentage of maximal Na^+,K^+-ATPase activity, maximal binding of [^{3}H]ouabain to Na^+,K^+-ATPase, maximal ^{86}Rb uptake activity, and maximal binding of [^{3}H]digoxin to anti-digoxin antibody. The maximal activities or bindings were obtained without any inhibitors. Assay conditions were described under Materials and Methods.

AP10Cβ and 23 nM ouabain. The specific activity of this enzyme preparation was 18.3 μmol of P_i min^{-1} (mg of protein)$^{-1}$ determined under the identical condition.

The binding of [^{3}H]ouabain to the rat brain ATPase preparation was almost completely inhibited by 100 units of the purified AP10Cβ and 1 μM ouabain (Figure 6B). A 50% displacement was observed with 9.2 units of the AP10Cβ and 42 nM ouabain. In addition, the displacement curves obtained with both AP10Cβ and ouabain were in parallel, spanning 2 orders of magnitude in concentration range.

In the study of the effect of the purified inhibitor on Na^+,K^+ pump activity in intact human erythrocytes, both AP10Cβ and ouabain showed concentration–dependent inhibition at lower concentrations relative to the inhibition of ligand–receptor binding or the inhibition of Na^+,K^+-ATPase activity (Figure 6C). A 50% inhibition of *ouabain-sensitive* ^{86}Rb uptake by erythrocytes was observed at 0.8 units of AP10Cβ and 5 nM ouabain. Again, the dose–response curves were in parallel. Approximately 86% of the total ^{86}Rb uptake was inhibited at 10 units of AP10Cβ and 0.1 μM ouabain.

Both purified AP10Cβ and ouabain weakly cross-reacted with anti-digoxin-specific antibody (Figure 6D). Although complete inhibition of the binding of [^{3}H]digoxin to specific sheep anti-digoxin antibody was not observed in the present study because of the limited amount of the purified compound, the cross-reactivity curve due to AP10Cβ paralleled that of ouabain.

In order to characterize further details of the interaction between the purified inhibitor and Na^+,K^+-ATPase, effects of K^+ on the inhibitory activity of AP10Cβ against Na^+,K^+-ATPase, ouabain binding to the enzyme, and ^{86}Rb uptake were studied in comparison with ouabain. As shown in Figure 7, inhibition of the ATPase activity and ^{86}Rb uptake into intact human erythrocytes by both purified AP10Cβ and ouabain were markedly diminished with increasing K^+ concentration. On the other hand, the displacement of [^{3}H]-

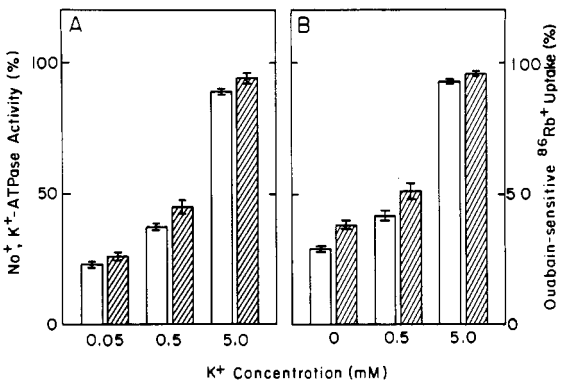

FIGURE 7: Effect of K^+ concentration on the inhibitory activity of purified AP10Cβ for Na^+,K^+-ATPase (A) and ^{86}Rb uptake activities (B). Na^+,K^+-ATPase activities were determined in 0.78 mL of assay medium, which is 60% of the original assay volume described under Materials and Methods, at 37 °C for 2 h for 0.05 mM K^+, for 1 h for 0.5 mM K^+, and for 30 min for 5 mM K^+. The concentrations of the constituents and other conditions were the same as the original method except for the concentrations of K^+, AP10Cβ (final concentration 7.6 units), and ouabain (23 nM). ^{86}Rb uptake activity in human erythrocytes was also determined in a reduced assay volume (50% of original assay volume described under Materials and Methods). The concentrations of the constituents were the same as the original method except for K^+ concentration, AP10Cβ (1.5 units), and ouabain (220 pM). Open bars indicate the activities observed in the presence of ouabain, and hatched bars indicate the activities in the presence of AP10Cβ. Activities are the mean ± SE of quadruplicate determinations.

ouabain binding by AP10Cβ and unlabeled ouabain was not affected by 0.5 mM KCl. It required 5 mM KCl to inhibit the ouabain binding displacement (Figure 8). In all three experiments, a physiological concentration of K^+ strongly reduced the inhibitory activities of both AP10Cβ and ouabain. In addition, the magnitudes of negative K^+ dependency on the inhibitory potencies of AP10Cβ and ouabain also resembled each other.

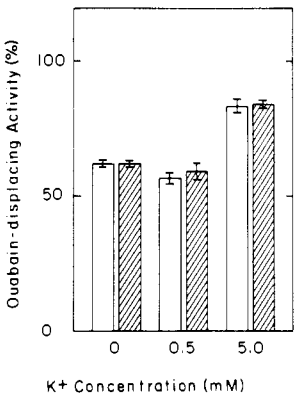

FIGURE 8: Effect of K^+ concentration on the ouabain displacing activity of the purified AP10Cβ. The ouabain displacing activities were determined in a reduced assay volume (20% of original assay volume described under Materials and Methods). The concentrations of the constituents were the same as the original method except for K^+ concentration and AP10Cβ concentration. Open bars indicate the activity due to 42 nM ouabain, and hatched bars indicate the activity due to 9.2 units of AP10Cβ. Activities are the means ± SE of quadruplicate determinations.

Table II: Effect of the Purified Na^+,K^+-ATPase Inhibitor AP10Cβ and Ouabain on Skeletal Muscle Sarcoplasmic Reticulum Ca^{2+}-ATPase Activity and Ca^{2+} Loading Rate[a]

compd	concn	% inhibn of Na^+,K^+-ATPase act.	Ca^{2+}-ATPase act. (μmol of P_i mg^{-1} min^{-1})	Ca^{2+} loading rate (μmol of Ca^{2+} mg^{-1} min^{-1})
control[b]		0	5.91 ± 0.27	12.58 ± 0.89
ouabain	20 nM	45	6.06 ± 0.25	11.40 ± 0.76
	200 nM	95	6.12 ± 0.15	11.68 ± 0.41
AP10Cβ	3 units	23	6.12 ± 0.15	12.11 ± 0.70
	23 units	81	5.94 ± 0.18	11.98 ± 0.91

[a] Values are the mean ± SE of quadruplicate determinations at 23 °C. [b] Deionized water was used as control.

The specificity of the inhibitor to Na^+,K^+-ATPase was further tested by evaluating the effect of AP10Cβ on the highly purified Ca^{2+} pump and Ca^{2+}-ATPase from longitudinal membranes of the sarcoplasmic reticulum from rabbit skeletal muscle. As indicated in Table II, both AP10Cβ and ouabain had no effect on the Ca^{2+}-ATPase activity. Even with 23 units of AP10Cβ which inhibits over 80% of Na^+,K^+-ATPase activity, no significant effect on Ca^{2+}-ATPase activity was observed. Although a small effect was observed for the Ca^{2+} loading rate by both AP10Cβ and ouabain, these effects were not dose dependent and were not statistically significant. The Ca^{2+} pumping efficiency in each of these trials was approximately 1.9–2.1 mol of Ca^{2+} transported into the vesicle per mole of ATP hydrolyzed. These results indicated that the purified Na^+,K^+-ATPase inhibitor AP10Cβ interacts with Na^+,K^+-ATPase in a highly specific manner.

Discussion

This study represents the isolation and characterization of a specific Na^+,K^+-ATPase inhibitor from bovine adrenal. The purification procedure consists essentially of three steps including extraction with methanol, flash chromatography, and six steps of successive HPLC using five different columns. The molecular mass of the purified inhibitor is estimated as less than 350. The purified inhibitor is suggested to be a nonpeptidic substance. The purified compound shows functional similarity to one of the cardiac glycosides, ouabain, in that the compound inhibits both canine kidney Na^+,K^+-ATPase and ^{86}Rb uptake into intact human erythrocytes, displaces [^{3}H]ouabain from the enzyme, and cross-reacts with specific anti-digoxin antibodies. Also like ouabain, the inhibitory potency of this compound is diminished by K^+. This inhibitor is specific for Na^+,K^+-ATPase as it has no effect on Ca^{2+}-ATPase and Ca^{2+} pump activities. These results strongly suggest that the purified inhibitor is an endogenous specific ligand for a cardiac glycoside receptor of Na^+,K^+-ATPase.

Several tissues have been used as sources for the purification and characterization of the Na^+,K^+-ATPase inhibitor. However, in many of these studies, it has not been possible to determine the structure of the inhibitors or to clarify the nature of their interaction with Na^+,K^+-ATPase because many of these studies were mainly focused on obtaining evidence for the existence of the inhibitors. Only a limited number of studies including our previous studies have revealed that several compounds such as unsaturated fatty acids (Bidard et al., 1984; Tamura et al., 1985; Kelly et al., 1986), lysophosphatidylcholines (Bidard et al., 1984; Kelly et al., 1986; Tamura et al., 1987a,b), dihydroxyeicosatrienoic acid (Schwartzman et al., 1985), dopamine (Clarkson & deWardener, 1985), dehydroepiandrosterone sulfate (Vasdev et al., 1985), lignan (Fagoo et al., 1986), and ascorbic acid (Ng et al., 1985) are endogenous substances with the inhibitory activity. However, they do not fulfill all of the criteria for an endogenous Na^+,K^+-ATPase inhibitor which should (i) be of low molecular weight, (ii) inhibits Na^+,K^+-ATPase, (iii) displaces [^{3}H]-ouabain bound to the enzyme, (iv) inhibits Na^+,K^+ pump activity, (v) cross-reacts with anti-digoxin antibody, and (vi) does not interfere with other ATPase. In the present study, bovine adrenals were chosen as a source for the purification of Na^+,K^+-ATPase inhibitor. Many fractions were found to possess some or all of the four types of activities including criteria ii–v. While all of the active substances have not been purified and characterized, the characteristics of most of the activities in the fraction eluted by 15–85% acetonitrile from the flash chromatography resembled those of unsaturated fatty acids and lysophosphatidylcholines (our unpublished data). The strongest inhibitory activity for ^{86}Rb uptake by erythrocytes in the fraction eluted by 0–15% acetonitrile from the flash chromatography was successfully purified by using a combination of normal and reverse-phase HPLC. As further proof, this substance satisfied all of the criteria mentioned above. Such a substance has never been isolated from mammlian tissues which interact with Na^+,K^+-ATPase in a manner so similar to ouabain.

AP10C, which eluted at a retention time of 34–42 min from ODS HPLC, was further purified by a combination of normal-phase NH_2 columns and a reverse-phase ODS column up to the third step of HPLC (Figures 1 and 2A,B). The fourth step of HPLC with the CN column apparently produced two activity peaks, designated AP10Cα (retention time of 2–6 min) and AP10Cβ (retention time of 16–26 min in Figure 2C). However, there was proof that individual rechromatography of each activity peak again produced two activity peaks with the same retention time as AP10Cα (2–6 min) and AP10Cβ (16–26 min). Although it is possible that AP10Cα and AP10Cβ are identical, both activities were individually purified to a single peak substance using an additional two steps of reverse-phase HPLC (Figure 3). The proportional relationship between the ratio of peak heights and inhibitory activities of ^{86}Rb uptake into red blood cells due to AP10Cα and AP10Cβ in the final step of HPLC also indicated strongly the similarity of the two substances. The structure of the purified inhibitor could not be obtained by (400 MHz) NMR and FAB mass spectrometric studies apparently due to an insufficient amount of the compound obtained from 1.5 kg of bovine adrenal

glands. It seems that the total amount of the purified AP10Cβ was less than 10 μg, which is considered to be the minimum quantity of ouabain for obtaining reasonable NMR signals by 8-h scans. On the basis of this assumption, the upper limit of 1 unit concentration of AP10Cβ used as an arbitrary concentration for the characterization studies was estimated to be less than 24 nM AP10Cβ. This figure was derived by assuming a molecular weight of 350 and a minimally detectable total weight of 10 μg for the inhibitor and based on a total amount of purified AP10Cβ (1702 units) from 1.5 kg of adrenal glands and a total volume (0.72 mL) of the assay mixture for the ^{86}Rb uptake assay. The estimate of the inhibitor concentration, given 1 unit of activity, can be made from an independent calculation. Assuming 3.8 nmol of the enzyme protein per milligram of total protein in the Jorgensen preparation [specific activity, 34.1 units/mg obtained under the optimal assay condition described under Materials and Methods except for the K^+ concentration which was 20 mM and the turnover number (9000/min) (Jorgensen, 1974b)], with 6.8 μg in the ATPase assay, the theoretical level of the Na^+,K^+-ATPase in the assay would be 25.8 pmol; thus, at 50% inhibition, at least 13 pmol of endogenous inhibitor would be required to produce this 50% inhibition. From Figure 6, 7.6 units of AP10Cβ were required for 50% inhibition. If the endogenous inhibitor were bound with the theoretical affinity, this 13 pmol and 7.6 units would correspond to about 4.6 ng ($M_r \sim 350$). Thus, as a reasonable lower limit using this approximation, 1 unit would approximate 0.6 ng of the inhibitor in the assay mixture of 1.3 mL and would lead to a concentration under 50% inhibition of Na^+,K^+-ATPase as low as 1.3 nM. Thus, an upper limit of 24 nM and a lower limit of 1.3 nM can be postulated as a probable range for the 1 unit concentration of the adrenal Na^+,K^+-ATPase inhibitor. Correspondingly, the concentration of AP10Cβ subjected to NMR analysis, which employed 1702 units of AP10Cβ, could have been as high as 106 μM but as low as 6 μM.

On the other hand, effects of the heat, acid, and alkaline treatments and various enzymatic digestion upon the inhibitory activity of the purified substance provided additional information with regard to the nature of the inhibitor. Since two of the proteolytic enzyme, particularly nonspecific protease (Pronase E), treatments were not effective to inactivate the activity, it was suggested that the inhibitory activity could be due to a nonpeptidic substance. The results of the treatments with phospholipase C also suggest that lysophospholipids should not be responsible for this inhibitory activity. Furthermore, the inhibitory activity was stable in boiling water for 10 min, whereas charring and acid hydrolysis with 6 N HCl almost completely destroyed the inhibitory activity, demonstrating that, at least, inorganic salts such as K^+ and Ca^{2+} are not responsible for the Na^+ pump inhibitory activity of this purified substance. At −20 °C and neutral pH, the inhibitory activity for ^{86}Rb uptake into erythrocytes was preserved for 9 months without losing any activity. The chemical nature of this inhibitor, therefore, can be summarized as heat stable, low molecular weight (molecular mass less than 350), nonpeptidic, and an acid- and alkaline-sensitive substance.

In the studies of inhibitory potencies of the purified compound and ouabain, practically parallel dose–response curves were observed in all four assays. In addition, the relative concentration ranges of AP10Cβ and ouabain were comparable in each assay although the absolute concentration of the two compounds could not be compared. Thus, the functional similarity between AP10Cβ and ouabain, parallel dose dependency within comparable relative concentration ranges, and the negative K^+ dependency seem to reflect a certain degree of structural similarity between the two compounds. In this regard, it is of interest to note that both inhibitors, AP10Cβ and ouabain, can be detected by both the ouabain displacement assay with a relatively high sensitivity and by the radioimmunoassay for digoxin with a low sensitivity relative to digoxin. These observations seem to indicate that the purified AP10Cβ has some degree of similarity to ouabain, rather than digoxin. Further, the observations obtained from the inhibition of ouabain binding to Na^+,K^+-ATPase as well as the K^+ dependency of AP10Cβ actions in the inhibition of Na^+,K^+-ATPase, Na^+ pump, and ouabain binding to the enzyme indicate that the purified inhibitor is a competitive ligand for binding to a specific cardiac glycoside receptor site in the ATPase. With regard to the K^+ dependency, it has been reported that a high concentration of K^+ inhibits the binding of a cardiac glycoside to Na^+,K^+-ATPase (Hansen, 1984). In addition, negative K^+ dependency on the inhibitory activity of AP10Cβ was opposite to that of vanadate (Beague et al., 1980).

Close similarity in numerous functional features between the purified substance AP10Cβ and ouabain prompted us to investigate the possibility that this substance was possibly derived from plant dietary sources. However, no seasonal variation of the inhibitory activity to ^{86}Rb uptake by the AP10C fraction was found in spite of marked differences in the dietary conditions in winter and summer seasons. Furthermore, no age-dependent difference of the inhibitory activity was observed among adrenal glands of newborn, calf, heifer, steer, and milking cows with greatly diminished milk-producing ability (our unpublished data). The adrenal glands of hogs, which are fed with grains, also contain the inhibitor (our unpublished results). These results suggest that the inhibitor purified from the adrenal is not ouabain or derived from plant dietary sources.

To examine the specificity of the purified inhibitor, the effects of AP10Cβ on Ca^{2+} pump and Ca^{2+}-ATPase from sarcoplasmic reticulum membranes of skeletal muscle were examined. Neither AP10Cβ nor ouabain exhibits any effect on Ca^{2+} pumping rate or Ca^{2+}-ATPase activity. These results demonstrate that the purified inhibitor is a specific inhibitor for Na^+,K^+-ATPase. Recently Haupert and his colleagues (Haupert & Sancho, 1979; Haupert et al., 1984; Carilli et al., 1985) have reported a low molecular weight Na^+,K^+-ATPase inhibitor in bovine hypothalamus which also inhibits Na^+ pump in human erythrocytes and ouabain binding to frog urinary bladder. This substance from hypothalamus was shown to have a high affinity and reversible binding to the Na^+,K^+-ATPase (Haupert et al., 1984). While these properties are very similar to those purified from bovine adrenal in the present study, the hypothalmic inhibitor does inhibit Ca^{2+}-ATPase from the sarcoplasmic reticulum of rabbit skeletal muscle with a potency identical with the inhibition of Na^+,K^+-ATPase. In contrast, the inhibitor isolated from bovine adrenal shows no inhibition to Ca^{2+}-ATPase and is apparently different from hypothalmic Na^+,K^+-ATPase inhibitor. Cloix et al. (1985) also found a nonpeptidic Na^+,K^+-ATPase inhibitor in human urine from relatives of hypertensive subjects. Although they have not shown any dose-dependent response curves in comparison to a reference compound such as ouabain, the dose–response curves of urinary inhibitor in the [^3H]ouabain displacement assay and Na^+,K^+-ATPase inhibition were approximately twice as steep as that of adrenal inhibitor. Therefore, urinal Na^+,K^+-ATPase inhibitor may be considered

to be different from the adrenal inhibitor.

Many of the adrenal-derived steroids have been examined for their inhibitory potencies against Na^+,K^+-ATPase and ouabain binding to the enzyme by methods similar to those employed in the present studies (Diamandis et al., 1985; Labella et al., 1985). However, the present inhibitor seems to exhibit a greater potency than other adrenal compounds. Thus, it is likely that the Na^+,K^+-ATPase inhibitor purified in the present study is different from other adrenal-derived steroids.

The present study clearly demonstrates that there is a highly specific endogenous Na^+,K^+-ATPase inhibitor in the bovine adrenal gland and that this inhibitor functions in a manner similar to that of ouabain. However, important questions remain unanswered as to its chemical structure, pathophysiological significance, and relation to circulating Na^+,K^+-ATPase inhibitor. Further efforts are being made to identify this Na^+,K^+-ATPase inhibitor and to evaluate the physiological significance of this compound.

Acknowledgments

We are indebted to Dr. Robert L. Post of the Department of Molecular Physiology and Biophysics for his assistance in the measurement of Na^+,K^+-ATPase and for his valuable discussion and to Dr. Makoto Inui for his assistance in the determination of Ca^{2+}-ATPase and Ca^{2+} loading rate and for his valuable discussion. We gratefully acknowledge Dr. Thomas M. Harris of the Department of Chemistry for ^1H NMR study and Drs. Brian J. Sweetman and Ian A. Blair for their assitance in the FAB mass analysis.

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In Vitro Protein Kinase C Phosphorylation Sites of Placental Lipocortin†

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Received January 25, 1988; Revised Manuscript Received March 24, 1988

ABSTRACT: Human placental lipocortin is a high-affinity substrate for rat brain protein kinase C in vitro with phosphorylation occurring on serine and threonine residues in a ratio of approximately 2 to 1. Comparison of the ability of various N-terminal-truncated derivatives of lipocortin to serve as phosphorylation substrates, and direct analysis of the N-terminal peptides cleaved from ^{32}P-labeled lipocortin, indicated that threonine-24, serine-27, and serine-28 were the phosphorylation sites. The possibility is discussed that a lysine residue near the carboxy side of the phosphorylation site was involved in lipocortin interaction with the catalytic site of protein kinase C.

Covalent modification of proteins by phosphorylation is one of the primary mechanisms through which extracellular signals modulate cellular function. Protein–tyrosine kinases and protein kinase C (PKC)[1] are implicated in a number of cellular events, including regulation of cell replication. The identification of cellular substrates for these kinases is an active area of current research. Recently, lipocortin (Wallner et al., 1986) has been identified as a high-affinity substrate for the EGF-stimulated protein–tyrosine kinase (De et al., 1986; Pepinsky & Sinclair, 1986; Haigler et al., 1987; Giugni et al., 1985; Sawyer & Cohen, 1985) and for PKC (Summers & Creutz, 1985; Michener et al., 1986; Khanna et al., 1986) both in vivo and in vitro.

The amino acid sequence of lipocortin (Wallner et al., 1986), which also has been called lipocortin I (Huang et al., 1986), p35 (De et al., 1986), calpactin II (Glenney, 1986a), and chromobindin 9 (Creutz et al., 1987), reveals that it belongs to a family of structurally related Ca^{2+}-binding proteins known as annexins (Geisow et al., 1987). This family of proteins, initially investigated as substrates for protein–tyrosine kinases, mediators of exocytosis, inhibitors of phospholipase A_2, or components of the cytoskeleton, undergo Ca^{2+}-dependent binding to certain phospholipids. The exact physiological roles of these proteins are not yet known. Lipocortin inhibits phospholipase A_2 in some in vitro assays (Waller et al., 1986), but this is by an indirect mechanism (Davidson et al., 1987; Haigler et al., 1987) and has not been shown to reflect a physiological function of the protein.

The complete amino acid sequences are known for four annexins: lipocortin (Wallner et al., 1986); calpactin I (Kristensen et al., 1986; Saris et al., 1986), which also has been called p36 (Saris et al., 1986), protein I (Gerke & Weber, 1984), and lipocortin II (Huang et al., 1986); protein II (Weber et al., 1987); and endonexin II (Schlaepfer et al., 1987; Kaplan et al., 1988), which also has been investigated as an anticoagulant protein (Funakoshi et al., 1987; Iwasaki et al., 1987). Each protein has two domains: a small N-terminal domain with only limited sequence similarity between proteins and a core domain with 40–60% sequence identity between proteins. The N-terminal and core domains are joined by a hinge region that is very sensitive to proteolysis (Haigler et al., 1987); Weber et al., 1987; Glenney, 1986b). The Ca^{2+} and phospholipid binding sites are located in the core domain (Glenney, 1986b; Schlaepfer & Haigler, 1987) while it is reasonable to speculate that the N-terminal domain confers a different biological activity to each protein.

Lipocortin and calpactin I are phosphorylated on tyrosine residues in the N-terminal domain by the EGF receptor/kinase (De et al., 1986; Haigler et al., 1987) and by pp60^{src} (Glenney & Tack, 1985), respectively. This raises the possibility that phosphorylation modulates an as yet undefined biological activity of these proteins. In addition, PKC phosphorylates serine-25 of calpactin I (Gould et al., 1986) and threonine-6 of protein II (Weber et al., 1987); both of these sites are in the N-terminal domains. Lipocortin has previously been shown to be a substrate for PKC in vitro (Summers & Creutz, 1985; Khanna et al., 1986) and in a stimulant-dependent manner in intact chromaffin cells (Michener et al., 1986). One study

† Supported by U.S. Public Health Service Grant GM357844.
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[1] Abbreviations: DNFB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenyl; EGF, epidermal growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/88/0427-4253$01.50/0