Lysophosphatidylcholines Containing Polyunsaturated Fatty Acids Were Found as Na^+,K^+-ATPase Inhibitors in Acutely Volume-Expanded Hog†

Masaaki Tamura,‡ Thomas M. Harris,§ Koichi Higashimori,‡ Brian J. Sweetman,‖ Ian A. Blair,‖ and Tadashi Inagami*,‡

With the technical assistance of Trinita Fitzgerald and Edward Price, Jr.

Departments of Biochemistry, Chemistry, and Pharmacology, Vanderbilt University, Nashville, Tennessee 37232

Received August 18, 1986; Revised Manuscript Received December 30, 1986

ABSTRACT: Na^+,K^+-ATPase inhibitors possessing inhibitory activities against the specific binding of ouabain to Na^+,K^+-ATPase and ^{86}Rb uptake into hog erythrocytes have been purified from the plasma of acutely saline-infused hog. The purifications were performed by a combination of Amberlite XAD-2 adsorption chromatography and four steps of high-performance liquid chromatography with four different types of columns. Fast atom bombardment (FAB) mass and proton NMR spectrometric studies identified the purified substances as γ-arachidoyl- [LPCA(γ), 34%], β-arachidoyl- [LPCA(β), 4%], γ-linoleoyl- (LPCL, 33%), and γ-oleoyl- (LPCO, 25%) lysophosphatidylcholine, expressed in molar ratio in the plasma. Small amounts of γ-docosapentaenoyl-, γ-eicosatrienoyl-, and γ-palmitoyllysophosphatidylcholine were also detected by both FAB mass and ^1H NMR spectrometric studies. Only γ-acyl-LPC's showed inhibitory activities on Na^+,K^+-ATPase and ouabain-binding activities. These LPC's were effective at 100 μM levels in attaining 50% inhibition of the enzyme activity. The inhibition of Na^+,K^+-ATPase activity due to these compounds was always more sensitive than that of both ouabain-binding and ^{86}Rb uptake activities. The ouabain-displacing activity in plasma due to these compounds increased with time during saline infusion. The maximal plasma level was approximately 10 times higher than that in the preinfusion plasma sample. Although these results suggest the γ-acyl-LPC's with long-chain polyunsaturated fatty acids are not simple competitive inhibitors to Na^+,K^+-ATPase, these compounds could be implicated in the pathogenesis of the circulation abnormality through the modulation of membrane enzyme.

Recognition of the presence of a receptor in Na^+,K^+-ATPase for cardiac glycosides such as ouabain and digitalis-derived compounds has prompted speculation of the existence of an endogenous ligand for the receptor. In support of this hypothesis, many investigations have reported the existence of putative Na^+,K^+-ATPase inhibitors in plasma of both clinically hypertensive subjects (Hamlyn et al., 1982; Graves & Williams, 1984) and experimentally hypertensive animals (Buckalew & Nelson, 1974; Huot et al., 1983; Pamnani et al., 1983; Castaneda-Hernandez & Godfraind, 1984) and implicated the inhibitors in the pathogenesis of essential hypertension (Grault et al., 1983; Vassallo et al., 1985). However, neither the structure nor the nature of the interaction of these inhibitors with Na^+,K^+-ATPase is clear. In the course of numerous attempts to identify these compounds, controversies have arisen. Some investigators have attributed the inhibitory activity to an acidic peptide (Gruber & Buckalew, 1978; Gruber et al., 1980) while others have proposed the activity to be due to nonpeptidic substances (Kojima, 1984; Kelly et al., 1985). It has also been postulated that this inhibitor interacts with an antibody to digoxin (Gruber et al., 1980; Kojima, 1984; Kelly et al., 1985). Studies in this field have been exceedingly complicated because different methodologies for the extraction and purification have been used and most of the studies are not complete. We have attempted to contribute to the clarification of this complicated problem by the complete purification and structural analysis of the inhibitors. We have recently demonstrated that multiple Na^+,K^+-ATPase inhibitors were detected in saline-infused hog plasma, using a method generally used for the purification of steroids or their conjugates (Tamura et al., 1985). We also demonstrated that inhibitory activity in the plasma of volume-expanded animals was attributable to unsaturated fatty acids (Tamura et al., 1985). In this paper we report complete purification and identification of the structure of the remaining inhibitory activities.

Materials and Methods

Chemicals. [γ-^{32}P]Adenosine 5′-triphosphate tetrakis(triethylammonium) salt (30.0 Ci/mmol), [12α-^3H]digoxin (19.0 Ci/mmol), $^{86}RbCl$ (2.55 mCi/mg), and [G-^3H]ouabain (18.0 Ci/mmol) were purchased from New England Nuclear. Antisera against digoxin conjugated to bovine serum albumin were from Miles Laboratories, Inc. (Elkhart, IN). Adenosine 5′-triphosphate (vanadium free), digoxin, ouabain, and Amberlite XAD-2 polymeric adsorbent were from Sigma Chemical Co. Organic solvents were of HPLC[1] grade. Other chemicals were of analytical grade.

† This work was supported by Research Grants HL14192, HL35323, GM-15431, RR-01688, and RR-05424 and Biomedical Research Support Grants from the National Institutes of Health.

* Address correspondence to this author.
‡ Department of Biochemistry.
§ Department of Chemistry.
‖ Department of Pharmacology.

[1] Abbreviations: HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholine; LPCA(β), β-arachidoyllysophosphatidylcholine; LPCA(γ), γ-arachidoyllysophosphatidylcholine; LPCD, γ-docosapentaenoyllysophosphatidylcholine; LPCE, γ-eicosatrienoyllysophosphatidylcholine; LPCL, γ-linoleoyllysophosphatidylcholine; LPCO, γ-oleoyllysophosphatidylcholine; LPCP, γ-palmitoyllysophosphatidylcholine; FAB, fast atom bombardment; ^1H NMR, proton nuclear magnetic resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

0006-2960/87/0426-2797$01.50/0

Preparation of Plasma. Plasma of volume-expanded hogs was prepared as described previously (Tamura et al., 1985). In brief, 10% (body weight) isotonic saline was infused to cross-bred hogs kept on regular dog chow and anesthetized with pentobarbital over 60 min. The infusion was continued thereafter at 40% of the initial rate for an additional 60–90 min. After stabilization of urine flow, the blood was collected from the femoral artery in a heparin-containing flask and centrifuged for 20 min at 4000*g* and 25 °C. The separated plasma was stored at −60 °C until use.

Assays. The capacities to inhibit Na^+,K^+-ATPase activity and to displace [^{3}H]ouabain binding to this enzyme were determined as described previously with partially purified rat brain Na^+,K^+-ATPase (Tamura et al., 1985). In the assay for Na^+,K^+-ATPase inhibitory activity, a 0.5-mL reaction mixture consisted of 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, 0.5 mM EGTA, 80 μg of the enzyme protein, 50 mM imidazole hydrochloride buffer (pH 7.4), and 18–30 nCi of [γ-^{32}P]ATP. After incubation at 37 °C for 30 min, the reaction was terminated by addition of 50 μL of ice-cold 55% trichloroacetic acid. The radioactivity of inorganic phosphate released was separated from unreacted [γ-^{32}P]ATP by addition of 0.5 mL of 5% (w/v) activated charcoal and counted by liquid scintillation counting. The ouabain-sensitive Na^+,K^+-ATPase activity was calculated as the difference between inorganic phosphate released in the absence and presence of 1 mM ouabain. The assay was performed at fixed concentrations of K^+ (20 mM) and ATP (4 mM).

The ouabain-displacing activity was determined by measuring the binding of [^{3}H]ouabain to rat brain Na^+,K^+-ATPase in competition with an inhibitor. A 0.5-mL reaction mixture contained the following constituents: 50 mM Tris-HCl buffer, pH 7.4, 0.5 mM EDTA, 80 mM NaCl, 4 mM $MgSO_4$, 2 mM ATP, 62.5 nCi of [^{3}H]ouabain, 80 μg of Na^+,K^+-ATPase preparation, and various concentrations of sample or ouabain standard (2 nM to 2 μM). After incubation at 37 °C for 60 min, the reaction was terminated by the addition of 3 mL of ice-cold 50 mM Tris-HCl buffer, pH 7.4. Bound [^{3}H]ouabain was trapped by Whatman GF/B filter (Whatman, Inc., Clifton, NJ), and the radioactivity on the filter was counted in aqueous counting scintillant (Amersham, Arlington Heights, IL). Ouabain-displacing activities are expressed as ouabain molar equivalents or nanograms of ouabain equivalent per milliliter of plasma.

Na^+,K^+ pump activity in hog erythrocytes was assayed by measuring ^{86}Rb-uptake activity according to the method of Diamandis et al. (1985). Blood (85 volumes) obtained from a local slaughterhouse was quickly mixed with 15 volumes of the solution containing 41.6 mM citric acid, 85.2 mM sodium citrate and 122 mM D-glucose. Red blood cells were separated by centrifugation for 5 min at 2000*g* and washed 3 times in 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. For the 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 to 0.1 μM). After preincubation for 2 h at 37 °C, 20 μL of 2 μCi of [^{86}Rb]RbCl in 15 mM KCl was added to each tube, and the reaction mixture was incubated for 1 h in a shaking water bath. 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 sedimenting the cells in the medium for 5 min at 2000*g*. Radioactivity incorporated into the red cells was counted in a γ counter (Micromedic System 2/200, Atlanta, GA).

Digoxin-like immunoreactivity was measured by radioimmunoassay according to the method of Gruber et al. (1980) with anti-digoxin antibodies. Free digoxin was separated from antibody-bound digoxin by adsorption to charcoal. The sensitivity of this radioimmunoassay system was 4 ng/tube.

Extraction of Na^+,K^+-ATPase Inhibitors. Volume-expanded hog plasma (600 mL) obtained after saline infusion for 2 h was treated with 5.4 L of acetone/methanol (1:1 v/v) at −20 °C for 16 h. The mixture was filtered through Whatman No. 1 filter paper and concentrated by a rotary evaporator. The resultant water phase was lyophilized.

Adsorption Chromatography. Amberlite XAD-2, a nonionic polymeric adsorbent, was prewashed with 20 volumes of methanol and distilled water and packed in a glass column (2.8 × 60 cm). Plasma extract obtained from 600 mL of plasma from the volume-expanded animal was suspended in an equal volume of distilled water and applied to the column preequilibrated with distilled water. After the column was washed with 3 column volumes of distilled water, the inhibitory activity was eluted with 3 volumes of methanol at a flow rate of 60 mL/min and at room temperature. Methanol in the eluate was evaporated by flash evaporation, and the remaining aqueous solution was lyophilized. This chromatography was repeated to obtain a sufficient amount of activity. The dry material was dissolved in a small amount of methanol for HPLC.

High-Performance Liquid Chromatography. HPLC on an aminopropyl (NH_2) columns (0.46 × 25 cm, Alltech, Deerfield, IL, and Du Pont, Wilmington, DE) was carried out with a decreasing linear concentration gradient of acetonitrile at a flow rate of 1 mL/min. Reverse-phase HPLC on a Zorbax octadecylsilane (ODS) column (0.46 × 25 cm, Du Pont, Wilmington, DE) and on a SynChropak octylsilane (C-8) column (0.41 × 25 cm, SynChrom, Linden, ID) were run with linear gradients of acetonitrile or 2-propanol in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. The absorbance was monitored at 214 nm.

Structure Analysis. ^{1}H NMR spectra were recorded on Bruker AM-400 and IBM NR-300 spectrometers operated at 400 and 300 MHz, respectively, with samples dissolved in [$^2H_4$]methanol. The spectra were standardized against the residual CD_2HOD signal in the solvent, which was assigned to 3.30 ppm. Assignments were made on the basis of chemical shifts, multiplicities, and peak areas and confirmed by homonuclear decoupling. In a typical data acquisition, 16 384 data points, 10 ppm sweep width, and a 40°–60° pulse were employed.

Fast atom bombardment (FAB) mass spectra of purified substances were obtained on a VG 70/250 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 to a resolving power of 2500, and spectra were obtained at an accelerating voltage of 6 kV. Scans were obtained at 10 s/decade by switching on alternate scans between the positive ion and negative ion modes. Purified samples (1–4 μg) in methanol (1–1.5 μL) were introduced on a stainless steel target with a thin film of neat glycerol as the matrix.

RESULTS

Purification of Ouabain-Displacing Activities. A crude extract was prepared from 1.4 L of plasma of acutely saline-infused hog as described previously (Tamura et al., 1985). An Amberlite XAD-2 column was used for adsorption chromatography of the crude extract as the second step of puri-

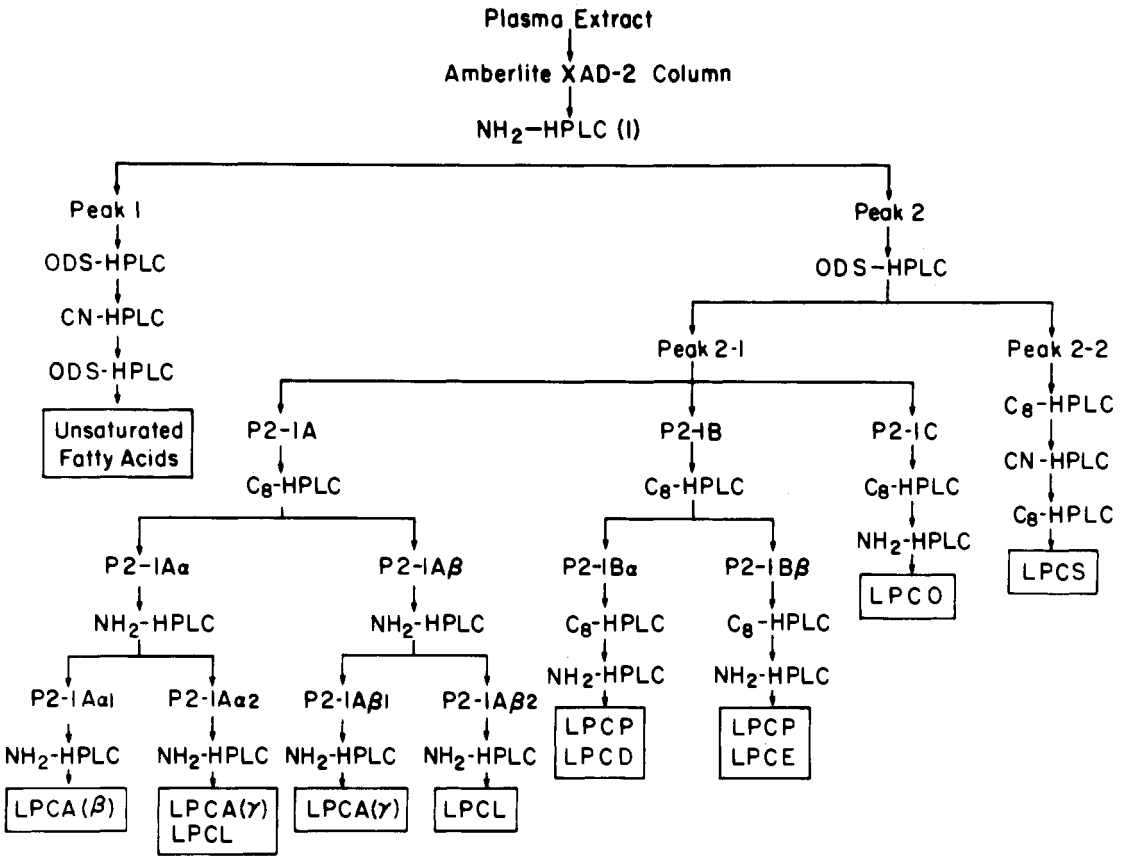

FIGURE 1: Scheme of purification of ouabain-displacing activities in volume-expanded hog plasma.

fication for removing substances that interfere with the assays. Active materials obtained from the Amberlite XAD-2 column chromatography separated into two distinct activity peaks by HPLC on an Alltech aminopropyl column (Figure 1). The first peak contained free fatty acid as reported previously (Tamura et al., 1985). The second peak from the aminopropyl column, with a retention time of 39 min, was further resolved into two broad activity peaks on an ODS column (Figure 2). The first activity peak, which eluted from the ODS column at 26–40 min and was designated P2-1, showed strong optical absorbance at 214 nm. Ouabain-displacing activity under P2-1 was seen to consist of at least three components as shown in Figure 2; however, separation of the three components could not be achieved by reverse-phase (Zorbax ODS column) or normal-phase HPLC [Alltech silica (Sil) (0.46 × 25 cm) and Zorbax cyanopropyl (CN) (0.46 × 25 cm) columns]. P2-1A (retention time of 26–32 min on the ODS column), peak 2-1B (retention time of 34–36 min), and P2-1C (retention time of 36–40 min) were further purified individually by HPLC on an octylsilane (C-8) column (Figure 3). As shown in Figure 3, each of the three fractions was resolved into two activity peaks. However, it was found that the trailing part of the activity of P2-1B (retention time of 31–34 min in Figure 3B) corresponded to P2-1C and the early eluting activity peak on the chromatogram of P2-1C (retention time of 21–24 min in Figure 3C) corresponded to P2-1B. Materials under these five activity peaks were purified individually by HPLC on a Zorbax NH_2 column. P2-1Aα and P2-1Aβ were further resolved into two activity peaks each (Figure 4). Thus, a total of seven highly purified active substances were obtained by rechromatography on a Zorbax NH_2 column (Figure 1). P2-2 was purified to homogeneity as reported (Tamura et al., 1986, 1987).

Structure Analysis of the Ouabain-Displacing Substances. Structures of the purified substances were identified by

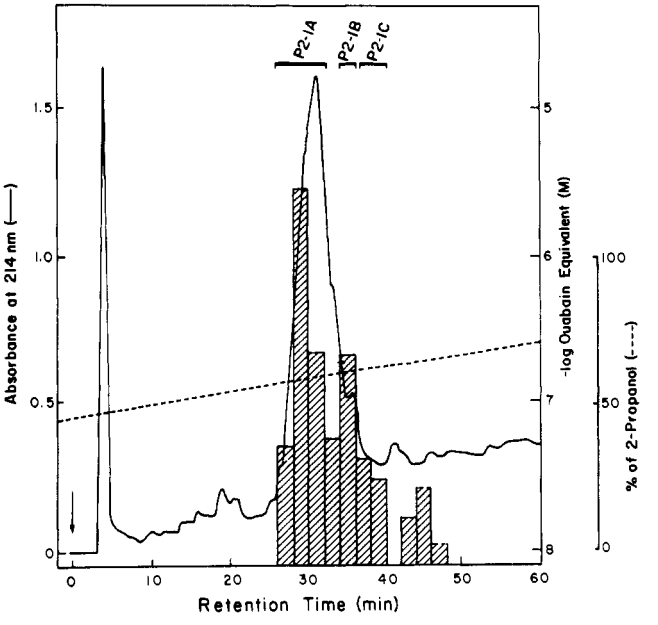

FIGURE 2: Elution profile of ouabain-displacing activities on an ODS column. The second activity peak from Alltech aminopropyl HPLC derived from 32 mL of plasma was fractionated by ODS HPLC with a linear gradient of 2-propanol (45–69%) in 0.1% TFA over 60 min. The arrow indicates the time of sample injection. Two-minute fractions were collected and monitored for ouabain-displacing activity. Active fractions under brackets were individually collected.

high-field ^1H NMR spectrometry (Figures 5 and 6) and FAB mass spectrometry (Figure 7). The NMR spectra provided clear evidence for all of the compounds being lysophosphatidylcholines. In particular, all of the compounds gave intense singlets at 3.20 ppm for the trimethylammonium cation and multiplets near 4.27 and 3.63 for the ethylene linkage in the choline fragment. Further examination of the signals for

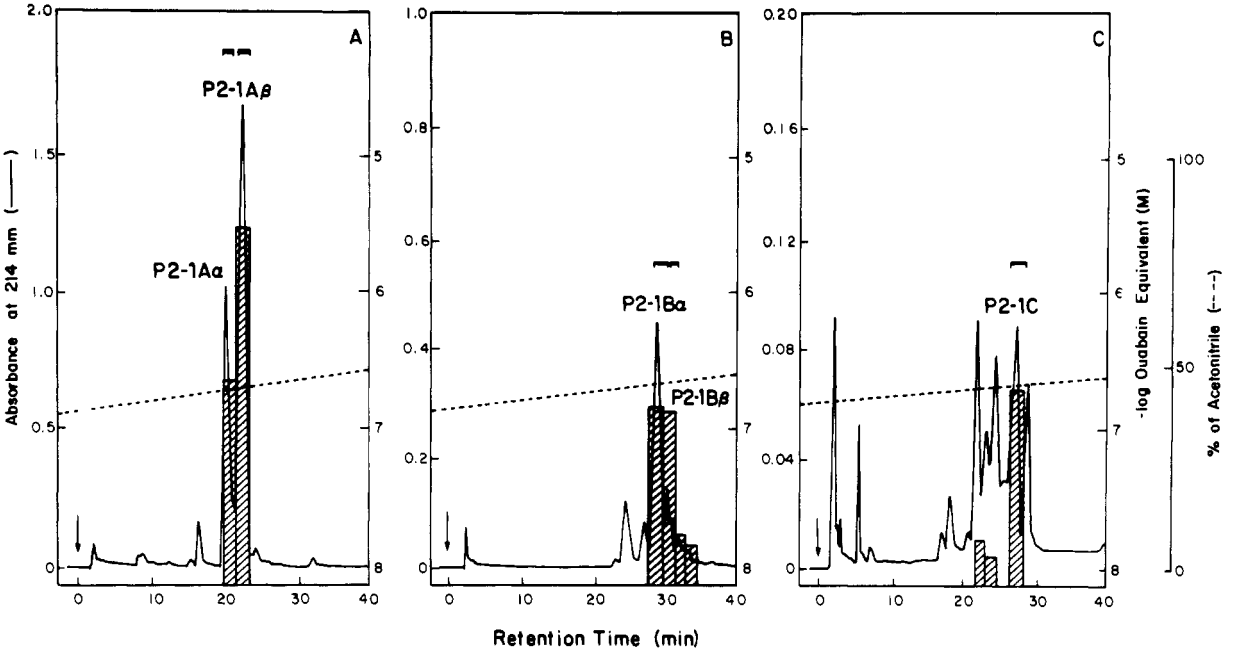

FIGURE 3: Elution profiles of ouabain-displacing activities by HPLC on a C-8 column. P2-1A fractions (retention time = 26–32 min), P2-1B fractions (34–36 min), and P2-1C fractions (36–40 min) indicated by the brackets in Figure 1 were further purified by HPLC on C-8 columns with a linear gradient of acetonitrile (38–50% for P2-1A and P2-1B, 40–52% for P2-1C) in 0.1% TFA over 50 min. Arrows indicate the time of sample injection. Two-minute fractions were collected, except for the active peak fractions, which were collected manually. Flow rates were uniformly 1 mL/min. Ouabain-displacing activity of these fractions is indicated in hashed bars.

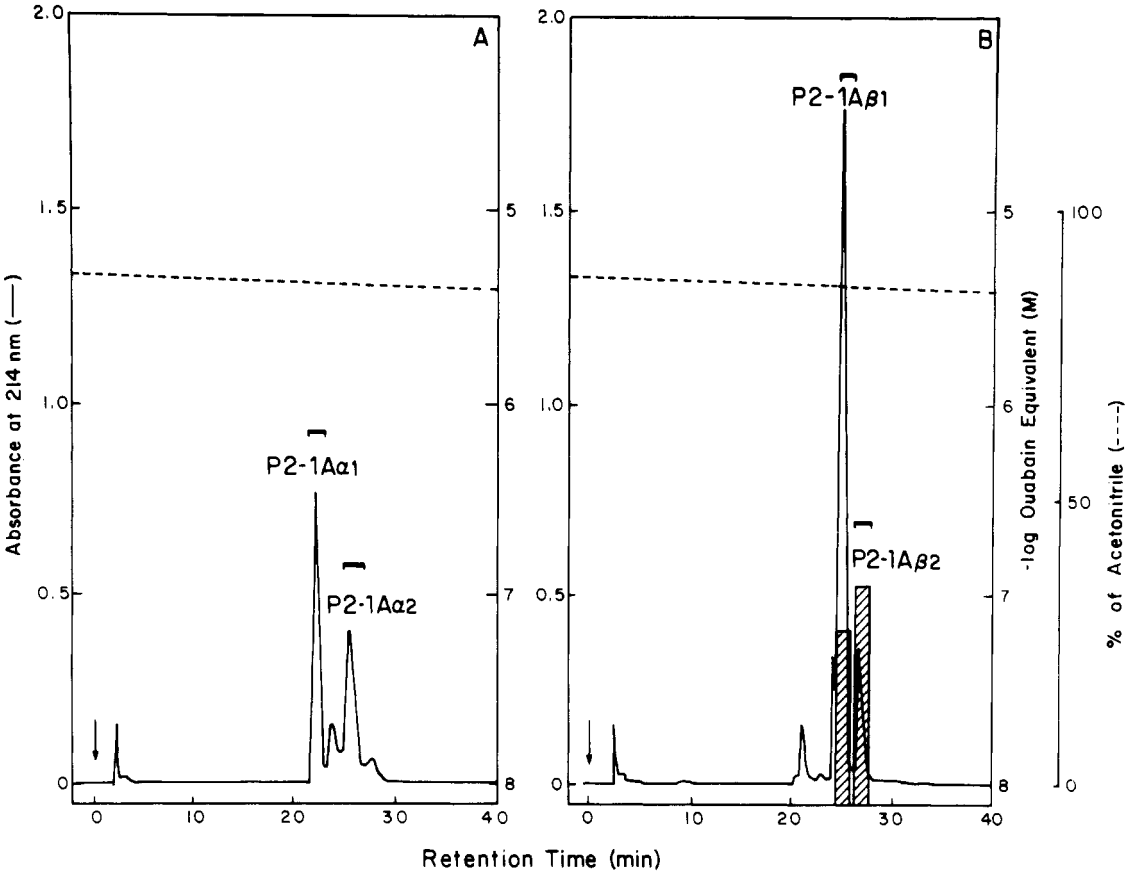

FIGURE 4: Elution profiles of ouabain-displacing activities in HPLC on a Zorbax NH_2 column. P2-1A fractions (retention time of 19–21 min) and P2-1A fractions (22–25 min) indicated by the brackets in Figure 2A were purified by HPLC on Zorbax NH_2 columns with a decreasing linear gradient of acetonitrile (81–87%) in distilled water over 40 min. The purified compounds under brackets were pooled. The ouabain-displacing activity was monitored as described in Figure 2.

the glycerol fragment revealed that the purified lysophosphatidylcholines fell into two classes (Figure 5). All but one gave three signals for the glycerol fragment in a 2:2:1 ratio at 3.89, 4.13, and 3.96 ppm. The remaining one, designated P2-1Aα1, gave signals at 3.69, 4.00, and 5.00 ppm. The two structural groups were assigned as the γ-acyl and β-acyl isomers, respectively, on the basis of the signal (of area 1.0) for the β-hydrogen on the glycerol being deshielded approximately 1 ppm by acylation of the hydroxyl group at that position. From the comparison of the signals of the acyl fragments, it

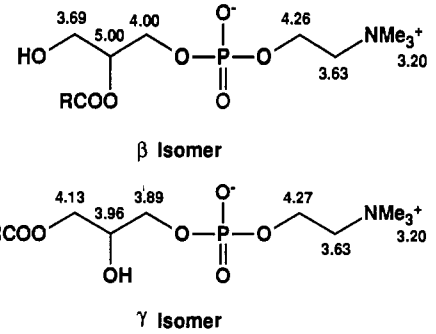

FIGURE 5: Assignment and chemical shifts of purified β- (and γ-) arachidoyl-, γ-linoleoyl-, and γ-oleoyllysophosphatidylcholines. Chemical shifts are referenced to the residual CD_2HOD signal in the solvent at 3.30 ppm.

was revealed that all of the acyl moieties of the purified compounds have one to four double bonds as follows: P2-1Aα1 and P2-1Aβ1, four double bonds; P2-1Aβ2, two double bonds; P2-1C, one double bond.

This information together with the nominal masses obtained from positive ion FAB mass spectra established the molecular mass and chain lengths of the fatty acid moieties. P2-1Aα1, P2-1Aβ1, P2-1Aβ2, and P2-1C produced only single protonated molecular ion peaks at m/z 544, 544, 520, and 522, respectively, while P2-1Aα2, P2-1Bα, and P2-1Bβ produced multiple protonated molecular ion peaks at m/z 520 and 544, 496 and 570, and 496 and 546, respectively. Fragmentation peaks for all of the purified compounds were detected at m/z 58, 86, 104, 125, 166, and 184, which are derived from the phosphocholine chain as shown in Figures 7 and 8. Negative ion FAB mass spectra obtained with all of the compounds did not show peaks due to $(M - H)^-$, but each did show an intense peak attributed to $MH^+ - CH_3 - H$ (data not shown). The negative ion spectra also showed intense peaks attributed to the anions of the fatty acid moieties. These characteristic fragmentation patterns obtained from positive and negative ion FAB mass spectra as well as protonated molecular ion peaks from all of the compounds established the structure of

Table I: Time Course of Ouabain-Displacing Activities during Saline Infusion[a]

	infusion period			
	0 min	30 min	60 min	120 min
peak 2-1	3.27	8.73	35.09	10.96

[a] Ouabain-displacing activities were extracted from plasma samples during time course studies on the effect of saline infusion and partially purified by Amberlite XAD-2 chromatography and then by HPLC on NH_2 and ODS columns. The active fraction was then subjected to the determination of the ouabin-displacing activity. Values are means of two experimental hogs and are expressed as nanograms of ouabain equivalents per milliliter of plasma. The hogs had been kept on dog chow before the infusion.

the purified compounds as γ- or β-acyllysophosphatidylcholines but did not distinguish between them. From the results obtained from both ^1H NMR and FAB mass spectrometric studies, the purified compounds were identified as follows: P2-1Aα1, β-arachidoyllysophosphatidylcholine [LPCA(β)]; P2-1Aα2, mixture of γ-arachidoyl- [LPCA(γ)] and γ-linoleoyl- (LPCL) lysophosphatidylcholine (1:1); P2-1Aβ1, LPCA(γ); P2-1Aβ2, LPCL; P2-1Bα, mixture of γ-palmitoyl- (LPCP) and γ-docosapentaenoyl- (LPCD) lysophosphatidylcholine (1:1); P2-1Bβ, mixture of LPCP and γ-eicosatrienoyllysophosphatidylcholine (LPCE) (1:1); P2-1C, γ-oleoyllysophosphatidylcholine (LPCO). The quantities of the LPC's recovered from the present experiment were corrected for their recovery and expressed as nanomoles per milliliter of the plasma as follows: LPCA(β), 1.3; LPCA(γ), 10.1; LPCL, 9.8; LPCO, 7.6; LPCP, 0.7; LPCD, 0.4; LPCE, 0.2. These LPC's accounted for approximately 48% of total ouabain-displacing activity in plasma collected after 120 min of saline infusion.

Relationship between Lysophosphatidylcholines and Acute Saline Infusion. In order to assess the relationship between LPC's and extracellular fluid volume expansion, the total ouabain-displacing activity under P2-1 was measured at time intervals during saline infusion. Two hogs kept on regular dog chow were used. Preinfusion plasma collected at time zero

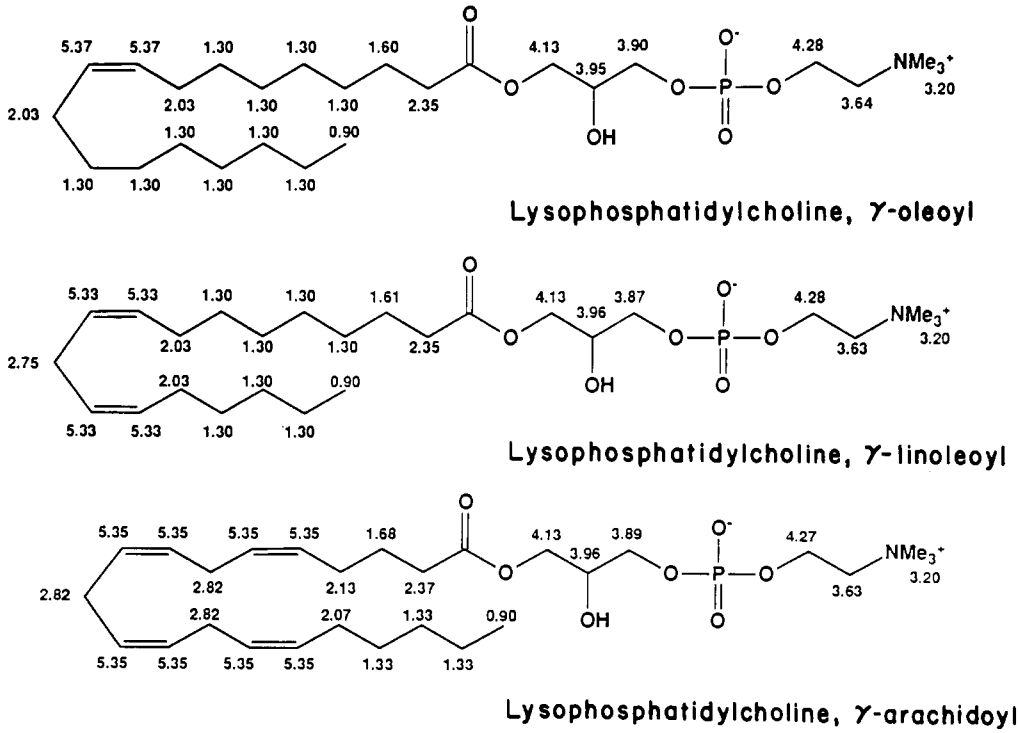

FIGURE 6: Assignment and chemical shifts of purified β- (and γ-) arachidoyl-, γ-linoleoyl-, and γ-oleoyllysophosphatidylcholines. Chemical shifts are referenced to the residual CD_2HOD signal in the solvent at 3.30 ppm.

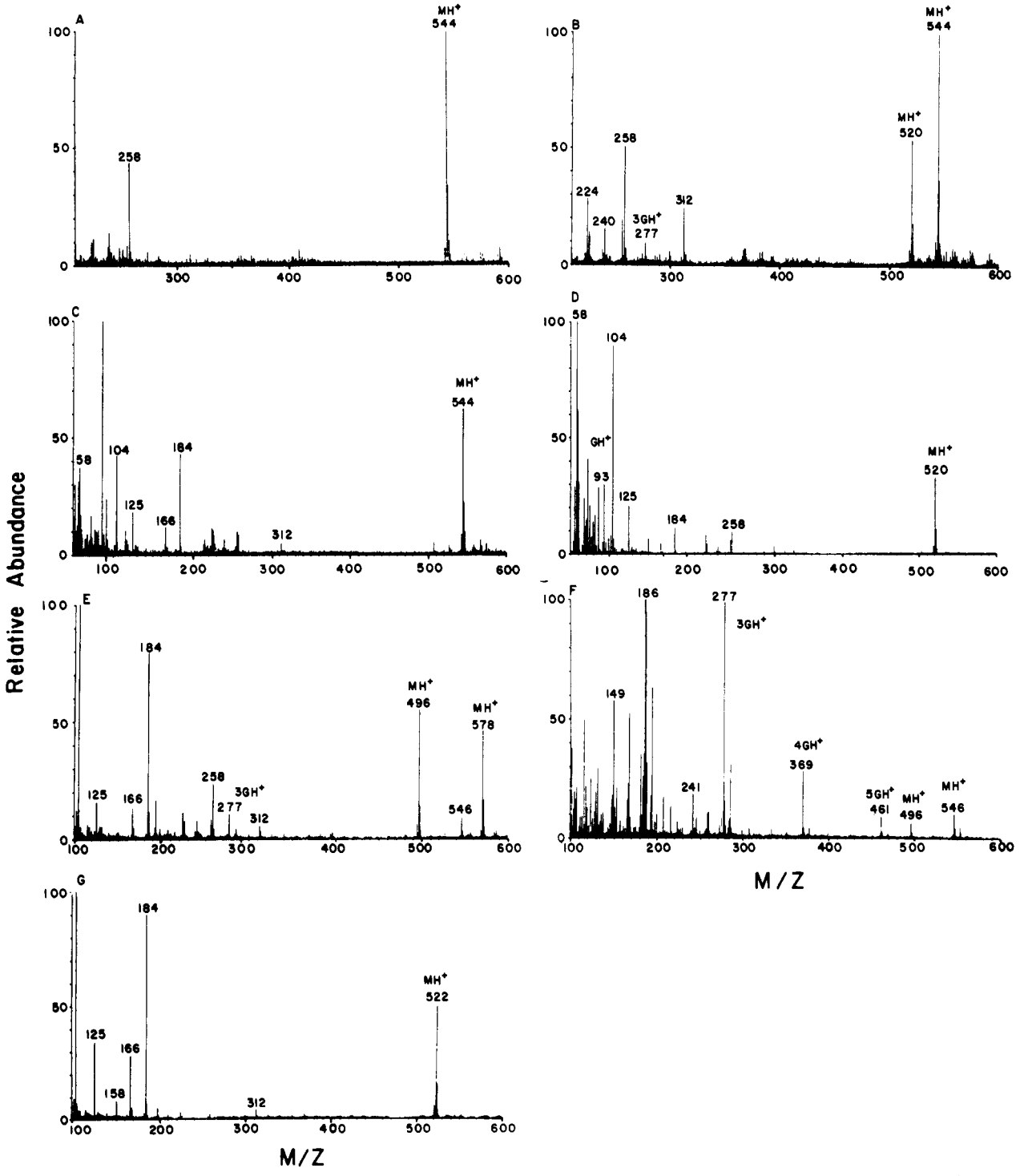

FIGURE 7: FAB mass spectrum of the purified substances: (A) P2-1Aα1; (B) P2-1Aα2; (C) P2-1Aβ1; (D) P2-1Aβ2; (E) P2-1Bα; (F) P2-1Bβ; (G) P2-1C. The ions at m/z 461, 369, 277, 185, and 93 are respectively protonated penta-, tetra-, tri-, di-, and monoglycerol ions from the matrix employed in FAB mass spectrometry.

was used as a control. As shown in Table I, the ouabain-displacing activity in P2-1 increased during the first 60-min period of saline infusion. The increased level was approximately 11 times higher than that of the preinfusion level. The ouabain-displacing activity in P2-1 began to decrease after 120 min of saline infusion.

Characterization of the Inhibitors. The inhibitory potencies of the purified compounds LPCA(β), LPCA(γ), LPCL, and LPCO against Na^+,K^+-ATPase activity, ouabain binding to the enzyme, ^{86}Rb uptake activity, and digoxin binding to anti-digoxin antibody were compared (Figures 9 and 10). In the study of Na^+,K^+-ATPase inhibitory activity, LPCA(γ), LPCL, and LPCO at 1 mM almost completely inhibited the ouabain-sensitive Na^+,K^+-ATPase (Figure 9A). In contrast, LPCA(β) was not inhibitory, even at 1 mM. The enzyme activity was increased by addition of LPCA(β) to 0.4–0.8 mM. The maximal increase in the activity was approximately 40%. Under the present conditions, a 50% inhibition of the ATPase activity was obtained at 286 μM LPCA(γ), 59 μM LPCL, and 105 μM LPCO. Ouabain inhibited this enzyme with an ID_{50} of 0.8 μM (Figure 9A).

The binding of [^{3}H]ouabain to the rat brain ATPase preparation was also completely inhibited at 1 mM LPCA(γ), LPCL, and LPCO. However, LPCA(β) did not affect this receptor binding at 1 mM. A 50% inhibition was observed at 521 μM LPCA(γ), 257 μM LPCL, and 336 μM LPCO

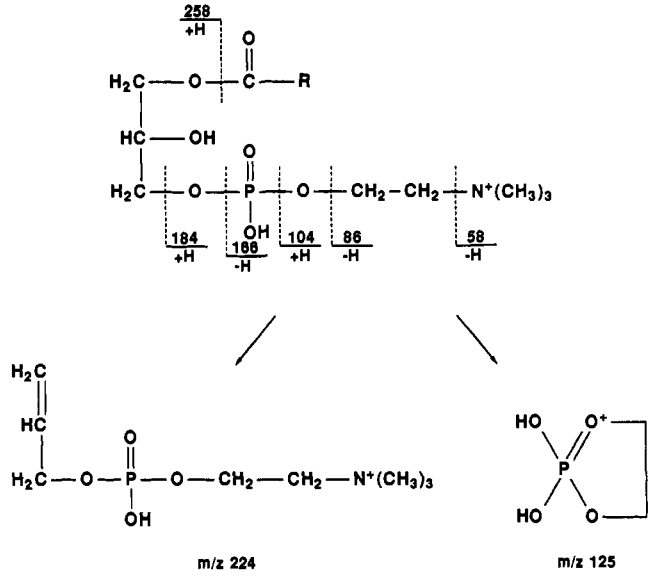

FIGURE 8: Sequence information derived from the fragmentations observed in the FAB mass spectrum (Figure 7) of the purified lysophosphatidylcholines.

(Figure 9B). Under these conditions, 50% binding of radioactive ouabain was displaced by 35 nM unlabeled ouabain.

In the study of ^{86}Rb fluxes into hog erythrocytes, LPCA(γ), LPCL, and LPCO showed concentration-dependent inhibition (Figure 9C). LPCA(β) showed negligible inhibition of ^{86}Rb influx even at high concentrations. Hemolysis was observed at higher concentration (over 400 μM) for all of the compounds. A 50% inhibition was observed at 11 nM ouabain without any cytolytic effect on the red blood cells. Approximately 80% of ^{86}Rb uptake flux was inhibited at 0.1 μM ouabain.

None of the purified compounds inhibited the binding of [^{3}H]digoxin to specific rabbit anti-digoxin antibody (Figure 10). Instead, increased binding of [^{3}H]digoxin to these compounds was observed for all of the purified compounds.

Discussion

We had demonstrated that there are at least two groups of humoral Na^+,K^+-ATPase inhibitors in acutely volume-expanded hog and that one of these is unsaturated fatty acids (Tamura et al., 1985). However, approximately half of the inhibitory activity in plasma has eluded attempts for its isolation and identification. We now extend these studies to the identification of the other type of Na^+,K^+-ATPase inhibitor in hog plasma that emerged upon acute expansion of extracellular fluid volume by saline infusion.

Several tissues were used as a source for the purification and characterization of Na^+,K^+-ATPase inhibitors (Gruber & Buckalew, 1978; Haupert & Sancho, 1979; Raghavan & Gonick, 1980; Crabos et al., 1984; Cloix et al., 1985; Fagoo & Godfraind, 1985; Kramer, 1985; Morgan, 1985). It is likely that the humoral inhibitors have partial structural similarity to cardiac glycosides such as digitalis and ouabain, since the inhibitors displace ATPase-bound ouabain (Lichtstein & Samuelov, 1980; Crabos et al., 1984) and cross-react with anti-digoxin antibody (Gruber et al., 1980; Kojima, 1984; Kelly et al., 1985). It is also likely that its physicochemical characteristics are similar to cardiac glycosides (Kelly et al., 1985). Accordingly, a strategy for the extraction and purification of steroids and their conjugates was employed in this study as well as previous studies (Tamura et al., 1985, 1987; Tamura & Inagami, 1986). While the possibility is not completely excluded that minor inhibitory activities might be discarded

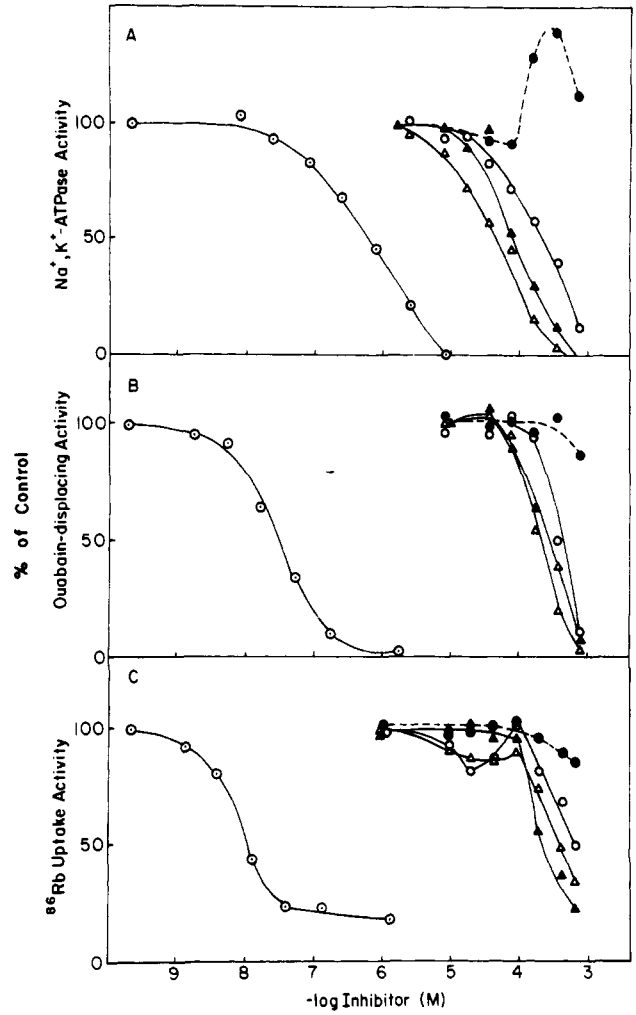

FIGURE 9: Inhibitory effect of purified LPCA(γ) (○), LPCA(β) (●), LPCL (△), and LPCO (▲) on Na^+,K^+-ATPase activity (A), on binding of [^{3}H]ouabain to Na^+,K^+-ATPase (B), and on ^{86}Rb uptake into hog erythrocytes (C). Dose–response curves of these activities were determined in comparison to ouabain (⊙) under the same assay conditions. Each point is the mean of duplicate determinations in two separate experiments and expressed as the percentages of maximal Na^+,K^+-ATPase activity, maximal binding of [^{3}H]ouabain to Na^+,K^+-ATPase, and maximal ^{86}Rb uptake activity.

in the early stage of purification, a steroid or steroid-like compound was not found as the major inhibitory component. Instead, a series of very unusual lysophosphatidylcholines (LPC's) containing long-chain polyunsaturated fatty acids, predominantly γ-arachidoyl or γ-linoleoyl, were found, which represent a new group of inhibitors. In addition, small amounts of LPC's with saturated fatty acids such as stearic (Tamura & Inagami, 1986; Tamura et al., 1987) and palmitic acids (this study) were also isolated (Figure 1). The ouabain-displacing activity due to these LPC's under P2 corresponds to approximately 95% of total activity in plasma before saline infusion and 48% of the total activity after 120 min of saline infusion. The remainder is accounted for by free fatty acids.

P2, which eluted at a retention time of 39 min by aminopropyl HPLC, produced two active peaks upon further purification by ODS HPLC (Figure 2). The second peak (P2-2) had been purified and identified as γ-stearoyllysophosphatidylcholine (Tamura & Inagami, 1986; Tamura et al., 1987). P2-1, with a retention trime of 26–40 min, yielded several active peaks on HPLC using a C-8 column with large pore size packing. This HPLC made it possible to isolate a series of LPC's. Additional purification of these LPC's was performed by Zorbax aminopropyl HPLC, and a series of

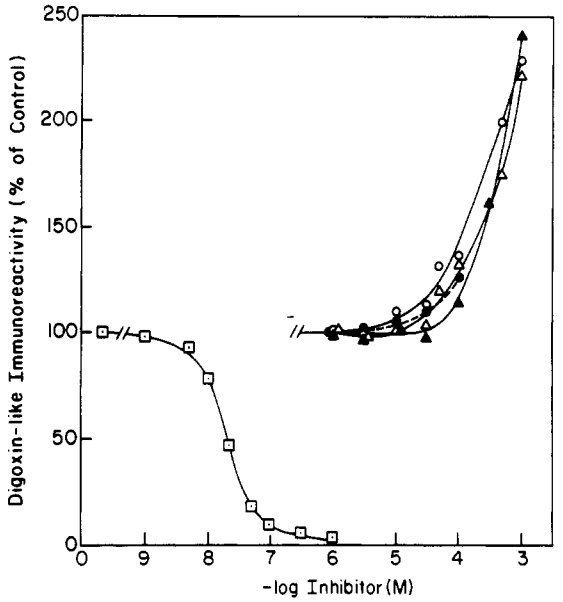

FIGURE 10: Cross-reactivity of purified LPCA(γ) (○), LPCA(β) (●), LPCL (△), and LPCO (▲) with anti-digoxin antibodies. Dose–response curves of the activities were determined in comparison to digoxin (▣) under the same assay conditions. Each point is the mean of duplicate determinations in two separate experiments and expressed as the percentage of maximal binding of [^{3}H]digoxin to anti-digoxin antibody.

LPC's were subsequently identified. In the identification procedures, FAB mass spectrometric and ^{1}H NMR studies indicated that four of seven purified activities (P2-1Aα1, P2-1Aβ1, P2-1Aβ2, and P2-1C) were homogeneous since only one protonated molecular ion peak was observed for each in the FAB mass spectrum and the locations, areas, and multiplicities of signals in the ^{1}H spectra were consistent with the materials being single substances. The remaining three purified materials (P2-1Aα2, P2-1Bα, and P2-1Bβ) were a mixture of PLC's. While these three samples were not pure enough for complete structural analysis by ^{1}H NMR, combined FAB mass and ^{1}H NMR spectrometric studies suggest that these three active substances consisted of LPC's containing long-chain fatty acids (22:5, 20:3, 18:2, and 16:0) in which all acyl moieties were linked to the γ-hydroxyl group of the glycerol moiety. Although it is assumed that all double bonds are in cis configurations, this point was not established except for arachidoyl compounds. In the case of the γ-arachidoyl compound, a comparison of the spectrum with that of arachidonic acid itself (in $CDCl_3$) showed very good agreement in peak location, size, and multiplicity. Therefore, all double bonds of arachidoyl compound were considered to be cis. Although the cis–trans configuration of the fatty acid moieties of the other purified compounds have not been determined, they also are presumed to be cis on the basis of comparisons with authentic fatty acids and also on the basis of existing knowledge that most of the double bonds of naturally occurring fatty acids are in the cis configuration.

It is noteworthy that γ-acyllysophosphatidylcholines containing long-chain polyunsaturated fatty acids were discovered to emerge in the hog plasma by acute extracellular volume expansion. In addition, this is, to the best of our knowledge, the first identification and characterization of arachidoyllysophosphatidylcholine. Generally, fatty acids linked to the γ-position of the glycerol backbone of phosphatidylcholine are saturated while those at the β-position are unsaturated. Phosphatidylcholine is considered to be a parent molecule for the LPC. Furthermore, most fatty acid moieties in LPC's are known to be long-chain saturated fatty acids. This study indicates that most of the LPC's purified from P2 are γ-acyl-LPC's and fatty acid moieties are predominantly polyunsaturated fatty acids (98% of P2-1 fraction and 92% of P2 fraction). It is unlikely that β-acyl-LPC's were chemically shifted to γ-acyl-LPC's during purification and storage for the following reasons. First, freshly prepared plasma was used for the purification. Second, organic solvents, mainly methanol, were used under N_2 gas during storage. Finally, the purification of P2-1 fractions was repeated in as short a time as possible, but only γ-acyl compounds were detected again by ^{1}H NMR analysis. However, the possibility that β-acyl-LPC's underwent a rapid transformation to γ-acyl-LPC's at a very early stage after their formation cannot be excluded.

Aside from the structural peculiarity, the ratio of unsaturated/saturated fatty acids of LPC's recovered from these experiment was unusual. Approximately 38% of the purified compounds were arachidoyl-LPC, and linoleoyl- and oleoyl-LPC's accounted for 33 and 25%, respectively. Palmitoyl- and stearoyl-LPC's accounted for 2 and 6%, respectively. However, it is unlikely that most of the LPC's containing saturated fatty acids had failed to be recovered during the extraction and purification procedures since a certain amount of LPCP and LPCS were recovered from the active P2 fraction. It was difficult to separate LPC's with saturated and unsaturated fatty acids from each other by the methods of organic solvent extraction and chromatography employed in the early steps of this purification. Thus, it is apparent that further study is necessary for the evaluation of the physiological significance of these LPC's. On the other hand, we can only conjecture as to the origin of these lysophospholipids. The characteristic fatty acid compositions of lipoprotein–lipid conjugates and plasma membranes of platelets suggest that LPC's rich in polyunsaturated fatty acids might be derived from these sources. It has been reported that the percentage of arachidonic acid in phospholipids of the lipoprotein–lipid conjugates (10%) and in the total lipids of platelets (24%) is much higher than those of other tissues such as vascular and adipose tissues (Picard et al., 1972; Safrit et al., 1972).

The observations that a series of purified LPC's, except LPCA(β), showed Na^+,K^+-ATPase inhibitory activity as well as ouabain-displacing activity are in agreement with our previous finding (Tamura & Inagami, 1986; Tamura et al., 1987) and that of Bidard et al. (1984), who reported that a commercially obtained mixture of LPC's, which consists mainly of saturated fatty acids, inhibits Na^+,K^+-ATPase and displaces ouabain from the ATPase. However, the concentrations exhibiting 50% inhibition in both ouabain-binding and Na^+,K^+-ATPase activity obtained in this study were higher than that from their experiment. This discrepancy could be explained by the difference of the Na^+,K^+-ATPase employed in the two studies. Indeed, the inhibitory potencies observed with the purified canine kidney Na^+,K^+-ATPase were approximately 10 times higher than those with the membrane preparation (our unpublished data). Although the LPC's [LPCA(γ), LPCL, and LPCO] purified in this study showed inhibition of ^{86}Rb uptake into hog red cells in dose-dependent manners, they also showed hemolysis at concentrations above 400 μM. Therefore, it is not clear whether the inhibition of ^{86}Rb uptake is due to hemolysis. It is also not clear whether the effects of LPC's in this range (400 μM) is of physiological significance since this concentration is very high. However, the ^{86}Rb uptakes were slightly inhibited at lower concentrations (10–40 μM) of LPC's with γ-arachidoyl and γ-linoleoyl whereas LPC with oleoyl did not show any effect at this concentration. These observations suggest that LPC's with

polyunsaturated fatty acids are different from LPC's with unsaturated fatty acids containing one double bond in terms of Na^+,K^+ pump inhibition due to LPC.

The finding that LPCA(β) does not inhibit any of these three activities at a similar concentration range to the other three compounds suggests that the linkage of fatty acid to the γ-position of the glycerol backbone is essential for the inhibition of Na^+,K^+ pump and Na^+,K^+-ATPase activities and ouabain binding to the enzyme. On the other hand, the observation that Na^+,K^+-ATPase activity was stimulated at high concentration of LPCA(β) whereas the ouabain binding was not affected at the same concentration may indicate that the interaction of LPC's and Na^+,K^+-ATPase is not simple competitive binding to the cardiac glycoside binding site in the enzyme. Indeed, the dose-dependent inhibitions of Na^+,K^+-ATPase by the purified γ-acyl-LPC's occurred invariably at lower LPC concentrations than those required for the inhibitions of ouabain binding to the enzyme. It was also observed that Ca^{2+},Mg^{2+}-ATPase including basal Mg^{2+}-ATPase activity is slightly inhibited at high concentration of LPC's (approximately 10% of the activity was inhibited by 400 μM γ-acyl-LPC's), whereas ouabain did not affect these ATPase activities under the same assay condition. In addition, the [^{3}H]ouabain displacement curves obtained with authentic unlabeled ouabain and LPC's were not parallel. These phenomena also suggest that inhibition of the Na^+,K^+ pump by LPC's is not by the same mechanism with ouabain.

However, for pathogenesis of hypertension and for the explanation of the Na^+ retention in the volume-dependent type of hypertension, LPC's can still be considered as candidates for endogenous inhibitors of Na^+,K^+-ATPase under certain pathophysiological conditions. In this relation it is worth pointing out that ^{86}Rb-uptake activities of hog erythrocytes were decreased after 120 min of saline infusion, and this decrease was presumably due to a membrane abnormality (our unpublished observation). It was also observed that a similar inhibitory activity increased in rat brain by Na^+ loading caused by prolonged drinking of 1.7% NaCl solution (our unpublished observation). Together with the present finding of an increase in abnormal LPC's in plasma after saline infusion, these observations suggest that LPC's with polyunsaturated long-chain fatty acids may be implicated in the modulation of the development of certain types of hypertension through their inhibition of a certain membrane-bound enzyme(s). Indeed, LPC has been implicated in certain pathological conditions including cardiac arrythmia subsequent to the development of cardiac ischemia (Sobel et al., 1978; Corr et al., 1982). During the ischemia, LPC was found to increase significantly in cardiac tissue. The accumulation of LPC has been observed in the region of atherosclerosis (Andreoli & Cazzulo, 1969; Portman & Alexander, 1969). LPC was also implicated in the modulation of several membrane enzymes (Fiscus & Schneider, 1966; Jones & Wakil, 1967) including Ca^{2+},-Mg^{2+}-ATPase (Martonosi et al., 1968). These observations support the hypothesis that LPC's play an important role in the modulation of membrane-bound enzymes. In contrast to the generation of LPC's in tissues as cited above, the present observation deals with increases in Na^+,K^+-ATPase inhibitory activity in plasma largely due to LPC. However, the number of animals tested is just two, and plasma concentrations of phospholipids as well as fatty acids in pre- and postinfusion animals were not individually determined in this study. Therefore, precise quantification of changes in plasma lipids and clarification of the pathophysiological role of LPC's in plasma will require further studies.

In regard to the specificity of LPC's, it is worth pointing out that the ouabain-displacing and Na^+,K^+-ATPase inhibitory activities have not been observed with other lysophosphatidylic acids such as lysophosphatidylethanolamines (Bidard et al., 1984). These observations indicate that the actions of LPC's on Na^+,K^+-ATPase is not simple nonspecific actions of lysophosphatidic acids in general. On the other hand, the ATPase activity is strongly modulated by certain types of detergents. This modulation has been shown to be affected by monovalent cations such as Na^+ and K^+ as well as substrate concentration (Huang et al., 1986). A variety of detergents have been found to inhibit or stimulate Na^+,K^+-ATPase under different conditions (Huang et al., 1986). It is possible that the action of LPC's on Na^+,K^+-ATPase may also involve the site where the detergents bind to the enzyme. The finding that LPC's with long-chain polyunsaturated and saturated fatty acids inhibit Na^+,K^+-ATPase and displace ouabain from the ATPase suggests that part of endogenous ouabain-like activity in plasma could be attributable to the LPC's. However, LPC did not cross-react with digoxin antibody even at millimolar concentrations. The lack of immunoreactivity with digoxin antibody is reasonable in view of vast structural differences between LPC and digoxin. The apparent increase of the binding of [^{3}H]digoxin to antibodies in the higher concentration range of LPC's may be due to interference of the lipids to the radioimmunoassay system.

LPC did not show ouabain-displacing activity and Na^+,K^+-ATPase inhibitory activity when a high concentration (40 mg/mL) of bovine serum albumin (fraction V, Sigma) was added in the assay system. This phenomenon is in agreement with a recent observation by Kelly et al. (1985) in which Na^+,K^+-ATPase inhibitory activities in all three active fractions from human plasma separated by HPLC on an ODS column were diminished by bovine or human serum albumin. It is well-known that most of LPC's, as well as phosphatidylcholine, are bound to plasma protein under physiological conditions. Binding by protein will reduce the effective concentration of free LPC's in plasma available for the inhibition of the ATPase. This is important in view of the ubiquity and key physiological roles of the ATPase. This binding may also protect various tissues from cytolytic action of LPC. Accordingly, it would be of interest to know whether plasma LPC levels increase in chronic renal failure where plasma Na^+,K^+-ATPase inhibitor activity has been reported to increase (Mayer et al., 1986). Whether LPC's will play pathological or physiological roles in the regulation of Na,K-ATPase is still unclear and requires further investigation.

A series of these studies indicate the multiplicity of the ouabain-displacing activity in the plasma of volume-expanded animals. Two groups of the components were separated by HPLC on an aminopropyl column. One of them has been found to consist of unsaturated fatty acids (Tamura et al., 1985). The other yielded two peaks upon HPLC with an ODS column. One of them has now been identified as LPC's with long-chain polyunsaturated fatty acids and palmitic acid. The other one has been identified as γ-stearoyllysophosphatidylcholine (Tamura & Inagami, 1986; Tamura et al., 1987). Further efforts are being made to evaluate the pathophysiological significance of these compounds. While this paper was in review, a paper by Kelly et al. (1986) appeared that reported observation of mass spectrometric signals corresponding to palmitoyl-LPC as a component in a peak fraction with Na^+,K^+-ATPase inhibitory activity isolated by HPLC from normal human plasma. These authors have also identified various free fatty acids in fractions exhibiting Na^+,K^+-ATPase

inhibitory activity. Our present identification of γ-palmitoyl-LPC as a component of the Na^+,K^+-ATPase inhibitory substance in plasma confirms their finding, even though the inhibitory activities were not completely purified to homogeneity in their study. The identification of free fatty acids by them is in agreement with our earlier observation (Tamura et al., 1985) that unsaturated free fatty acids account for a sizable portion of Na^+,K^+-ATPase inhibitory activity in plasma. The present identification of LPC's with unsaturated fatty acids as another group of the ATPase inhibitors and that their concentrations are increased upon plasma volume expansion in experimental animals further extend our knowledge on the plasma ATPase inhibitors.

Registry No. LCPA(γ), 63163-02-0; LPCL, 5655-12-9; LPCO, 3542-29-8; LPCA(β), 67341-29-1; LPCP, 14863-27-5; LPCD, 107557-99-3; LPCE, 107558-00-9; ATPase, 9000-83-3.

REFERENCES

Andreoli, V. M., & Cazzullo, C. L. (1969) *Life Sci. 8* (*Part 2*), 327–334.

Bidard, J.-N., Rossi, B., Renaud, J.-F., & Lazdunski, M. (1984) *Biochim. Biophys. Acta 769*, 245–252.

Buckalew, V. M., Jr., & Nelson, D. B. (1974) *Kidney Int. 5*, 12–22.

Castaneda-Hernandez, G., & Godfraind, T. (1984) *Clin. Sci. 66*, 225–228.

Cloix, J.-F., Crabos, M., Wainer, I. W., Ruegg, Y., Seiler, M., & Meyer, P. (1985) *Biochem. Biophys. Res. Commun. 131*, 1234–1240.

Corr, P. B., Snyder, D. W., Lee, B. I., Gross, R. W., Keim, C. R., & Sobel, B. E. (1982) *Am. J. Physiol. 243*, H187–H195.

Crabos, M., Wainer, I. W., & Cloix, J.-F. (1984) *FEBS Lett. 176*, 223–228.

Diamandis, E. P., Papanastasiou-Diamandi, A., & Soldin, S. J. (1985) *Clin. Biochem. (Ottawa) 18*, 48–55.

Fagoo, M., & Godfraind, T. (1985) *FEBS Lett. 184*, 150–154.

Fiscus, W. G., & Schneider, W. C. (1966) *J. Biol. Chem. 241*, 3324–3330.

Grault, M. H., Vasdev, S. G., Longerich, L. L., Fernandez, P., Prabhakaran, V., Dawe, M., & Maillet, C. (1983) *N. Engl. J. Med. 309*, 1459.

Graves, S. W., & Williams, G. H. (1984) *J. Clin. Endocrinol. Metab. 59*, 1070–1074.

Gruber, K. A., & Buckalew, V. M., Jr. (1978) *Proc. Soc. Exp. Biol. Med. 159*, 463–467.

Gruber, K. A., Whitaker, J. M., & Buckalew, V. M., Jr. (1980) *Nature (London) 287*, 743–745.

Hamlyn, J., Ringel, R., Schaeffer, J., Levinson, P. D., Hamilton, B. P., Lowarski, A. A., & Blaustein, M. P. (1982) *Nature (London) 300*, 650–652.

Haupert, G. T., & Sancho, J. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4658–4660.

Huang, W. H., Kakar, S. S., & Askari, A. (1985) *J. Biol. Chem. 260*, 7356–7361.

Huot, S. J., Pamnani, M. B., Clough, D. L., Buggy, J., Bryant, H. J., Harder, D. R., & Haddy, F. J. (1983) *Hypertension (Dallas) 5* (*Suppl. I*), 94–100.

Jones, P. D., & Wakil, S. J. (1967) *J. Biol. Chem. 242*, 5267–5273.

Kelly, R. A., O'Hara, D. S., Canessa, M. L., Mitch, W. E., & Smith, T. W. (1985) *J. Biol. Chem. 260*, 11396–11405.

Kelly, R. A., O'Hara, D. S., Mitch, W. E., & Smith, T. W. (1986) *J. Biol. Chem. 261*, 11701–11711.

Kojima, I. (1984) *Biochem. Biophys. Res. Commun. 122*, 129–136.

Kramer, H. J., Heppe, M., Weiler, E., Backer, A., Liddiard, C., & Klingmuller, D. (1985) *Renal Physiol. 8*, 80–89.

Lichtstein, D., & Samuelov, S. (1980) *Biochem. Biophys. Res. Commun. 96*, 1518–1523.

Martonosi, A., Donley, J., & Halpin, R. A. (1968) *J. Biol. Chem. 243*, 61–70.

Morgan, K., Lewis, M. D., Spurlock, G., Collins, P. A., Foord, S. M., Southgate, F. K., Scanlon, M. F., & Mir, M. A. (1985) *J. Biol. Chem. 260*, 13595–13600.

Pamnani, M. B., Huot, S. J., Buggy, J., Clough, D. L., & Haddy, F. J. (1983) *Hypertension (Dallas) 3* (*Suppl. II*), 96–101.

Picard, J., Veissieve, D., Voyer, F., & Bereziat, G. (1972) *Clin. Chim. Acta 36*, 247–250.

Portman, O. W., & Alexander, M. (1969) *J. Lipid Res. 10*, 158–165.

Raghavan, S. R. V., & Gonick, H. C. (1980) *Proc. Soc. Exp. Biol. Med. 164*, 101–104.

Safrit, H. F., Weiss, H. J., & Phillips, G. B. (1972) *Lipids 7*, 60–67.

Sobel, B. E., Corr, P. B., Robinson, A. K., Goldstein, R. A., Witkowski, F. X., & Klein, M. S. (1978) *J. Clin. Invest. 62*, 546–553.

Tamura, M., & Inagami, T. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 523.

Tamura, M., Kuwano, H., Kinoshita, T., & Inagami, T. (1985) *J. Biol. Chem. 260*, 9672–9677.

Tamura, M., Inagami, T., Kinoshita, T., & Kuwano, H. (1987) *J. Hypertens.* (in press).

Vassallo, P. M., Perez, J. G., Getino, M. A., Marrero, F., & Battaner, E. (1985) *Life Sci. 37*, 835–840.