Calcium Binding to Rat Heart Plasma Membranes: Isolation and Purification of a Lipoprotein Component with a High Calcium Binding Capacity†

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ABSTRACT: A calcium binding lipoprotein component of rat heart plasma membrane was isolated, purified, and characterized. The lipoprotein complex had an apparent molecular weight of 71 400. One mole of lipoprotein contained 90 mol of phospholipid and 1 mol of a 12 300 molecular weight protein. The maximum calcium binding capacity was 4.27 μmol/mg of protein, which corresponded to 52 mol of calcium per mol of lipoprotein complex. Calcium binding was competitively inhibited by a variety of metal ions and experimental antiarrhythmic and anesthetic agents.

Plasma membranes from most mammalian tissues possess single or multiple types of high capacity binding sites for calcium (Feldman and Weinhold, 1977; Hui et al., 1976; Sulakhe et al., 1973; Madeira and Carvalho, 1972; Madeira and Antunes-Madeira, 1973; Hemminki, 1974; Shlatz and Marinetti, 1972; Mikkelsen and Wallach, 1974). Calcium is known to be required for intercellular communication, intercellular adhesion, and monovalent cation transport processes in plasma membrane (Mikkelsen and Wallach, 1974). Calcium binding activity in skeletal and heart muscle plasma membrane may represent an essential component of calcium transport which occurs during excitation–contraction coupling. G. A. Langer has supported a model for excitation–contraction coupling in heart muscle, which proposes that the immediate source of "contractile dependent" calcium is located on external sites of the plasma and T tubular membranes (Langer, 1974, 1976). These concepts have stimulated scientific interest in exploring the chemical and physical nature of muscle cell membranes, in search of isolating high capacity calcium binding components. MacLennan and co-workers have had considerable success in isolating and purifying the functional components of the rabbit skeletal muscle sarcoplasmic reticulum membrane (MacLennan et al., 1971, 1972; Ostwald and MacLennan, 1974; MacLennan, 1975). At least six distinct components have been isolated by membrane extraction with KCl and sodium deoxycholate.

Previous studies from this laboratory (Feldman and Weinhold, 1977) have demonstrated a high calcium binding capacity in rat heart plasma membranes. A large variety of experimental antiarrhythmic and anesthetic agents were shown to competitively inhibit the calcium binding activity. This communication describes the isolation, purification, and characterization of a calcium binding lipoprotein component of rat heart plasma membrane.

Materials and Methods

Female rats (180–250 g) were obtained from Holtzman Co., Madison, Wis. $^{45}CaCl_2$ was purchased from New England Nuclear Corp. (sp act. = 400 Ci/mol). Sodium deoxycholate was purchased from Difco Laboratories, Detroit, Mich. DEAE-cellulose (DE-32) was purchased from Whatman. Agarose (0.5m) was purchased from Bio-Rad Laboratories. Sephadex (G-25) was purchased from Pharmacia Fine Chemicals, Inc. Propranolol (racemic) was obtained from Sigma Chemical Co. The experimental antiarrhythmic and anesthetic drugs (UM 424 [1-dimethylisopropylamino-3-(2-phenylphenoxy)propan-2-ol chloride]; UM 272 [*N,N*-dimethyl-1-isopropylamino-3-(1-naphthyloxy)propan-2-ol]) were synthesized in the laboratory of Dr. R. E. Counsell, Department of Medicinal Chemistry, University of Michigan. The compounds were prepared as iodide salts. All other biochemicals were purchased from Sigma Chemical Co. Precoated silica gel 60 TLC[1] plates were purchased from E. M. Laboratories, Elmsford, N.Y.

Plasma Membrane Isolation. Plasma membranes were prepared from rat heart by methods previously developed in this laboratory (Feldman and Weinhold, 1977). Briefly, the procedure involves homogenization of fresh rat heart in 50 mM $CaCl_2$–10 mM histidine–Tris (pH 7.6). A crude membrane preparation obtained by low-speed centrifugation (270*g*) was washed with 10 mM histidine–Tris (pH 7.6) by repeated suspension and centrifugation (270*g*) until the wash supernatant was colorless. The washed membrane material was suspended in cold deionized water (pH 7.8) and collected by centrifugation at 10 000*g* for 30 min and resuspended in 0.25 M sucrose–10 mM histidine–Tris (pH 7.6). Purification was achieved by centrifugation on a discontinuous sucrose gradient (0.3 M/1.32 M/1.75 M). The plasma membrane fraction was collected at the 1.32 M/1.75 M interface, washed once with 0.25 M sucrose–10 mM histidine–Tris (pH 7.6), and stored as a suspension in the sucrose–histidine solution at −40 °C. This membrane preparation was previously shown to be relatively free from sarcoplasmic reticulum and mitochondrial membranes (Feldman and Weinhold, 1977).

Plasma Membrane Solubilization. Purified plasma membranes were added to 50 mM Tris-HCl (pH 7.6) that contained 2.5 mM (0.1%) sodium deoxycholate and 1.0 M NaCl so that a final protein concentration of 0.4–0.5 mg/mL was obtained.

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[1] Abbreviations used: TLC, thin-layer chromatography; UM 424, 1-dimethylisopropylamino-3-(2-phenylphenoxy)propan-2-ol chloride; UM 272, *N,N*-dimethyl-1-isopropylamino-3-(1-naphthyloxy)propan-2-ol; Tris, tris(hydroxymethyl)aminomethane.

The mixture was homogenized by motor-driven Teflon pestle and stirred for 2 h at 4 °C. The extract was collected by centrifugation at 100 000*g* for 60 min and dialyzed overnight against 25 volumes of 50 mM Tris-HCl (pH 7.6)–2.5 mM deoxycholate.

Calcium Binding Analysis. Calcium binding was measured by equilibrium dialysis using an eight-chamber dialysis block (Bel-Art Model 375), with 0.5-mL volume per half chamber. One side of each chamber was filled with 0.3 mL of 50 mM Tris-HCl (pH 7.6) containing 2.5 mM deoxycholate and plasma membrane protein. The opposite side of each dialysis chamber contained 0.3 mL of $^{45}CaCl_2$ (10 000–15 000 cpm/nmol) dissolved in deionized H_2O at a final concentration of 25–430 μM. In experiments designed to measure the ability of an inhibitor to prevent calcium binding, the agent was added to the $CaCl_2$ side of the dialysis chamber. The final volume of each dialysis cell was 0.6 mL. The two halves of the dialysis block were separated by a single sheet of cellulose dialysis membrane (5 × 5 cm; Bel Art No. 299), previously soaked overnight in deionized water. Dialysis was performed by vertical rotation for 24 h at 4 °C at a constant speed of 10 rpm (Scientific Industries Rotator, Model 150). Duplicate aliquots of 100 μL were removed from each half chamber with a 100-μL Eppendorf pipet and the radioactive ^{45}Ca was measured by liquid scintillation in Aquasol (New England Nuclear).

Calcium binding constants were determined according to Klotz et al. (1946). In this method the inverse amount of calcium bound to 1 mg of protein was plotted vs. the inverse concentration of unbound calcium. Normally, five to eight individual data points were used to construct a single plotted line. Each experimental plot of data points was considered acceptable if the linear correlation coefficient was greater than 0.95, as calculated by the least-squares method of linear analysis. Calcium binding inhibition analysis was performed according to the slope intercept method described by Dixon and Webb (1964). The inhibition constant (K_I) was calculated from the interception point of the plotted lines on the calcium concentration axis, according to the equation $K_I = [I]/(K_{APP} \div K_D) - 1$. For the experiments described in this communication, the maximum acceptable deviation from a true interception of the least-squares lines was ±5%.

Control experiments in the absence of protein revealed that calcium equilibrium was established after 17 h. In the presence of 10 μg/mL lipoprotein sample, maximal calcium binding was established after 20 h and was maintained for as long as 36 h. Equivalent binding results were achieved when calcium was initially added to the protein side of the dialysis cell. The amount of calcium bound to the lipoprotein (at 343 μM $CaCl_2$) is proportional to protein concentration up to 15 μg/mL. An average of 4.0% variability in sampling precision was observed with the Eppendorf 100-μL pipet. Therefore, all calcium binding measurements were conducted at 10–15 μg of protein/mL to maintain the amount of bound calcium equal to 20–40% of the amount of unbound calcium, minimizing the effect of Eppendorf pipet variability.

The calcium content of the membrane protein samples, inhibitor solutions, and 50 mM Tris-HCl buffer (pH 7.6) containing 2.5 mM deoxycholate was measured by atomic absorption spectrophotometry (Perkin-Elmer Model 306), at 422 nm in the presence of 10 mM $LaCl_3$. The Tris-HCl buffer with deoxycholate contained 21.2 μM calcium, which would contribute a net increase of 10.6 μM at dialysis equilibrium. This concentration was added to the calculation of calcium specific radioactivity for all binding experiments, although it produced only a 2% change in the calcium K_D value of the purified lipoprotein. Quadruplicate measurements of the calcium content of the various membrane protein fractions were made after digestion with 70% $HClO_4$ for 2 h at 180 °C. No significant calcium content was detected above that contributed by the Tris-HCl–deoxycholate buffer. The concentration of calcium measured in the metal ion inhibitor solutions (KCl, NaCl, $MgCl_2$) contributed a maximum of 5 μM calcium at the highest concentrations of inhibitor (100 mM).

Chemical Analysis. Protein was estimated by the method of Lowry et al. (1951). When protein samples contained deoxycholate, the modification of Bensadoun and Weinstein (1976) was adopted. Membrane phospholipids were extracted by the method of Folch et al. (1957). Total phospholipids were extracted into 20 volumes of chloroform–methanol (2:1 v/v) and washed by gel filtration on Sephadex G-25, as described by Radin (1967). Organic phosphorus was estimated as described previously by Weinhold and Villee (1965). Sialic acid was estimated by the method of Jourdian et al. (1971).

Phospholipid Analysis. Phospholipids were separated by thin-layer chromatography (TLC) according to the general procedure of Skipsky et al. (1964). Precoated silica gel-60 TLC plates were developed in a solvent mixture containing chloroform–methanol–acetic acid–water (25:15:4:0.5 by volume). The individual phospholipids were visualized by exposure to iodine vapor and identified by comparison with standards applied to the same TLC plate. The amount of phospholipid was determined by measuring organic phosphorus.

Molecular Weight Analysis. Lipoprotein molecular weight was determined by gel filtration on Agarose 0.5m. Stokes radii were calibrated according to Siegel and Monty (1966). The stokes radius value was converted to the corresponding molecular weight for a globular protein. The locations of bovine serum albumin and ovalbumin were determined by ultraviolet absorption at 280 nm. Cytochrome *c* was measured by visible absorption at 540 nm.

Apoprotein Molecular Weight Analysis. Apoprotein molecular weight was estimated by dodecyl sulfate–polyacrylamide gel electrophoresis, using Bio-Phore (Bio-Rad) precast gels (7.5% acrylamide, 5.5 × 100 mm). A Tris-acetate–dodecyl sulfate buffer (0.2 M/0.2 M/0.1%, pH 6.6) was used. The protein was localized by staining with Coomassie brillant blue R-250, and the molecular weight calibration was performed according to Dunker and Kenyon (1976).

Results

Lipoprotein Purification. The membrane extraction procedure was designed to achieve maximal solubilization of extrinsic and intrinsic membrane proteins. Approximately 70% of the membrane protein was extracted in the presence of 2.5 mM deoxycholate and 1.0 M NaCl. A typical DEAE-cellulose elution profile for the plasma membrane extract is shown in Figure 1. Calcium binding analyses (Table I) revealed that a protein species exhibiting a high maximal binding capacity was eluted after 1.0 M NaCl was applied to the column. Fraction IV (B_{max} = 826 nmol/mg protein) was collected and further purified by agarose gel filtration. The elution profile of DEAE-cellulose fraction IV on agarose 0.5m is shown in Figure 2. A high concentration of organic phosphorus eluted simultaneously with protein fraction II, about 40 mL past the void volume.

A summary of the results of the lipoprotein purification (agarose fraction II) is presented in Table II. Calcium binding analysis revealed a high maximal binding capacity (B_{max} = 4.27 μmol/mg protein) coupled with a relatively high calcium

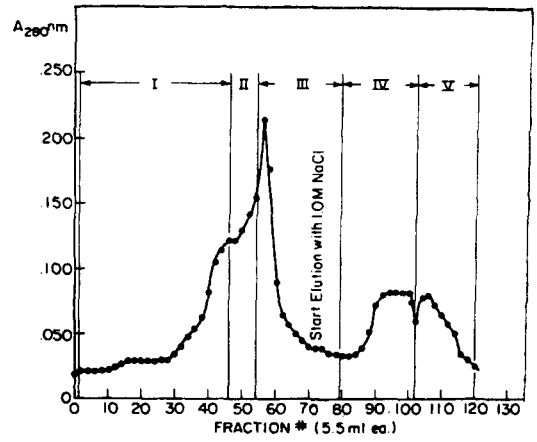

FIGURE 1: DEAE-cellulose elution profile of deoxycholate extract from rat heart plasma membrane. A sample of the dialyzed deoxycholate extract of rat heart plasma membrane was prepared as described under Materials and Methods. Approximately 270 mg of protein (1.5 mg/mL) was applied to a DEAE-cellulose column (1.75 × 47 cm) previously equilibrated with 50 mM Tris-HCl at pH 7.6 (Tris buffer). The column was washed with 200 mL of Tris buffer followed by a 300-mL linear gradient of NaCl (50 to 500 mM) prepared in Tris buffer. Two column volumes of 1.0 M NaCl was then required to elute the lipoprotein complex (fraction IV). All the fractions were concentrated by ultrafiltration (Amicon PM-10 filter) and dialyzed against 1000 volumes of Tris buffer containing 2.54 mM deoxycholate. Precipitated material was removed by centrifugation.

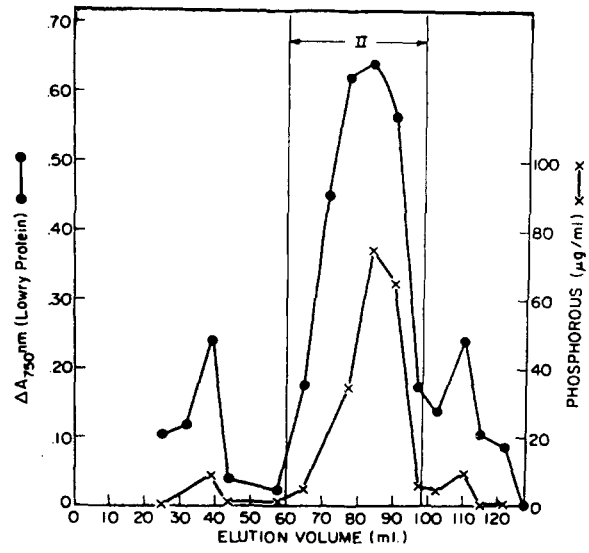

FIGURE 2: Agarose 0.5m elution profile of lipoprotein component from rat heart plasma membrane. A sample of DEAE-cellulose fraction IV containing 4.2 mg of protein (0.66 mg/mL) was applied to a column of agarose 0.5m (1.65 × 85 cm) previously equilibrated with Tris buffer containing 2.54 mM deoxycholate. The protein elution profile was measured by the method of Lowry et al. (1951) without conversion to actual protein concentration. The organic phosphorus elution profile was measured by perchloric acid digestion procedure according to Weinhold and Villee (1965). The lipoprotein which eluted after the void volume (fraction II) was concentrated by ultrafiltration and stored at −40 °C for 2–3 months.

TABLE I: Calcium Binding Analysis of DEAE-Cellulose Fractions.[a]

Fraction	K_D (μM)	B_{max} (nmol/mg of protein)
I	185	32
II	252	52
III	221	69
IV	250	826
V	126	236

[a] The calcium binding activity of the DEAE-cellulose fractions, prepared as described in Figure 1, was determined by equilibrium dialysis as described in Materials and Methods.

affinity (K_D = 74 μM). The total protein recovered as agarose fraction II represents 0.5% of the initial plasma membrane protein. However, this lipoprotein species represents approximately 33% of the calcium binding capacity of the plasma membrane. The lipoprotein contains 7.35 μmol of phospholipid phosphorus per mg of protein. The amount represents almost 15% of the plasma membrane phospholipid. Sialic acid content of the lipoprotein species is considerably higher than found in the plasma membrane preparation (120 nmol/mg protein vs. 18 nmol/mg protein).

Molecular Weight Analysis. A molecular weight analysis of agarose fraction II is presented in Figure 3. The lipoprotein was eluted from an agarose 0.5m column at a position corresponding to a Stokes radius of 33.5 Å and an apparent molecular weight of 71 400. Furthermore, the protein and phospholipid components of the lipoprotein remained firmly associated during the gel filtration procedure, confirming the lipoprotein nature of the complex. The symmetrical shape of the elution profile and the relative lack of contamination by other phospholipid and protein species suggest a relatively high degree of purity. Dodecyl sulfate–polyacrylamide gel electrophoresis revealed the presence of a single, low molecular weight (12 300) protein species (Figure 3). This protein was also present in samples of plasma membrane and in the deoxycholate solubilized extract of plasma membrane. These results support the concept of the plasma membrane origin of the protein. No organic phosphorus was present in the same low-molecular-weight region of a dodecyl sulfate gel prepared with the lipoprotein species, not treated with the Coomassie stain. Thus, the lipoprotein was completely dissociated in the presence of 1.0% dodecyl sulfate to produce a single low-molecular-weight apoprotein species.

Calcium Binding Analysis. Equilibrium dialysis in the presence of deoxycholate was performed to measure the lipoprotein calcium binding activity (Figure 4A). Only one type of calcium binding site was detected within the range of calcium concentrations used in these experiments (50–430 μM). It was determined that *d,l*-propranolol, a β-adrenergic antagonist and antiarrhythmic agent, produces a potent competitive inhibition of calcium binding to the lipoprotein (K_I = 26 μM). Additional inhibition results are presented in Table III. The experimental antiarrhythmic agents UM 424 and UM 272 (Kniffen et al., 1976; Shuster et al., 1973) also cause a competitive inhibition of calcium binding. UM 424 is five times more potent than UM 272, as seen previously in studies of plasma membrane preparations (Feldman and Weinhold, 1977). The monovalent cations Na^+ and K^+ are 33 times less potent inhibitors than the divalent cation Mg^{2+}. This result is also similar to results obtained with the plasma membrane preparation. The lipoprotein is apparently more selective toward Ca^{2+} than Mg^{2+}, in comparison to the plasma membrane, as the Ca^{2+} dissociation constant (K_D = 74 μM) is six times smaller than the Mg^{2+} inhibition constant (K_I = 440 μM). Since all the agents tested produce a direct competitive inhibition of calcium binding, it appears that they may be attracted to the same cation binding site on the lipoprotein.

Phospholipids were extracted from the lipoprotein by the procedure described by Radin (1967). An analysis of the cal-

TABLE II: Purification Analysis of Lipoprotein from Rat Heart Plasma Membranes.[a]

Fraction	Calcium binding (no Mg^{2+}-ATP) K_D (μM)	Calcium binding (no Mg^{2+}-ATP) B_{max} (nmol/mg)	Protein (mg)	Phospholipid (μmol)	Sialic acid (μmol)	Phospholipid/ protein (μmol/mg)	Sialic acid/ protein (μmol/mg)
Plasma membranes	350 ± 61	66.3 ± 1.4	400	97.6	7.14	0.24	17.9
Deoxycholate-soluble extract	244	121	270	90	9.90	0.33	36.7
DEAE-Cellulose fraction IV	250	826	4.4	16.8	ND	3.82	ND
Agarose (0.5m) fraction II	74	4270	1.94	14.25	0.23	7.35	120

[a] The various chemical analyses were performed as described in Materials and Methods.

TABLE III: Inhibition of Calcium Binding to Purified Lipoprotein.[a]

Inhibitor	K_I[b]
d,l-Propranolol	26 μM
UM 424	24 μM
UM 272	120 μM
$MgCl_2$	440 μM
NaCl	14.4 mM
KCl	14.4 mM

[a] Calcium binding activity was measured by equilibrium dialysis (Materials and Methods) with a lipoprotein concentration of 12.9 μg of protein/mL in the sample side of the dialysis chamber. For a competitive inhibitor, K_I represents one-half of the concentration required to produce a 50% inhibition of calcium binding if the free calcium concentration is equal to its K_D concentration. [b] All competitive.

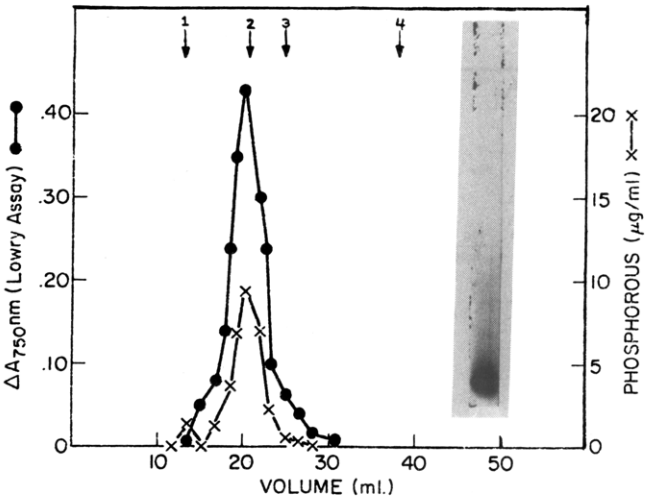

FIGURE 3: Lipoprotein molecular weight measurement by agarose 0.5m gel filtration. Gel filtration was performed in a column (1 × 57 cm) of agarose 0.5m equilibrated in 50 mM Tris-HCl (pH 7.6) containing 2.54 mM deoxycholate. The original sample contained 190 μg of protein and 44 μg of lipid phosphorus (1.42 μmol). The protein and phosphorus elution profiles were measured as described in Figure 2. The agarose column was calibrated in a separate experiment, with the indicated molecular weight markers, using the same type buffer: (1) blue dextran 2000; (2) bovine serum albumin; (3) ovalbumin; (4) cytochrome c. Dodecyl sulfate–polyacrylamide gel electrophoresis of lipoprotein. Samples containing 70–150 μg of protein were pretreated by boiling for 10 min in the presence of 1.0% dodecyl sulfate and 5.0% β-mercaptoethanol. The electrophoresis was performed as described in Bio-Rad Bulletin 1038. The protein band was stained with a solution containing 0.1% Coomassie brillant blue, 8.0% glacial acetic acid, and 25% ethanol.

cium binding activity of the phospholipid extract in the presence of 1.27 mM deoxycholate (Figure 4B) indicated that 2 mol of phospholipid bind a maximum of 1 mol of calcium. The apparent calcium dissociation constant (K_D = 92 μM) is similar to that observed for the lipoprotein (K_D = 74 μM). In order to determine the type(s) of phospholipid species involved in the lipoprotein calcium binding activity, the phospholipid extract was analyzed by thin-layer chromatography (Table IV). The lipoprotein contains a mixture of phospholipid species that is similar to the phospholipid composition of the cardiac tissue homogenate and the plasma membrane preparation. There is an apparent increase in lysophosphatidylethanolamine and lysophosphatidylcholine content of the lipoprotein. Phosphatidylserine represents only 4.4% of the total. Thus, a single type of acidic phospholipid species is not responsible for the calcium binding activity of the lipoprotein.

Discussion

A lipoprotein component of rat heart plasma membrane, with a high calcium binding capacity, has been isolated and purified. The lipoprotein complex has an apparent molecular weight of 71 400, and the apoprotein subunit has a molecular weight of 12 300. The lipoprotein complex contains 7.35 μmol of lipid phosphorus per mg of protein. The maximum calcium binding capacity is 4.27 μmol/mg of protein with an apparent dissociation constant for calcium of 74 μM.

MacLennan and co-workers have previously characterized the protein components of skeletal muscle sarcoplasmic reticulum (MacLennan, 1975; Ostwald and MacLennan, 1974; MacLennan et al., 1972; MacLennan and Wong, 1971). Many of these components interact strongly with calcium and are assumed to be required for the calcium binding and uptake properties of the membrane. Only two of the six or more isolated components are associated with phospholipid. The Ca^{2+}-ATPase contains about 70 molecules of a wide variety of lipids and phospholipids. A low-molecular-weight proteolipid species contains less than 1 mol of phospholipid per 10 mol of protein. It was observed that *neither* of these components possesses a significant calcium binding activity. Calsequestrin (mol wt = 44 000) and the "high affinity" calcium binding protein (mol wt = 55 000) exhibit fairly high maximal binding capacities for calcium (B_{max} = 970 and 450 nmol/mg of protein, respectively). However, MacLennan (1975) reported that neither of these two proteins contains phospholipid.

In recent years, sodium deoxycholate has become widely recognized for its ability to selectively solubilize extrinsic and intrinsic membrane components without causing major conformational changes or protein denaturation. At moderate concentrations, below the critical micelle concentration (4–6 mM), deoxycholate will release lipid–protein complexes from membranes, in the form of mixed detergent–lipid–protein micelles. Only at concentrations above the critical micelle concentration will deoxycholate produce pure detergent–lipid

TABLE IV: Phospholipid Composition of Plasma Membrane and Purified Lipoprotein.[a]

Phospholipid	Homogenate		Plasma membrane		Lipoprotein	
	% total	Sp act.	% total	Sp act.	% total	Sp act.
Phosphatidic acid + cardiolipin	35	17.8	16.8	41	14	1030
Phosphatidylethanolamine	23	11.71	23.5	57.3	16	1178
Phosphatidylserine	3.3	1.68	4.0	9.8	4.4	324
Lysophosphatidylethanolamine	1.1	0.56	7.0	17.1	11.7	861
Phosphatidylcholine	33	16.8	40	97.6	36	2650
Sphingomyelin + lysophosphatidylcholine	3.7	1.89	6.0	14.6	9.5	700

[a] Phospholipid composition was determined by thin-layer chromatography (Materials and Methods). Specific activity = nmol of phosphorus/mg of protein. Each sample contained 5–10 μg of lipid phosphorus. The lysophosphatidylethanolamine fraction may have contained phosphatidylinositol.

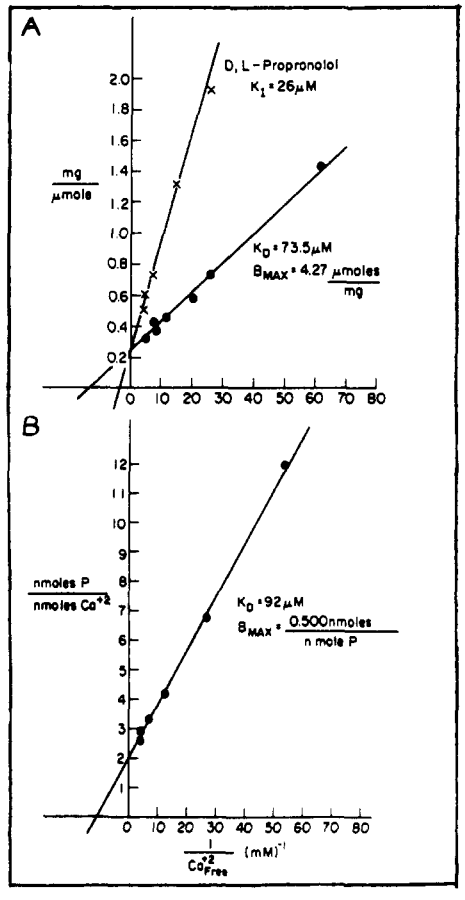

FIGURE 4: Klotz plot analysis of calcium binding to the purified lipoprotein (A). Calcium binding of the lipoprotein was measured by equilibrium dialysis as described under Materials and Methods. The protein concentration was 12.9 μg/mL. Calcium was added within a concentration range of 41.6–416 μM (sp act. = 17 500 cpm/nmol). Klotz plot analysis of calcium binding to phospholipid extracted from the purified lipoprotein (B). A total phospholipid extract of the lipoprotein was prepared as described in Materials and Methods. The chloroform was removed by evaporation under nitrogen at room temperature. The phospholipids were dissolved in 50 mM Tris-HCl (pH 7.6) containing 2.54 mM deoxycholate at a final phosphorus concentration of 0.19 μmol/mL, by a brief sonication at 4 °C. Calcium binding activity was measured as described in Materials and Methods.

and detergent–protein micelles. The concentration of deoxycholate used by MacLennan et al. (1972) was above the critical micelle concentration and thus may have produced soluble membrane proteins with low phospholipid content. Our approach was to select appropriate conditions of pH, ionic strength, and deoxycholate concentration in order to achieve a maximal solubilization of the protein and lipid components, while producing a minimal amount of lipoprotein dissociation. This was achieved by maintaining the concentration of deoxycholate at 2.5 mM, below the critical micelle concentration of 4–6 mM.

A comparison of the calcium B_{max} of the lipoprotein (4.27 μmol/mg of protein) with the B_{max} of the plasma membrane (66 nmol/mg), and assuming that the lipoprotein accounts for approximately 0.5% total protein in the plasma membrane, indicates that the lipoprotein may account for 33% of the calcium binding activity of the plasma membrane. If the calcium binding activity of the phospholipid extract from the lipoprotein can be assumed to represent the calcium binding properties of the phospholipid in the lipoprotein, then the contribution of the phospholipid to the calcium binding activity of the lipoprotein can be calculated. If the apparent phospholipid concentration of 7.35 μmol/mg of protein were to bind a maximum of 0.5 μmol of calcium per μmol of phospholipid (Figure 4B), then the phospholipid component may account for up to 86% of the total calcium binding capacity of the lipoprotein.

Assuming a monomeric molecular weight of 12 300 for the protein component of the lipoprotein (see Figure 4), 1 mg of lipoprotein protein would contain 8.13 nmol of monomeric protein species. Based on a lipid phosphorus content of 7.35 μmol/mg of protein, there appears to be approximately 90 mol of phospholipid per mol of protein monomer. Assuming an average phospholipid molecular weight of 760, 90 mol of phospholipid would equal a molecular weight of 68 400. Based upon a lipoprotein molecular weight of 71 400, as estimated by agarose gel filtration, 1 mol of lipoprotein will likely contain 1 mol of 12 300 molecular weight protein and 90 mol of phospholipid. Based upon the maximum calcium binding capacity of 0.5 mol/mol of lipid phosphorus of the phospholipid extract, 90 mol of phospholipid would bind 45 mol of calcium. Since the phospholipid may account for up to 86% of the calcium binding activity of the lipoprotein, 1 mol of lipoprotein can be calculated to bind 52 mol of calcium. MacLennan (1975) has reported that calsequestrin binds up to 43 mol of calcium per mol of protein, and the 55 000 molecular weight protein binds 25 mol of calcium per mol of protein (MacLennan, 1975). Thus, the molar binding ratio of the lipoprotein from rat heart plasma membrane is similar to the binding ratios of proteins isolated from rabbit skeletal muscle sarcoplasmic reticulum. However, the high calcium binding capacity of the lipoprotein appears to be due to the presence of a high concentration of firmly bound phospholipid.

The accumulated results now available in the scientific literature appear to provide sufficient evidence to support the

concept that "acidic" phospholipid species such as cardiolipin, phosphatidic acid, phosphatidylserine, and possibly the monoacyl forms of phosphatidylcholine and phosphatidylethanolamine may account for the majority of the calcium binding activities of biological membranes (Seimya and Ohki, 1973; Hauser et al., 1975; Tyson et al., 1976). Lansman and Haynes (1975) have proposed that calcium binding to phosphatidic acid in membranes involves the interaction of one calcium ion with two monophosphate groups. Seimya and Ohki (1973) and Hauser et al. (1975) have found similar molar binding ratios for phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine. The maximal calcium binding capacity of 0.5 mol per mol of lipid phosphorus, which was determined for the total phospholipid extract of the lipoprotein, would suggest that calcium interacts equally well with the phosphate ester group of each phospholipid molecule in the extract. However, it is more likely that the molar binding ratio represents an averaged contribution of all the phospholipid species present in the lipoprotein extract.

Papahadjopoulos (1972) has recently reviewed the literature which describe studies of the action of local anesthetic agents on phospholipid model membranes. There is now sufficient evidence to conclude that organic amine local anesthetics can directly inhibit the binding of calcium to "acidic" phospholipid molecules in biological membranes. This action may account for the ability of local anesthetic agents to interfere with muscle cell membrane depolarization, preventing nerve stimulation of cell metabolism (Covino, 1972). The concentrations of the experimental antiarrhythmic and anesthetic agents required to produce a competitive inhibition of calcium binding to the lipoprotein were four to six times lower than those required to inhibit the calcium binding activity of rat heart plasma membrane (Feldman and Weinhold, 1977). The increased inhibitory activity of these agents upon the lipoprotein may be due to the higher phospholipid content of this complex in comparison with the plasma membrane preparation.

The apparent calcium affinity of the lipoprotein complex ($K_D = 74\ \mu M$) may raise some doubt as to its physiological role in the plasma membrane. Intracellular calcium (free) concentrations range from 10^{-8} to 10^{-5} M. This is not compatible with a regulatory function, if the lipoprotein is located on the intracellular side of the membrane. However, the extracellular concentration of calcium may range from 1 to 3 mM. At this concentration, the lipoprotein would be nearly saturated in the absence of any competing cations. Thus if the lipoprotein is located at the extracellular side of the membrane, it could function as a membrane storage site for calcium, prior to transport into the muscle cell. Additional studies are necessary to determine the physiological role of the lipoprotein.

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