Regulation of a Candidate Aminophospholipid-Transporting ATPase by Lipid†

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Received April 27, 1993; Revised Manuscript Received July 29, 1993®

ABSTRACT: The effect of lipid environment on the activation of a vanadate-sensitive Mg^{2+}-ATPase purified from human erythrocytes was studied in detergent–lipid–protein mixed micelles. ATPase activity was stimulated maximally by phosphatidylserine. Other anionic diacylglycerophospholipids (phosphatidic acid, cardiolipin, phosphatidylglycerol, and phosphatidylinositol) supported 25–100% of the phosphatidylserine-stimulated activity. Another aminophospholipid, egg PE, supported 38% of the phosphatidylserine-stimulated activity. The phosphoinositides (phosphatidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate) also stimulated the ATPase; however, activity decreased with increasing lipid phosphorylation. Monoacyl negatively charged lipids (lysophosphatidylserine, fatty acids) and zwitterionic lipids (phosphatidylcholine and sphingomyelin) did not activate the enzyme. ATPase activation was dependent on phospholipid fatty acyl chain composition: ATPase activity increased with increasing PS acyl chain length, and the optimal fatty acid composition was one saturated and one unsaturated fatty acid. However, the long, unsaturated acyl chain requirement could be satisfied by nonactivating lipids. The characteristics of this ATPase are similar to those of the Mg^{2+}-ATP-dependent aminophospholipid flippase, suggesting that it may be associated with the transporter.

Transmembrane phospholipid asymmetry is a characteristic and essential feature of biological membranes. The aminophospholipids, phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE), are located primarily on the cytoplasmic face of cell membranes, while the choline-containing phospholipids, sphingomyelin (SM) and phosphatidylcholine (PC), are located preferentially on the external face of the plasma membrane or the lumenal face of internal organelles. This distribution is maintained in part by a PS- and PE-specific transporter, or "flippase", which transports amine-containing phospholipids to the cytofacial monolayer [for recent reviews, see Devaux (1991) and Schroit and Zwaal (1991)]. Aminophospholipid transport requires Mg^{2+}-ATP (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985, 1989) and is sensitive to sulfhydryl reagents (Daleke & Huestis, 1985; Connor & Schroit, 1988; Truong et al., 1993), arginine reagents (Daleke, 1990), Ca^{2+} (Bitbol et al., 1987), and vanadate (Bitbol et al., 1987). The substrate specificity of the flippase is strict and tolerates few changes in glycerol backbone or headgroup structure. Inversion of glycerol stereochemistry (Martin & Pagano, 1987) or substitution of the diacylglycerol backbone with ceramide (Morrot et al., 1989) reduces transport considerably. Transport is dependent on headgroup structure: PS is transported at a rate 10-fold faster than PE (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Zachowski et al., 1986) and chemical modification of the serine headgroup reduces transport activity (Morrot et al., 1989). Transport is relatively insensitive to lipid acyl chain length (Daleke & Huestis, 1985; Morrot et al., 1989), although fluorescent acyl chain substituents alter significantly the rate of transport (Colleau et al., 1991; Tilly et al., 1990). These characteristics indicate that the transporter is associated with a vanadate and sulfhydryl reagent sensitive Mg^{2+}-ATPase with a high structural specificity for PS.

Although transport activity has been characterized well in intact cells and cell membranes, the protein(s) responsible remain unidentified. Two proteins have been proposed as candidate transporters: a 32 kDa PS-binding, sulfhydryl-containing erythrocyte protein in the band 7 region (Schroit et al., 1987; Connor & Schroit, 1988) and a 120-kDa Mg^{2+}-ATPase (Zachowski et al., 1989; Morrot et al., 1990). Using radiolabeled photoaffinity phospholipid probes and a radiolabeled cysteine reagent, Schroit and co-workers have demonstrated that the 32-kDa protein binds PS selectively (Schroit et al., 1987) and contains reactive cysteine residues (Connor & Schroit, 1988). The recently purified erythrocyte Rh(D) protein (de Vetten & Agre, 1988; Agre & Cartron, 1991) has several characteristics in common with the 32-kDa protein and the aminophospholipid transporter. Similarities in physical properties (Connor et al., 1992) and the ability of anti-Rh antibodies to immunoprecipitate the 32-kDa protein (Schroit et al., 1990) have supported the proposal that the two proteins may be identical (de Vetten & Agre, 1988; Connor & Schroit, 1988; Schroit et al., 1990). However, Rh_{null} cells, which are deficient in the expression of Rh proteins (Daleke et al., 1992; Chérif-Zahar et al., 1993), transport PS normally (Smith & Daleke, 1990; Schroit et al., 1990). Furthermore, the amino acid sequence of the Rh(D) protein possesses no apparent ATP binding sites (Chérif-Zahar et al., 1990), implying that

† Supported by grants from the NIH (GM47230) and the American Heart Association, Indiana Affiliate. M.L.Z. is a predoctoral fellow of the American Heart Association, Indiana Affiliate.

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® Abstract published in *Advance ACS Abstracts*, October 15, 1993.

[1] Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; CL, cardiolipin; lyso-PS, brain lysophosphatidylserine; DPPS, dipalmitoylphosphatidylserine (di-16:0 PS); DOPS, dioleoylphosphatidylserine (di-18:1 PS); POPS, 1-palmitoyl-2-oleoylphosphatidylserine (16:0, 18:1 PS); SOPS, 1-stearoyl-2-oleoylphosphatidylserine (18:0, 18:1 PS); DLiPS, dilinoleoylphosphatidylserine (di-18:2 PS); di-6:0 PS, dihexanoylphosphatidylserine; di-8:0 PS, dioctanoylphosphatidylserine; di-10:0 PS, didecanoylphosphatidylserine; di-12:0 PS, dilauroylphosphatidylserine; di-14:0 PS, dimyristoylphosphatidylserine; PIP, phosphatidylinositol 4-phosphate; PIP_2,phosphatidylinositol 4,5-bisphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid; $C_{12}E_9$,poly(oxyethylene)-9-lauroyl ether; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid).

0006-2960/93/0432-12257$04.00/0

if an Rh-like protein is involved in flippase activity, it must be associated with a separate ATPase. An alternative candidate transporter has been proposed by Devaux and coworkers (Zachowski et al., 1989; Morrot et al., 1990; Devaux, 1991), who have postulated that the chromaffin granule ATPase II, a 115-kDa ATPase of unknown function, may be a flippase. This protein has been purified (Moriyama & Nelson, 1988) and shows characteristics similar to those of an ATPase purified from bovine brain synaptic vesicles (Xie et al., 1988). A human erythrocyte protein with properties in common with these ATPases has been purified (Morrot et al., 1990; Daleke et al., 1990, 1992). These proteins are PS-stimulated, vanadate-, and NEM-inhibited Mg^{2+}-ATPases. Both the 32-kDa protein and the ATPase may be components of a larger flippase complex; however, neither protein has been reconstituted nor demonstrated to possess flippase activity.

Numerous transport proteins and membrane-binding enzymes require phospholipids to attain full enzymatic activity. Although two membrane-binding enzymes have been described which have strict phospholipid requirements [protein kinase C for PS (Lee & Bell, 1989; Newton, 1993) and β-hydroxybutyrate dehydrogenase for PC (Cortese et al., 1989)], most membrane-bound enzymes lack this specificity. For example, the prothrombinase complex (Gerads et al., 1990), Na^+,K^+-ATPase (Kimelberg & Papahadjopoulos, 1972), plasma membrane Ca^{2+}-ATPase (Ronner et al., 1977; Carafoli, 1991), and the glucose transporter (Tefft et al., 1986) are stimulated nonspecifically by acidic phospholipids. Selective stimulation of the ATPase activity of a putative flippase by its substrate (PS) is a reasonable criterion for its identification.

In the present work we characterize the lipid-dependence of the vanadate-sensitive, PS-stimulated Mg^{2+}-ATPase isolated from human erythrocytes. Other acidic phospholipids provide only partial stimulation of the enzyme, and evidence is presented indicating that the protein has two distinct phospholipid requirements. Similarities in structural requirements for activation of the ATPase with known requirements for translocase activity suggest that the ATPase may be associated with the aminophospholipid flippase.

MATERIALS AND METHODS

Materials. Bovine brain phosphatidylserine (PS), egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), 1-palmitoyl-2-oleoylphosphatidylglycerol (PG), bovine liver phosphatidylinositol (PI), egg phosphatidic acid (PA), bovine heart cardiolipin (CL), bovine brain lysophosphatidylserine (lyso-PS), dihexanoylphosphatidylserine (di-6:0 PS), dioctanoylphosphatidylserine (di-8:0 PS), didecanoylphosphatidylserine (di-10:0 PS), dilauroylphosphatidylserine (DLPS), dimyristoylphosphatidylserine (DMPS), dipalmitoylphosphatidylserine (DPPS), 1-palmitoyl-2-oleoylphosphatidylserine (POPS), and dioleoylphosphatidylserine (DOPS) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Sphingomyelin (SM), oleic acid, cholesterol, stearylamine, and dioleoylglycerol were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) were purchased from Boehringer Mannheim (Indianapolis, IN). Purity of commercial lipids was verified by thin layer chromatography on silica gel HL (Analtech, Inc., Newark, DE) eluted with 48:45:7:5 chloroform/methanol/water/30% ammonium hydroxide or 65:25:5 chloroform/methanol/98% formic acid. All other chemicals were reagent grade.

ATPase Purification. A vanadate-sensitive, PS-stimulated ATPase was purified from human erythrocytes using a modification of the methods of Moriyama and Nelson (1988), Xie et al. (1988), and Morrot et al. (1990). Outdated units of human erythrocytes were obtained from the Indiana University Medical Center Blood Bank, Indianapolis, IN. Erythrocytes were washed with isotonic saline (0.15 N NaCl) and lysed in phosphate buffer (5 mM phosphate, pH 8) containing EGTA (0.1 mM), pepstatin A (0.7 μg mL^{-1}), and leupeptin (0.5 μg mL^{-1}). Lysed cells were washed three times and solubilized by adding an equal volume of $C_{12}E_9$ (2%). Insoluble material was removed by centrifugation (30000*g*, 30 min), and the supernatant was treated with ammonium sulfate (45%). A floating precipitate was isolated by centrifugation (72000*g*, 20 min) and resuspended in Tris buffer (10 mM), pH 7.4, containing glycerol (10%), $C_{12}E_9$ (0.1%), dithiothreitol (1 mM), EGTA (0.1 mM), pepstatin A (0.7 μg mL^{-1}), and leupeptin (0.5 μg mL^{-1})(buffer A). The resuspended protein was centrifuged (141000*g*, 80 min) to remove insoluble material and bound to a Q Sepharose anion exchange column equilibrated with buffer A. Protein was eluted with a NaCl gradient (0–0.5 N) in buffer A. Fractions containing vanadate-sensitive, PS-stimulated ATPase activity (eluted at 0.2–0.27 M NaCl) were pooled and stored frozen (–70 °C). Proteins were analyzed by SDS–polyacrylamide gel electrophoresis (8%; Laemmli, 1970) and visualized by silver stain (Poehling & Neuhoff, 1981). Enzyme from four separate preparations was used in the following experiments. The protein composition by polyacrylamide gel electrophoresis was similar for each preparation, and the specific activity varied from 22 to 99 nmol min^{-1} mg^{-1}.

ATPase Assay. Lipids were stored in chloroform solution at –20 °C, and stock concentrations were determined by phosphate analysis (Bartlett, 1959). Solvent was removed from aliquots of lipid with a nitrogen stream. Lipid concentrates were prepared by dissolving the dry lipid in $C_{12}E_9$ (5%). ATPase activity was measured at 37 °C in 100 μL of HEPES (25 mM), $MgCl_2$ (5 mM), EGTA (0.5 mM), ouabain (0.1 mM), and $C_{12}E_9$ (0.09%) in the presence of ATP (2.5 mM) and 0.33–1 unit of enzyme (1 unit = 1 nmol of phosphate released min^{-1}). The reaction was quenched by the addition of 0.8 mL of malachite green·Cl (1 mM), ammonium molybdate (1% w/v), and flame photometer diluent (2% v/v, Bacharach, Inc., Pittsburgh, PA), in HCl (1 N). After 10 s, 100 μL of citric acid (34%) was added, and the tube was vortexed. The optical density at 660 nm (Lanzetta et al., 1979) was measured after a 30-min incubation at room temperature. Enzyme activity was measured in triplicate unless otherwise noted. Experimental error, expressed as the standard deviation, is indicated in each figure and was typically of smaller magnitude than the size of the symbols. Data were fit to the Michaelis–Menten equation by a nonlinear least-squares routine.

RESULTS

Figure 1 shows the protein composition of a preparation of the vanadate-sensitive Mg^{2+}-ATPase from human erythrocytes. The most abundant proteins migrate with molecular mass of 120, 100, 89, 50, and 31 kDa. ATPase activity of this preparation, measured in the presence of PS (see below), was sensitive to Ca^{2+}, *N*-ethylmaleimide, and vanadate (IC_{50} = 64, 45, and 1.8 μM, respectively; Figure 2). These inhibition constants are similar to previously reported values for the human erythrocyte (Morrot et al., 1990) and bovine chromaffin granule (Moriyama et al., 1991) ATPases. Ouabain,

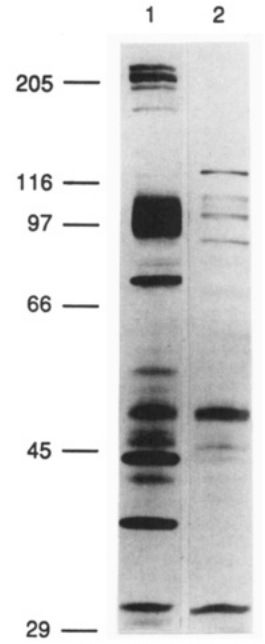

FIGURE 1: Polyacrylamide gel electrophoresis of the partially purified PS-stimulated, vanadate-sensitive, Mg^{2+}-ATPase from human erythrocytes. The ATPase was purified from erythrocyte membrane proteins (lane 1) by a combination of detergent solubilization, ammonium sulfate precipitation, and anion exchange chromatography (lane 2). Proteins were separated by SDS–PAGE (8%) and visualized by silver stain. Molecular mass markers are indicated in kilodaltons.

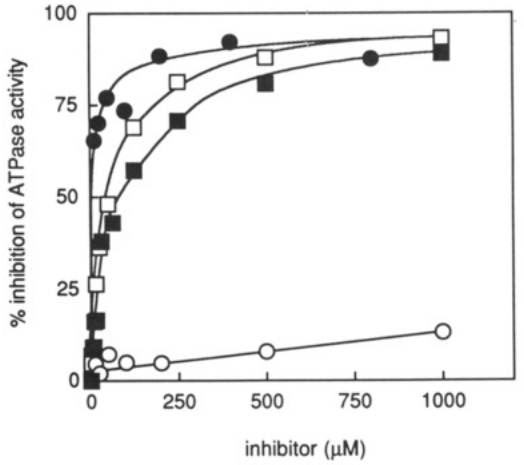

FIGURE 2: The ATPase is sensitive to flippase inhibitors. Inhibition of ATPase activity in the presence of PS (500 μM) by ouabain (○), vanadate (●), *N*-ethylmaleimide (□), and calcium (■). Values are normalized to the maximal activity in the presence of brain PS (22.3 nmol min^{-1} mg^{-1}). Data represent the average of duplicate determinations.

a potent inhibitor of the Na^+,K^+-ATPase, had no effect on PS-stimulated ATPase activity (Figure 2).

The concentration and time dependence of ATP hydrolysis in the presence of bovine brain PS was measured to determine optimal conditions for subsequent experiments examining the effect of lipid composition on ATPase activity. In the presence of saturating concentrations of bovine brain PS (500 μM, see below) the ATPase exhibited a K_m for ATP of approximately 250 μM (Figure 3A). The rate of ATP hydrolysis under these conditions was linear for at least 1 h (Figure 3B). All ATPase measurements described below were performed for 1 h in the presence of the indicated phospholipid and saturating concentrations of ATP (2.5 mM).

Stimulation of the partially purified ATPase by lipid was

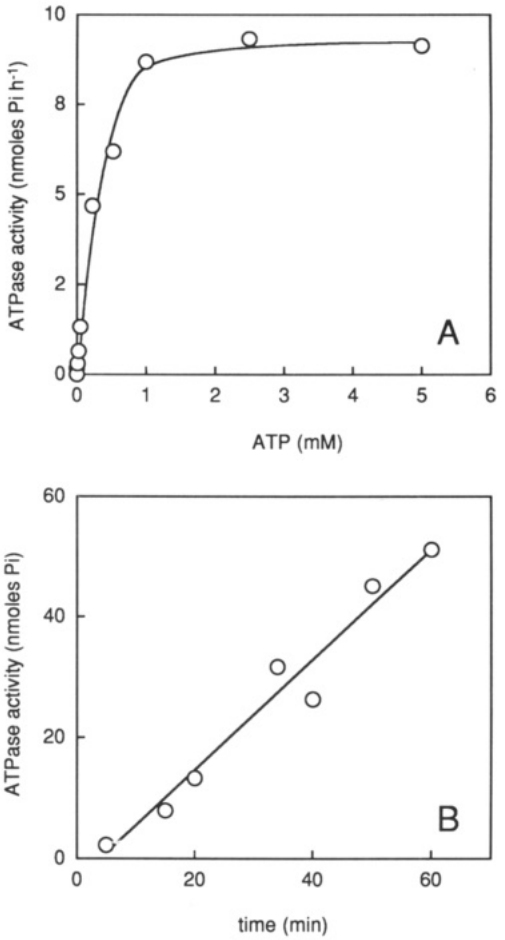

FIGURE 3: Concentration and time dependence of ATP hydrolysis in the presence of PS. (A) ATPase activity in the presence of the indicated concentration of ATP and PS (500 μM) and (B) time course of ATP hydrolysis in the presence of ATP (2.5 mM) and PS (500 μM). Data represent single measurements (B) or the mean ± standard deviation of triplicate measurements (A).

headgroup-specific. PS maximally stimulated the enzyme, exhibiting a V_{max} of 20 nmol h^{-1} and an apparent K_m of 87 μM (Figure 4; Table I). PE partially stimulated the ATPase. Although the maximal activity observed in the presence of PE was 39% of maximal PS activity, the apparent K_m (88 μM) was similar. Other anionic phospholipids supported ATPase activity partially. PA, CL, and lyso-PS generated 24–36% of the activity stimulated by PS (Figure 4; Table I) and demonstrated a wide range of apparent affinities (20–179 μM) for the enzyme. PG stimulated the enzyme at concentrations greater than 50 μM and reached a maximal activity that was 30% of that observed with PS (Figure 4; Table I). ATPase stimulation by PG was cooperative: when fit to a modified Hill equation, the calculated Hill coefficient was 3.5. Maximal stimulation of the enzyme by PI was equivalent to that of PS; however, PI displayed a much higher apparent K_m (389 μM, Figure 5, Table I). The zwitterionic phospholipids, PC and SM, did not support significant ATPase activity (Figure 4, Table I). Other anionic lipids (oleic acid), neutral lipids (dioleoylglycerol and cholesterol), and positively charged lipids (stearylamine) did not stimulate the enzyme (data not shown).

Stimulation of the ATPase by PI was dependent on the phosphorylation state of the headgroup (Figure 5). PIP and PIP_2 stimulated the enzyme 24% and 13%, respectively, as well as PI. The apparent K_m of the enzyme for the lipid

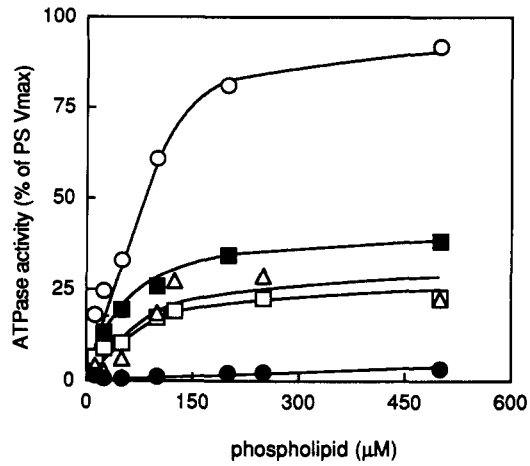

FIGURE 4: Headgroup selectivity of the ATPase. ATPase activity was measured in the presence of bovine brain PS (○), egg PC (●), egg PA (□), egg PE (■), and PG (△). Activity is normalized to the maximal activity in the presence of brain PS (22 nmol min^{-1} mg^{-1}). Data represent the mean ± standard deviation of triplicate measurements (PS, PC, PE, PG) or mean ± range of duplicate measurements (PA). Curves are nonlinear least-squares fits to the Michaelis–Menten equation.

Table I: Kinetic Parameters of Vanadate-Sensitive ATPase Activity in the Presence of Lipid[a]

lipid	K_m (μM)	V_{max} (% brain PS)	V_{max}/K_m
brain PS	87	100	1.2
egg PE	88	39	0.4
PG	47	30	0.6
PA	51	24	0.5
PI	389	100	0.3
PIP	18	24	1.3
PIP_2	8	13	1.6
CL	20	27	1.4
lyso-PS	179	36	0.2
PC		3	
SM		15	

[a] ATPase activity was measured in the presence of $C_{12}E_9$ (0.1% w/v) and various concentrations (0–500 μM) of the indicated lipid. Michaelis–Menten parameters were determined by least-squares fit analysis.

decreased sharply with increasing negative charge (from 389 to 8 μM, Table I), suggesting that the affinity for the inositides increases with increasing phosphorylation.

PS-stimulated ATPase activity was dependent on the length of saturated acyl chains. A homologous series of saturated phosphatidylserines with increasing acyl chain length (di-6:0 PS–di-16:0 PS) varied significantly in ability to stimulate the ATPase (Figure 6A). The apparent K_m of the enzyme for the lipids was unaffected by acyl chain length (~100 μM) and was similar to the K_m of the enzyme for POPS (not shown). However, catalytic efficiency (V_{max}/K_m) increased with increasing acyl chain length (up to 14 carbons) due to an increase in V_{max}. These results indicate that PS-stimulated activity, but not PS binding, is dependent on lipid acyl chain length.

The dependence of ATPase activity on fatty acyl unsaturation was studied using phosphatidylserines containing fatty acids with 16 or 18 carbons and varying degrees of unsaturation (Figure 7, Table II). Of the unsaturated phosphatidylserines tested, brain PS (mostly 18:0, 18:1) and SOPS (18:0, 18:1) stimulated the enzyme optimally. DOPS (di-18:1) and DLiPS (di-18:2) provided less stimulation than SOPS, indicating that the optimal PS acyl chain composition is one saturated and one unsaturated fatty acid. The addition of a single unsaturated fatty acid (18:1) increased ATPase activity from 43%

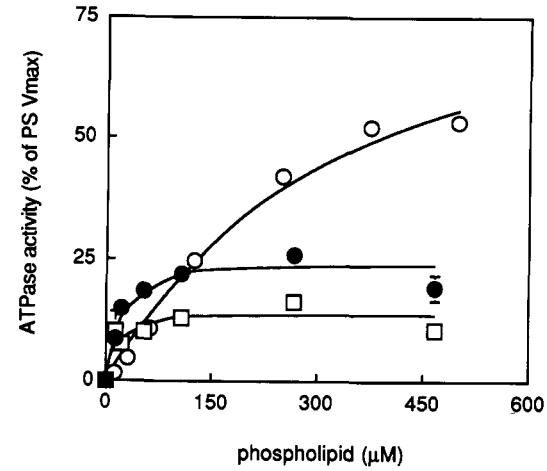

FIGURE 5: ATPase stimulation by PI is dependent on the phosphorylation state of the lipid. ATPase activity was measured in the presence of PI (○), PIP (●), and PIP_2 (□) in two separate experiments. Activity is normalized to the maximal activity in the presence of brain PS (PI, 99 nmol min^{-1} mg^{-1}; PIP, PIP_2, 29 nmol min^{-1} mg^{-1}). Data represent the mean ± standard deviation of triplicate measurements. Curves are nonlinear least-squares fits to the Michaelis–Menten equation.

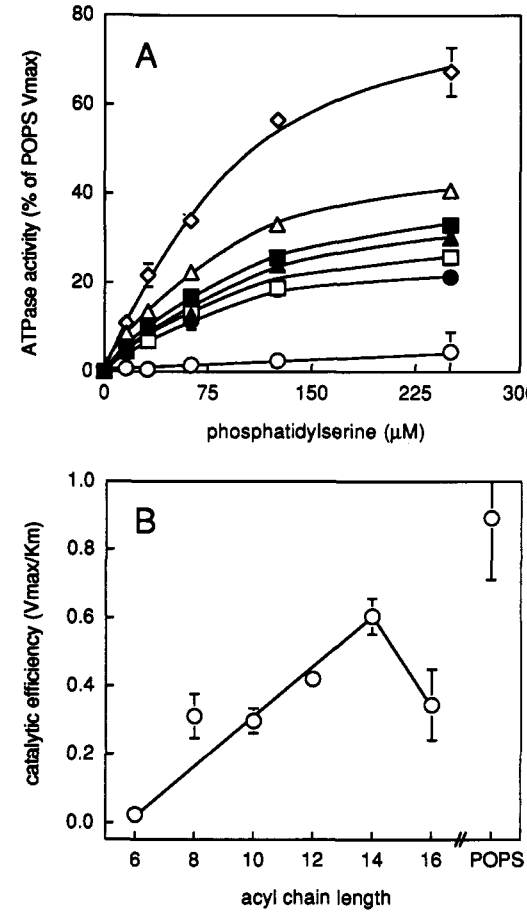

FIGURE 6: ATPase activity, but not PS affinity, is proportional to PS acyl chain length. (A) ATPase activity (% of POPS) in the presence of di-6:0 PS (○), di-8:0 PS (●), di-10:0 PS (□), di-12:0 PS (■), di-14:0 PS (△), and di-16:0 PS (▲) and POPS (◇). (B) Catalytic efficiency (V_{max}/K_m) of the ATPase toward saturated PS. Activity is normalized to the maximal activity in the presence of POPS (29 nmol min^{-1} mg^{-1}). Data represent the mean ± standard deviation of triplicate measurements. Curves are nonlinear least-squares fits to the Michaelis–Menten equation.

(DPPS, di-16:0) to 70% (POPS, 16:0, 18:1) of the activity in presence of SOPS. DOPS and DLiPS were 76% and 61%,

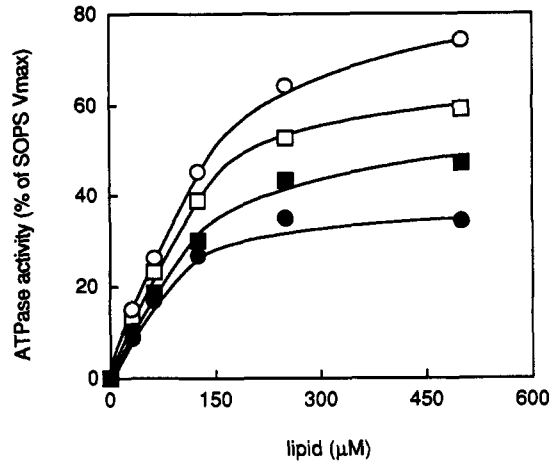

FIGURE 7: Role of PS acyl chain unsaturation in the activation of the ATPase. ATPase activity was measured in the presence of SOPS (○), DPPS (●), DOPS (□), or DLiPS (■). Curves are least- squares fits to the Michaelis–Menten equation. Activity is normalized to the maximal activity in the presence of SOPS (65 nmol min^{-1} mg^{-1}). Data represent the mean ● standard deviation of triplicate measurements. Curves are nonlinear least-squares fits to the Michaelis–Menten equation.

Table II: Acyl-Chain Dependence of Unsaturated PS-Stimulated ATPase Activity[a]

PS	acyl chain length	V_{max} (% brain PS)
brain PS	mostly 18:0, 18:1	100
SOPS	18:0, 18:1	99
DOPS	18:1, 18:1	76
POPS	16:0, 18:1	70
DLiPS	18:2, 18:2	61
DPPS	16:0, 16:0	43

[a] ATPase activity was measured in the presence of $C_{12}E_9$ (0.1% w/v) and various concentrations (0–500 μM) of the indicated lipid. Michaelis–Menten parameters were determined by least-squares fit analysis.

respectively, as effective as SOPS in stimulating enzyme activity at saturating concentrations, indicating that a single unsaturated acyl chain was sufficient for maximal activity. As shown above, ATPase activity was dependent on saturated fatty acyl chain length: POPS stimulated the ATPase 70% as well as SOPS. The requirement for fatty acyl unsaturation is restricted to monounsaturated fatty acids: DLiPS is less effective than DOPS in activating the ATPase.

The requirement for acyl chain unsaturation was not restricted to the PS component. The addition of DOPC to POPS or DPPS micelles, up to a molar ratio of 1:2, slightly increased ATPase activity (Figure 8). Similarly, the addition of DPPC increased marginally the activity of the ATPase in the presence of POPS or DPPS. However, at higher mole ratios (1:1) PC fatty acyl unsaturation significantly accentuates PS-stimulated ATPase activity (Figure 9). The addition of PC (di-8:0 PC, DLPC, or POPC) to micelles containing PS of identical acyl chain composition had no effect on ATPase activity, while POPC increased ATPase activity in the presence of short chain saturated PSs (di-8:0 PS and DLPS). These results indicate that ATPase activity, in the mixed micelle system employed here, was enhanced by the presence of long chain, unsaturated phospholipids.

DISCUSSION

The search for candidate aminophospholipid transporters has relied on identification by several well established properties of the flippase: vanadate sensitivity, cysteine

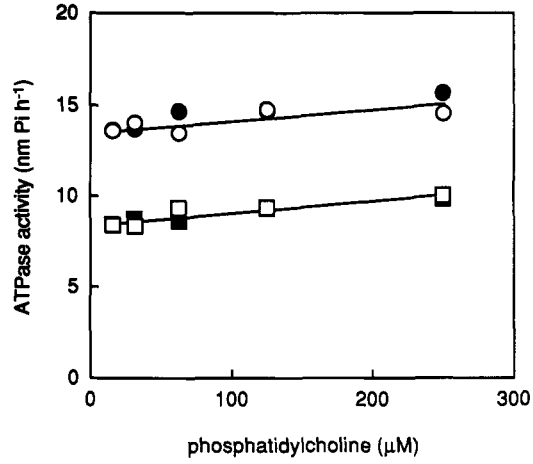

FIGURE 8: Effect of PC acyl chain unsaturation on PS-stimulated ATPase activity. ATPase activity was measured in the presence of POPS (500 μM, ○, ●) or DPPS (500 μM, □, ■) and the indicated concentration of DOPC (○, □) or DPPC (●, ■). Data represent the mean ± standard deviation of triplicate measurements.

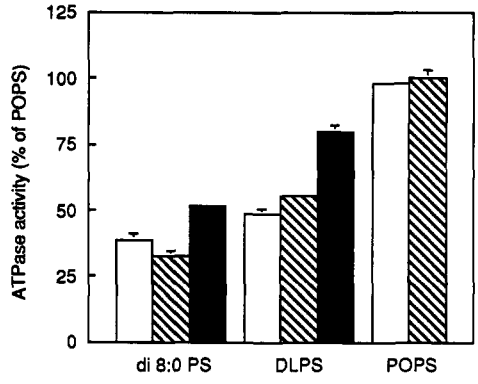

FIGURE 9: Requirement for acyl chain unsaturation can be fulfilled by nonactivating lipids. ATPase activity was measured in the presence of the PS (lightly stippled bars, 175 μM), PS + PC of the identical acyl chain length (darkly stippled bars, 175 μM each), or PS + POPC (solid bars, 175 μM each). Activity is normalized to the maximal activity in the presence of POPS (29 nmol min^{-1} mg^{-1}). Data represent the mean ± standard deviation of triplicate measurements.

reactivity, ATPase activity, and a high specificity for PS. PS binding and sulfhydryl modification have been used to identify a potential 32-kDa flippase that may be part of the Rh complex (de Vetten & Agre, 1988; Connor et al., 1992; Connor & Schroit, 1988; Schroit et al., 1990). We and others (Zachowski et al., 1989; Morrot et al., 1990; Daleke et al., 1990, 1992) have chosen to identity the flippase based on one of its two putative enzymatic activities: vanadate-sensitive, PS-stimulated ATPase activity. This strategy has lead to the identification and partial purification (Morrot et al., 1990; Daleke et al., 1990, 1992) of an ATPase that possesses characteristics anticipated of the aminophospholipid flippase.

The ATPase studied here demonstrates a high degree of specificity for PS as its lipid cofactor. Selective stimulation of the enzyme by PS and partial activation by PE parallels the selectivity of transport exhibited by the aminophospholipid flippase. PS is transported approximately 10-fold faster than PE in erythrocytes (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985) and estimates of the relative affinities of these lipids for the aminophospholipid translocase through competition studies indicate that the flippase binds PS with approximately 9.3-fold higher affinity than PE (Zachowski et al., 1986). The ATPase purified here has qualitatively similar characteristics. Although the relative affinities of PS and PE for the ATPase are similar, maximal activity (and

catalytic efficiency) in the presence of PS is 2.5-fold greater than in the presence of PE. This apparent disparity may reflect a difference in assay conditions. Translocase activity was measured in buffers of higher ionic strength (~150 mM NaCl; Zachowski et al., 1986) than the ATPase described here (~25 mM NaCl). In support of this argument, increasing the ionic strength of the ATPase assay buffer increases the K_m of the enzyme for PE but not for PS (M. Zimmerman and D. Daleke, unpublished observations). Alternatively, the differences in relative K_ms may reflect the nature of our enzyme preparation; multiple proteins are present in the ATPase mixture, and more than one ATPase may be present. Net neutral and zwitterionic amphipaths do not stimulate the ATPase, indicating an absolute specificity for anionic lipids, consistent with the inability of the aminophospholipid flippase to transport PC or SM.

PS-supported activity reflects the structure of the PS headgroup and not the net negative charge of the lipid. Other anionic phospholipids are less effective in stimulating ATPase activity: PS supports greater ATPase activity (3–4-fold) than PG, PA, CL, or lyso-PS. PI supports a high degree of ATPase activity; however, the catalytic efficiency of the enzyme toward PI is 4-fold less than for PS (Table I). Stimulation of the ATPase by lipid is not dependent on the magnitude of the negative charge. Indeed, increasing the net charge per lipid decreases ATPase activation (Figure 5, Table I). The catalytic efficiency of the ATPase in the presence of PIP and PIP_2 is higher than in the presence of PS (Table I), indicating that, at low concentrations of phospholipid, the polyphosphoinositides are more effective activators than PS. However, these lipids may not be significant regulators of the ATPase *in situ* because, compared to PS, they constitute a minor fraction of the erythrocyte phospholipid pool (Allan & Michell, 1978). Similarly, the catalytic efficiency of the ATPase in the presence of CL is greater than that in the presence of PS, but CL is absent from human erythrocytes.

Anionic lipid stimulation is restricted to diacylphospholipids; fatty acids and other negatively charged amphipaths do not stimulate the ATPase. This selectivity distinguishes the ATPase from other transmembrane transporters, including ATPases. The Ca^{2+}-ATPase, Na^+,K^+-ATPase, and the glucose transporter are stimulated by other anionic lipids (fatty acids, detergents) and phospholipids, in addition to PS (Kimelberg & Papahadjopoulos, 1972; Ronner et al., 1977; Tefft et al., 1986; Carafoli, 1991).

The diacyl lipid requirement may reflect the necessity for an appropriate hydrophobic environment. Longer, more unsaturated, PSs are the preferred cofactors, although the affinity of PS for the enzyme is independent of acyl chain length or degree of unsaturation. Acyl chain content alone is not sufficient for ATPase activation; cardiolipin has four fatty acyl chains per lipid and activates the enzyme 3.7-fold less than diacyl-PS. Another membrane-binding protein, protein kinase C, demonstrates a similar unsaturated fatty acid requirement (Bolen & Sando, 1992). In model membranes, protein kinase C activity is dependent on PS, diacylglycerol, and the presence of at least one unsaturated fatty acid, although the fatty acid can be present on a nonactivating lipid, such as PC (Bolen & Sando, 1992). Similarly, mixtures of saturated PSs (di-C8:0 PS or DLPS) and unsaturated PC (POPC) show an increase in ATPase activity over saturated PS alone or saturated PS in the presence of acyl chain matched PC. Thus, the unsaturated fatty acyl requirement can be partially fulfilled by nonactivating lipids. The acyl chain dependence of the ATPase described here contrasts with the acyl chain independence of the erythrocyte aminophospholipid flippase (Daleke & Huestis, 1985). Although a direct comparison of the transport rates of the lipids used in these studies has not been performed, recent studies have shown that the transport of spin- or fluorescent (NBD)-labeled PSs is dependent on the length of the linker group (Colleau et al., 1991). Furthermore, PSs containing a C_6-NBD fluorophore substituent in the *sn*-2 position are transported at a rate dependent on the fatty acyl chain length and unsaturation in the *sn*-1 position (Comfurius et al., 1990). Finally, the ATPase measurements in the present studies were performed in detergent–phospholipid mixed micelles, and the acyl chain dependence may reflect a lipid environment significantly different from that experienced by the flippase in an intact membrane.

The ATPase purified here has properties identical to those of the human erythrocyte ATPase purified by Morrot et al. (1990), yet shows qualitative differences from the chromaffin granule ATPase II (Moriyama et al., 1991). The chromaffin granule enzyme is active in the absence of PS, has a specific activity (in the presence of PS) that is 350–1000-fold higher than the erythrocyte enzyme, and is not activated by PE, PI, or PA (Moriyama et al., 1991). Although both enzymes are PS-stimulated and NEM- and vanadate-sensitive, these data indicate that regulation of the chromaffin granule ATPase II and the human erythrocyte ATPase preparation is significantly different. These dissimilarities may reflect the presence of additional ATPases in each preparation or tissue- and species-dependent differences in the properties of the two enzymes.

The forgoing properties of the ATPase are consistent with a role for the ATPase in flippase activity and provide a model for the regulation of a putative aminophospholipid transporter. Partial, nonspecific, stimulation by anionic lipids indicates that the ATPase/flippase may contain anionic lipid binding sites which must be filled to activate the enzyme. These anionic lipid binding sites would likely be localized to the cytofacial side of the protein, where there is a preponderance of endogenous negatively charged phospholipids. The specificity for PS indicates that the enzyme may possess a separate PS-binding site, perhaps the flippase substrate binding site. Acyl chain selectivity may reflect a requirement of fatty acyl length and unsaturation to fully express enzyme activity. However, positive identification of the flippase will require reconstitution and demonstration of phospholipid transporting activity. Further purification, characterization, and reconstitution of the ATPase described here is currently in progress.

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