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Role of Specific Acidic Lipids on the Reconstitution of Na^+-Dependent Amino Acid Transport in Proteoliposomes Derived from Ehrlich Cell Plasma Membranes†

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ABSTRACT: The effect of acidic phospholipids on the activity of a Na^+-dependent amino acid transporter (A system) from Ehrlich ascites cell plasma membranes was examined. Plasma membranes were solubilized in cholate/urea and reconstituted with Ba^{2+}-precipitated asolectin (soybean phospholipid free of anionic phospholipids) replenished with different acidic phospholipids. In the absence of added acidic phospholipids, transport activity was very low. However, three acidic lipids [cardiolipin > phosphatidic acid (PA) > phosphatidylinositol] were capable of restoring transport activity (in the order given) to proteoliposomes made from Ba^{2+}-precipitated asolectin, while other acidic phospholipids (phosphatidylserine and phosphatidylglycerol) were much less active in this respect. For restoration of optimal activity, PA containing at least one unsaturated fatty acyl moiety, particularly in the β position, was required. PA containing only saturated fatty acids in the β and γ positions was largely inactive. No difference in restoration of function was observed on varying the saturated fatty acyl chain length in PA from 10 carbons to 18 carbons. The specific effects of PA on the A-system transporter were not shared by the Na^+-independent amino acid exchange system (L system) or the glucose transport system. Treatment with poly(ethylene glycol) 8000 was shown to reduce the nonspecific permeability of the reconstituted proteoliposomes and to enhance Na^+-dependent amino acid transport.

Any "in vitro" study of solute translocation with isolated membrane proteins is coupled to the need to restore a suitable phospholipid environment. In contrast to binding studies or conventional enzyme assays, the requirements for reconstitution of transport include a need to form a compartment of finite volume and differential permeability, which will sustain ion gradients and/or the accumulation of translocated solutes.

Variability in the restoration of a native (or near native) conformation of the protein, as well as the disposition of the protein in the reconstituted membrane, would substantially affect the rate of transport.

Even if all the inherent catalytic (translocating) activity of the transporter were theoretically recovered, any variability in the size of the vesicles or their nonspecific permeability in different preparations would be reflected in differences in the apparent transport rate per unit amount of reconstituted protein.

Despite these stringent requirements, many investigators have reported the reconstitution of a wide variety of solute transporters from plasma membranes of mammalian cells (Kasahara & Hinkle, 1977; Bardin & Johnstone, 1978; Im & Spector, 1980; McCormick et al., 1984; Fafournoux et al., 1989). The level of reconstituted transport activity, however, rarely approaches that of the original system in the intact cell. It is self-evident that many of the variables in the reconstitution process still require systematic investigation to advance our understanding of how transporters work and the factors required for reconstitution of near-native activity.

One of the unresolved questions in the reconstitution of functional membrane proteins is the role, if any, of specific phospholipids. Studies on reconstitution of many transport activities have shown that heterologous phospholipids can provide an adequate environment for normal functioning of the transporter [for reviews, see Sandermann (1978), McElhaney (1982), and Yeagle (1989)]. While homologous lipid sources have been shown to provide optimal recovery of activity, asolectin (a plant phospholipid mixture) has been successfully used by many investigators to reconstitute transport activity from mammalian as well as bacterial systems (Villegas et al., 1977; Wu et al., 1981; McCormick et al., 1984; Wilson et al., 1985; Maloney & Ambudkar, 1989). Attempts to show highly selective lipid requirements for restoration of specific transport activities have met with only modest success. While acidic lipids have been implicated in the restoration of function of a number of plasma membrane transporters, requirements for specific acidic lipid species for this purpose have been less clearly established (Wheeler & Whittam, 1970; Niggli et al., 1981; Philipson & Nishimoto, 1984). Overall, most of the data from reconstitution studies have been consistent with the conclusion that the lipid environment must fulfill general conditions such as adequate lipid fluidity, and in some cases a suitable bilayer surface charge, with relatively little evidence for stringent requirements for specific lipid species (Knowles, 1976; Dean & Tanford, 1977; George et al., 1989; Yeagle, 1989). Such a conclusion would be in line with current observations that a variety of detergents (Lund et al., 1989) are excellent substitutes for phospholipids in the restoration of Ca^{2+}-ATPase activity.

Our recent attempts to obtain optimal conditions for recovery of Na^+-dependent amino acid transport from a detergent-solubilized membrane fraction from Ehrlich ascites cells have revealed a number of unexpected requirements,

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including a phosphatidic acid (PA)[1] requirement and an optimal vesicle size (McCormick et al., 1984, 1985; McCormick & Johnstone, 1988). It has been shown that the existence of a potentially functional transporter may be masked by virtue of an inadequate liposome volume, with the activity being restored by volume expansion alone (McCormick et al., 1985). In this paper, we report observations which provide additional evidence for specific lipid requirements, including a dependence on specific head groups and fatty acyl moieties, to recover Na^+-dependent amino acid transport activity from detergent-solubilized Ehrlich cell plasma membranes.

Materials and Methods

Materials

Cholic acid was purchased from Anachemia, Montreal, Canada, and recrystallized 3 times from ethanol before use (Kagawa & Racker, 1971). Ultrapure urea was purchased from Schwarz/Mann and asolectin from Associated Concentrates, Woodside, NY. Sephadex G-50 was purchased from Pharmacia, Dorval, Quebec. The radioisotopes [1-^{14}C]AIB (40 mCi/mmol) and L-[4,5-^{3}H]leucine (60 Ci/mmol) were purchased from ICN Canada, and 3-*O*-[^{14}C]methyl-D-glucose (55 mCi/mol) was from NEN Dupont Canada. Rubidium-86 (4.27 mCi/mg) was purchased from Amersham Canada. Egg PA and cardiolipin were purchased from Serdary Research Laboratories Inc., London, Ontario. PI was purchased from Supelco Canada Ltd. Other lipids were purchased from Sigma, St. Louis, MO. Specific PA's were generated from the corresponding PC by treatment with phospholipase D. All phospholipids were analyzed by TLC to check the purity before use. Precoated TLC plates (SIL G-25), manufactured by Macherey-Nagel, were purchased from Sybron/Brinkmann, Canada. Poly(ethylene glycol) 8000 was purchased from Fisher Scientific Co.

Methods

Solubilization of Plasma Membranes. Plasma membranes were prepared from Ehrlich acites cells and stored at −70 °C in 15% dimethyl sulfoxide as described (Colombini & Johnstone, 1974; McCormick et al., 1985). The membranes were solubilized by suspension (1–2 mg of protein/mL) in 2.5% cholic acid (w/v), 4 M urea, 100 mM NaCl, 0.1 mM EDTA, and 10 μM PMSF, in 5 mM Tris, pH 7.4, and incubating with constant stirring at 4 °C for 30 min (McCormick et al., 1985; McCormick & Johnstone, 1988). The urea solution was prepared immediately before use. Following incubation, the solubilized membrane was centrifuged at 120000*g* for 60 min, the pellet was discarded, and the supernatant was dialyzed overnight against 100 volumes of K^+ buffer (100 mM KCl, 5 mM Tris, pH 7.4, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$) containing 0.25% cholic acid and 1 μM PMSF. The dialyzed supernatant was stored at 4 °C until used for reconstitution.

Reconstitution. Asolectin (native or Ba^{2+}-precipitated) (10 mg) was suspended in 0.25 mL of K^+ buffer and vortexed 5–6 min under N_2 followed by bath sonication at 22 °C (Model G 112 SP 1G, Laboratory Supplies, Co. Inc.) for 2.5–3.5 min to clarity. The lipid suspension was mixed with 1–2 mL of the solubilized membrane suspension (containing 1.5–2.0 mg of protein) and applied to a sephadex G-50 column (1.5 cm × 28 cm), preequilibrated in K^+ buffer. The column was eluted with K^+ buffer or with K^+ buffer in which the anion was varied (see text), and the turbid fractions, eluting in the void volume, were collected, pooled, and frozen in dry ice/ethanol, followed by thawing at 22 °C. The freeze/thawed liposomes were collected by centrifugation at 40000*g* (20 min) and resuspended in a final volume 150 μL of K^+ buffer.

Liposome Fusion Induced by PEG. Vesicles of pure PA were made as descried by Gains and Hauser (1984) by suspending 2.6 μmol of egg PA in 0.5 mL of K^+ buffer (Ca^{2+} + Mg^{2+} free) containing 5 mM EDTA. After being vortexed well and sonicated for 3 min, the suspension was freeze/thawed 3 times in dry ice/ethanol to give a translucent white dispersion in which some of the particles were just visible. Asolectin proteoliposomes made from Ba^{2+}-precipitated asolectin (McCormick & Johnstone, 1988) were resuspended in 1 mL of (Ca^{2+} + Mg^{2+})-free K^+ buffer containing 5 mM EDTA. The two groups of liposomes were mixed, added to PEG-8000 to give a final PEG concentration of 28% (w/v), and incubated for 10 min with constant stirring at 22 °C. The suspension was diluted 30 times with (Ca^{2+} + Mg^{2+})-free K^+ buffer and centrifuged at 40000*g* for 20 min. The pellet was resuspended in K^+ buffer in a final volume of 150 μL, of which 25 μL was used for each transport assay.

Preparation of Phospholipids. Barium-precipitated asolectin, which does not contain PA or cardiolipin, was prepared by using the procedure of Van Dijck et al. (1978), recovering the neutral lipids selectively in the soluble fraction (McCormick & Johnstone, 1988). In contrast with earlier reports (Kagawa & Racker, 1971), our analyses of native asolectin by two-dimensional TLC showed that PA comprised ~12% of the total lipid while cardiolipin represented ~1% of the lipid. PA was obtained commercially or prepared from PC by hydrolysis with phospholipase D followed by extraction and purification by silica gel chromatography. Phospholipids were stored under N_2 at −70 °C. Prior to use, the phospholipids were dried down under N_2 and lyophilized overnight to remove all traces of solvent.

Na^+-Dependent Amino Acid Uptake. Amino acid transport activity was measured as reported earlier (McCormick et al., 1985; McCormick & Johnstone, 1988). Proteoliposomes (25-μL suspension) were diluted into 125 μL of Na^+ buffer (0.1 mM Ca^{2+}, 0.1 mM Mg^{2+}, 5 mM Tris, pH 7.4, and 100 mM NaCl) containing 0.12 mM [1-^{14}C]AIB (9.4×10^4 dpm/nmol). Samples of 25 μL were removed at intervals, diluted to 2 mL with Na^+ buffer at 4 °C containing 0.1 mM unlabeled AIB, and rapidly filtered under vacuum through Whatman GF/B filters. The filters were washed with 10 mL of Na^+ buffer (4 °C) and dried, and the retained radioactivity was measured. As control, uptake in K^+ buffer was measured.

3-O-Methyl-D-glucose Uptake. To measure the initial velocity of [^{14}C]MG uptake, 12.5 μL of a proteoliposome suspension (50–70 μg of protein) was added to 60 μL of K^+ buffer containing 0.12 mM [^{14}C]MG (3.7×10^4 dpm/nmol). After 15-s incubation at 22 °C, 1 mL of K^+ buffer (4 °C) containing 5 mM $MgCl_2$ was added, and the suspension was immediately applied to a glass fiber filter (Whatman GF/B) under vacuum. The filter was washed with 5 mL of K^+ buffer containing 5 mM $HgCl_2$ (4 °C) and dried and the retained radioactivity measured. The uptake of MG was also measured in the presence of 2 μM cytochalasin B. The measurements were made in triplicate. Nonspecific binding was determined by adding 12.5 μL of proteoliposomes to 1 mL of K^+ buffer at 4 °C containing [^{14}C]MG and 5 mM $HgCl_2$ to block glucose transport. The suspension was immediately filtered and

[1] Abbreviations: PA, phosphatidic acid; PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetic acid; MG, 3-methyl-D-glucose; TLC, thin-layer chromatography; PI, phosphatidylinositol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; Me-AIB, (methylamino)isobutyric acid.

Table I: Effect of PEG on AIB Uptake and Intravesicular Volume[a]

conditions	Na^+-dependent AIB uptake [pmol min^{-1} (mg of protein)$^{-1}$]	intravesicular volume (μL/mg of protein)	% of control volume
native asolectin proteoliposomes (control)	73 ± 27 (5)	4.4 ± 1.5 (5)	100
native asolectin proteoliposomes treated with PEG	147 ± 2(2)[b]	4.3 (2)[b]	98
Ba^{2+}-precipitated asolectin proteoliposomes	18 ± 2.9 (3)	2.6 ± 0.7 (3)	59
Ba^{2+}-precipitated asolectin proteoliposomes treated with PEG	45 ± 5(3)	2.4 (3)	55
Ba^{2+}-precipitated asolectin proteoliposomes and PA liposomes fused with PEG	88 ± 20 (3)	2.5 ± 0.6 (3)	57

[a] Proteoliposomes were prepared from native asolectin or Ba^{2+}-precipitated asolectin and treated (where indicated) with 28% PEG 8000 for 10 min at 22 °C. Where noted, liposomes made from pure PA were added to Ba^{2+}-precipitated proteoliposomes prior to treatment with PEG. The amount of PA added represented 20% of the lipid used in the preparation of PA-free proteoliposomes. After PEG treatment, the proteoliposomes were diluted 30 times with K^+ medium and centrifuged to remove the PEG. Control proteoliposomes (PEG free) were treated identically. The pelleted vesicles were resuspended in K^+ medium, and [1-^{14}C]AIB uptake was measured in either Na^+ or K^+ medium (see Methods). Volume measurements were made from the equilibrium distribution of [^{14}C]MG. The values given are means ± SD. The values in parentheses are the number of determinations. [b] Average value.

processed as described above. Nonspecific binding was measured in duplicate.

Uptake of [^{14}C]MG at equilibrium and steady-state ^{86}Rb uptake (McCormick et al., 1984) in the presence of valinomycin were used as measures of the intravesicular volume of proteoliposomes, and the results are expressed as microliters of solute per milligram of protein. For these measurements, the incubations were carried out as above except that the duration of incubation was 60 min for MG uptake and 10 min for ^{86}Rb uptake, both at 22 °C. Volume measurements were made in triplicate.

Measurement of L-*Leucine Efflux.* Reconstituted proteoliposomes made from native and Ba^{2+}-precipitated asolectin were treated with 28% PEG for 10 min at room temperature, diluted 30-fold with K^+ buffer to remove PEG, and then incubated with 0.1 mM L-[4,5-^{3}H]leucine (3.7×10^2 dpm/pmol) for 30 min at 22 °C to preload the liposomes. A 25-μL aliquot of the proteoliposomes (at 22 °C) was diluted to 1 mL in K^+ buffer containing a competing amino acid (see Figure 4) and incubated at 22 °C. Samples were taken at intervals, diluted with ice-cold buffer, filtered, and washed as described for AIB uptake (see above).

TLC of Phospholipids. Phospholipids were analyzed by TLC using chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5) as solvent (Ryu & MacCoss, 1979). The TLC plate was dried under a stream of nitrogen gas or in air at room temperature, and individual lipids were visualized by spraying with ammonium molybdate. For quantification of individual phospholipids, the spots were scraped from the plate, extracted, and digested with perchloric acid. The silica gel was removed by centrifugation and the P_i content of the supernatant measured according to Lowry and Tinsley (1974). A clear area of the silica gel of the same dimensions was scraped from the plate and treated identically to serve as a blank.

To determine whether specific phospholipids had been incorporated into vesicles after PEG treatment, fused proteoliposomes were recoverd by centrifugation and the lipids analyzed by TLC. To extract the lipids, 50 μL of liposome suspension was suspended in 250 μL of chloroform, then mixed with 250 μL of methanol and 200 μL of water, and shaken. The mixture was separated into two phases by centrifugation. The organic solvent phase was concentrated under N_2, and the residue was applied to a TLC plate and analyzed as above.

Protein and Phospholipid Determinations. Protein concentration was determined by a modified Lowry procedure (Markwell et al., 1981) using bovine serum albumin as standard. Phospholipid was determined by the Lowry and Tinsley method (Lowry & Tinsley, 1974). The acid digestion time was prolonged 2–3 h to ensure quantitative conversion of lipid phosphorus to inorganic phosphate.

Table II: Analysis of Phospholipid Content of Vesicles after Treatment with PEG[a]

component	% total lipid in pellet[b]: asolectin proteoliposomes	PA liposomes fused with Ba^{2+}-precipitated asolectin proteoliposomes	Ba^{2+}-precipitated asolectin proteoliposomes
PA	10–13	12.3	
cardiolipin	1		
PE	33	34.8	46
PC	37	34.0	43
unknown	10–15	18.2	10

[a] Proteoliposomes made from Ba^{2+}-precipitated asolectin were treated with a population of pure PA vesicles and 28% PEG for 10 min at room temperature. As control, native asolectin proteoliposomes were treated with 28% PEG in an identical fashion. Then the suspensions were diluted 30-fold with K^+ buffer and centrifuged. The proteoliposome pellet was suspended in K^+ buffer; the lipids were extracted and subjected to quantitative TLC analysis as described under Materials and Methods. Lipid phosphorus was used to assess total lipid and the percent of total in each fraction. [b] Values for the components are given as a percent of the total lipid content.

Results

Dependence of Transport on PA and Effect of PEG. An earlier paper (McCormick & Johnstone, 1988) showed that vesicles made from Ba^{2+}-precipitated asolectin (designated as PA-free asolectin) are incapable of supporting Na^+-dependent AIB transport and that PA replacement restored activity. However, the addition of PA to the phospholipid micelle prior to reconstitution resulted in an expansion of vesicle volume, a factor which can markedly influence transport activity (McCormick et al., 1985). To circumvent the effects of volume expansion, and to concentrate on the effects of specific lipids, we adopted a procedure in which PA was introduced into proteoliposomes by PEG treatment. Liposomes containing pure PA are small (~600 Å) (Gaines & Hauser, 1984) and do not sediment under our centrifugation conditions. It was observed that when a liposome suspension made with pure PA was incubated with proteoliposomes made with Ba^{2+}-precipitated asolectin in the presence of 28% PEG 8000, the pelleted proteoliposome population retained its original volume as measured by MG (Table I) or ^{86}Rb uptake (not shown) and had an increased PA content (Table II). Na^+-dependent transport activity increased markedly upon PA restoration in the presence of PEG (Table I). The data in Table I and Figure 1 show a 5-fold increase in Na^+-dependent AIB uptake after treatment with PEG and PA compared to the uptake in PA-free proteoliposomes. PEG and PA primarily affect the

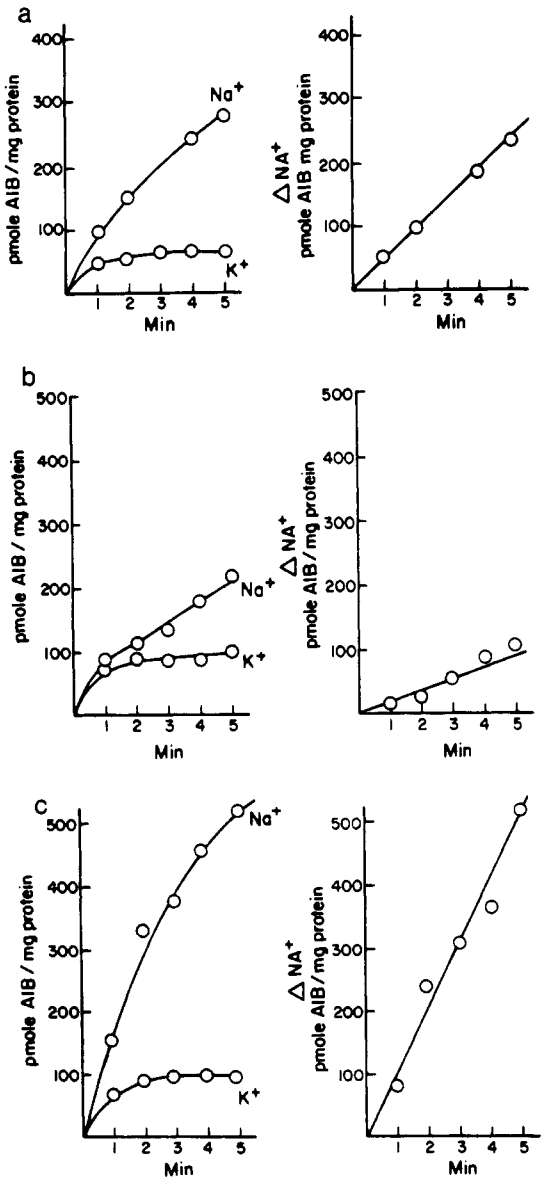

FIGURE 1: Effect of PA and PEG on transport of AIB in reconstituted proteoliposomes. Proteoliposomes prepared from Ba^{2+}-precipitated asolectin were treated with 28% PEG 8000 for 10 min at 22 °C (a). Control proteoliposomes from Ba^{2+}-precipitated asolectin, not treated with PEG (b). Liposomes made from pure PA were added to PA-free proteoliposomes prior to treatment with PEG (c). The amount of PA added represented 20% of the lipid used in the preparation of proteoliposomes with Ba^{2+}-precipitated asolectin. After 10-min incubation, the proteoliposomes were diluted 30 times with K^+ medium and centrifuged to remove the PEG. The pelleted proteoliposomes were resuspended in K^+ medium, and [1-^{14}C]AIB uptake was measured in either Na^+ or K^+ medium (see Methods).

Na^+-dependent component of AIB uptake (Figure 1).

Effect of PEG on Proteoliposome Permeability. The data in Figure 1 and Table I show that PEG treatment of Ba^{2+}-precipitated or total asolectin proteoliposomes doubles the apparent Na^+-dependent transport activity, suggesting that PEG has a direct effect on the proteoliposomes. This increase in transport activity occurs in the absence of volume enlargement as measured by 3MG uptake.

Further studies showed that PEG treatment reduced the passive permeability of reconstituted proteoliposomes made with Ba^{2+}-precipitated or native asolectin. The time required to attain equilibration of AIB in Na^+-free media was clearly increased after PEG treatment (Figure 2). Assuming that the approach to equilibrium in Na^+-free medium is a first-order process, the data show that the half-time for equilibration

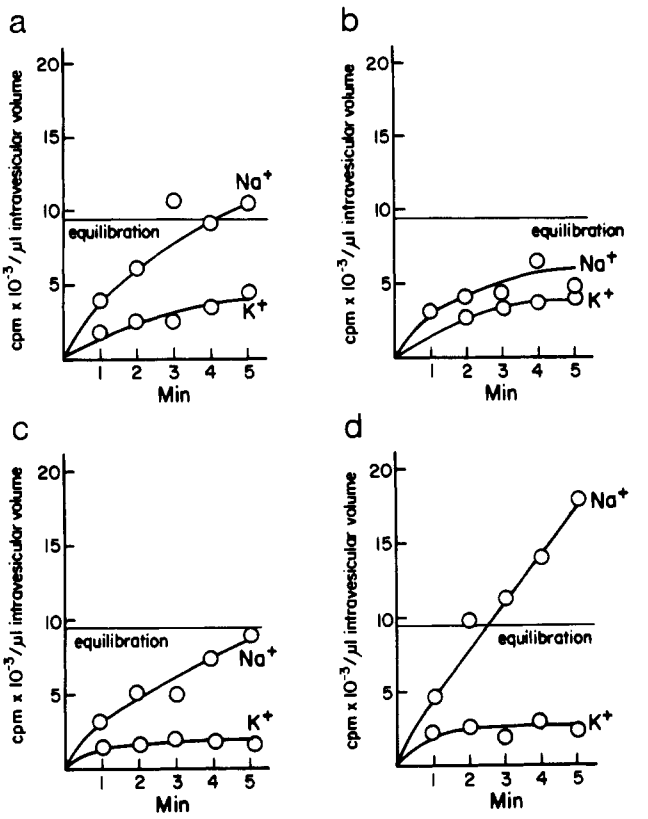

FIGURE 2: Effects of PEG and PA on the rate of equilibration of AIB. Proteoliposomes made from Ba^{2+}-precipitated asolectin were either untreated or incubated with PEG in the presence or absence of PA liposomes, as described under Materials and Methods. The proteoliposomes were incubated at room temperature with 0.1 mM [^{14}C]MG to achieve a steady state. The intravesicular volume of the proteoliposome was calculated from the relationship μL of intravesicular volume = (cpm in washed proteoliposomes)/(cpm/μL of medium). A parallel incubation was carried out at room temperature with 0.1 mM [1-^{14}C]AIB in K^+ medium where samples were taken at intervals before steady state was reached. The line drawn horizontal to the abscissa shows the equilibrium point in each set of proteoliposomes. Uptake of AIB in Na^+ medium is given for comparative purposes as are the results obtained with proteoliposomes made from total asolectin. Proteoliposomes were made with (a) total asolectin, (b) Ba^{2+}-precipitated asolectin, (c) Ba^{2+}-precipitated asolectin + PEG, and (d) Ba-precipitated asolectin + PEG + PA.

Table III: Passive Permeability of PEG-Treated Proteoliposomes: Effects of PA Content[a]

type of proteoliposome	$t_{1/2}$ for AIB uptake in Na^+-free medium (min)
native asolectin	6.6
Ba^{2+}-precipitated asolectin	6.4
Ba^{2+}-precipitated asolectin treated with PEG	10.3
Ba^{2+}-precipitated asolectin fused with PA liposomes by PEG treatment	9.8

[a] Proteoliposomes, made with native or Ba^{2+}-precipitated asolectin, were treated with 28% PEG or with PA liposomes and PEG as described under Methods. The available intravesicular volume was determined by incubation with [^{14}C]MG for 60 min at room temperature. Uptake of [^{14}C]AIB was determined in Na^+-free medium with samples taken at intervals before attainment of steady state. $t_{1/2}$ was calculated from the relationship $U_t = U_\infty(1 - e^{-kt})$ where U_t = the uptake at time t and U_∞ is the uptake at equilibrium (determined from 3MG uptake) when the intravesicular [AIB] equals that in the medium. Each value given is an average of two closely agreeing values (difference ≤10%) from the experiments performed with different membrane preparations.

of AIB is increased by ~50% after PEG treatment (Table III).

It is likely that this decrease in passive permeability is responsible for the increased carrier-mediated Na^+-dependent

Table IV: Effect of PA and Other Acidic Lipids on Reconstitution of AIB Transport[a]

condition	relative activity	relative volume
native asolectin	100	100
PA-free asolectin	19	61
PA-free asolectin + PS	20	80
PA-free asolectin + PG	35	84
PA-free asolectin + PI	56	80
PA-free asolectin + egg PA	94	90
PA-free asolectin + cardiolipin	100	90

[a] Different acidic phospholipids were mixed with Ba^{2+}-precipitated asolectin prior to reconstitution. The added acidic lipids represented 20% of the total lipid. The mixed lipid suspension was added to detergent-solubilized membranes, and proteoliposomes were reconstituted as described under Methods. All measurements of [^{14}C]AIB uptake were made in Na^+ and K^+ media over a time course lasting 5 min at room temperature. The initial rate of Na^+-dependent uptake (as described in Figure 1) was used to determine the relative activity based on the control uptake in native asolectin proteoliposomes. Volume measurements were made with [^{14}C]MG as in Tables I and III. The values given are representative of at least two separate experiments with different membrane preparations.

AIB uptake seen with PEG, since PEG-treated proteoliposomes would maintain the initial Na^+ gradients better, for a longer duration, as well as show less backflow of accumulated solute. To determine whether the reduction of passive permeability is restricted to AIB influx, we examined the rate at which leucine was lost from proteoliposomes. Experiments showed that the rate constant for leucine efflux was reduced 32 ± 8% (three determinations) after treatment of proteoliposomes with 28% PEG. The mechanism by which PEG causes these permeability changes is unknown.

Effect of PA on Kinetic Parameters of AIB Transport. Since PA replenishment (by PEG treatment) increases transport without changing the sealed volume available for solute accumulation (Table I), PA may affect carrier function directly. Using PEG-treated proteoliposomes, we determined the influence of PA on the K_m values for AIB uptake. With and without PA, the K_m values were estimated to be 0.34 ± 0.08 (SD) mM and 0.23 ± 0.01 mM, respectively. Since these values are close to those obtained with proteoliposomes reconstituted from total asolectin (0.35 mM), it is evident that neither PA nor PEG substantially affects the K_m values. Therefore, PA mainly influences the maximum velocity of uptake.

Specificity of PA Effects. Experiments were also carried out in which PA was replaced by several other acidic phospholipids. The results in Table IV show that acidic phospholipids were not all equally effective in reconstituting transport activity. Only bovine heart cardiolipin was as effective as PA. As shown earlier, neither PG nor PS was highly effective in replacing PA (McCormick & Johnstone, 1988). Addition of PI partly restored activity, but on a molar basis, PI was less effective than either PA or cardiolipin. Thus, full recovery of activity appears to depend on specific acidic lipids, suggesting a direct interaction between these lipids and the amino acid carrier.

Effects of Fatty Acyl Composition on Reconstitution of Transport Activity. In the experiments cited above, the PA was obtained from natural sources and therefore contained both unsaturated and saturated fatty acids of varying chain length in positions β and γ, respectively. To examine the effect of fatty acyl chain length on reconstitution, a series of synthetic PA molecules containing only saturated fatty acids was used and compared with synthetic PA's containing only unsaturated fatty acids (Table V, Figure 3). With synthetic PA containing

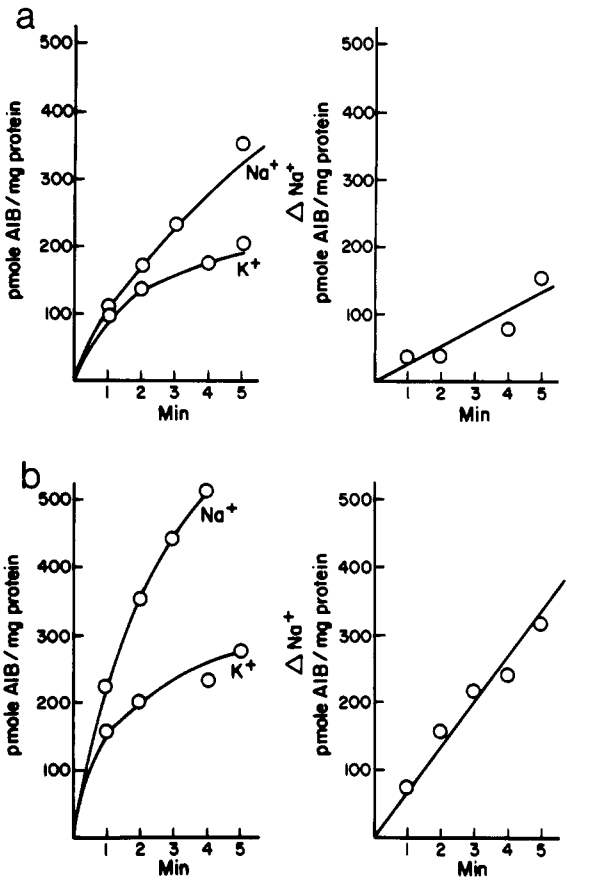

FIGURE 3: Effect of PA containing saturated and unsaturated fatty acyl moieties on the reconstitution of AIB transport. Ba^{2+}-precipitated asolectin containing 20% dipalmitoyl-PA (a) or 20% dioleoyl-PA (b) was used for reconstitution of Na^+-dependent amino acid transport. AIB transport in the proteoliposomes was measured in K^+ and Na^+ as described under Materials and Methods.

Table V: Fatty Acyl Content of PA and Restoration of AIB Transport[a]

	relative transport activity	relative vesicle volume
control (native asolectin proteoliposomes)	100	100
Ba^{2+}-precipitated asolectin	20	77
Ba^{2+}-precipitated asolectin + egg PA	94	95
Ba^{2+}-precipitated asolectin + didecanoyl-PA	44	120
Ba^{2+}-precipitated asolectin + dipalmitoyl-PA	38	146
Ba^{2+}-precipitated asolectin + distearoyl-PA	38	60
Ba^{2+}-precipitated asolectin + dioleoyl-PA	76	161
Ba^{2+}-precipitated asolectin + β-oleoyl-γ-stearoyl-PA	77	
Ba^{2+}-precipitated asolectin + β-linoleoyl-γ-palmitoyl-PA	77	
Ba^{2+}-precipitated asolectin + β-palmitoyl-γ-oleoyl-PA	43	
Ba^{2+}-precipitated asolectin + β-stearoyl-γ-oleoyl-PA	44	

[a] All phospholipid suspensions were added to the solubilized membranes prior to reconstitution as described (McCormick & Johnstone, 1988). PA represented 20% of the total phospholipids added. All data are representative of at least two experiments carried out with different membrane preparations on separate occasions.

only saturated fatty acids, the increase in vesicle volume was variable, but no species of fully saturated PA brought about a major increase in transport activity. For example with dipalmitoyl-PA, the vesicle volume, but not activity, increased more than with egg PA. No difference in reconstitution of transport activity was seen when the saturated PA acyl chain length was varied from 10 to 18 carbons. In contrast, when

PA with purely unsaturated fatty acids was used (dioleoyl-PA), recovery of activity approached that seen with egg PA. These results show that PA is important for recovery of activity and that the acidic lipid must contain at least one unsaturated fatty acyl chain (Table V and Figure 3).

Interestingly, the position of the fatty acid in PA has measurable effects on the efficacy of PA in restoring transport activity. A PA with an unsaturated fatty acid in the normal β position is more effective than one with an unsaturated chain in the γ position in restoring activity (Table V). Such a result also suggests a specific interaction between acyl chains and carrier proteins.

PA and Reconstitution of Other Solute Transporters. We have shown earlier that both the glucose transporter and the Na^+-independent L-system transporter may be reconstituted under our conditions (McCormick et al., 1984). To assess whether the PA requirement also pertains to these transporters, we examined the leucine and glucose transport activities in vesicles reconstituted with Ba^{2+}-precipitated or native asolectin. The data in Figure 4 show that exchange–efflux of leucine did not require PA for recovery of transport activity in reconstituted proteoliposomes. Furthermore, there was no evidence for a PA requirement to restore cytochalasin B sensitive glucose uptake. The specific glucose uptakes [in pmol min^{-1} (mg of protein)$^{-1}$] were 404 and 610 in proteoliposomes made from native and PA-free asolectin, respectively.

Role of Anions in Reconstitution. Much attention has been dedicated to determine the conditions required for optimal reconstitution of transport activity. As shown earlier (McCormick et al., 1985), vesicle volume is an important factor, and volume enlargement may contribute to the enhancement of A-system-mediated transport. In view of the fact that proteoliposomes prepared in Na^+ are smaller than those prepared in K^+ and the presence of several anionic lipids bring about enlarged volume, we also examined a variety of inorganic anions to determine whether freeze/thawing in salts of these anions alters recovery of transport activity. We tested KBr, KI, KNO_3, K_2SO_4, and KCNS as replacement for KCl during reconstitution. With the exception that prior exposure to KI resulted in less activity, reconstituted vesicles made in the presence of the other anions (as their K salts) did not show major differences in activity or volume from those made with KCl.

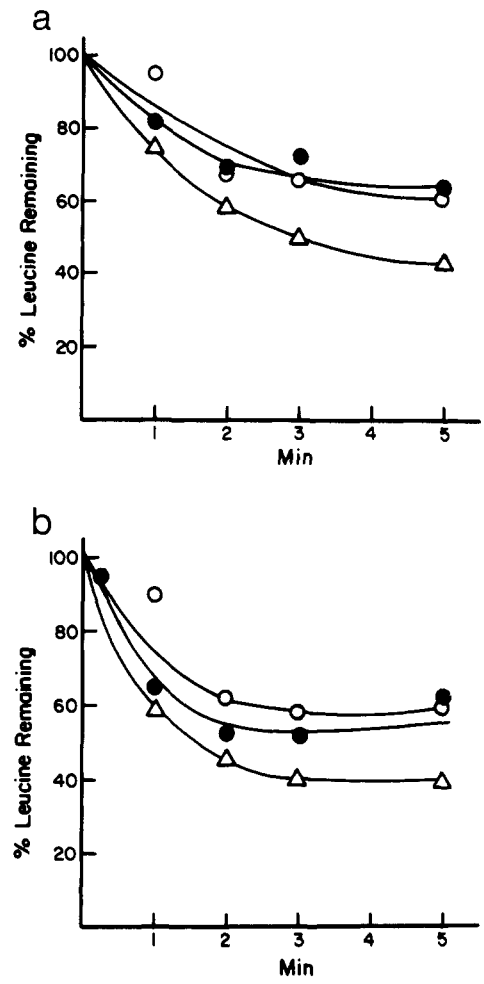

FIGURE 4: Exchange of leucine is not influenced by PA content of liposomes. Proteoliposomes, prepared from Ba^{2+}-precipitated or native asolectin, were treated with 28% PEG and washed as described in Figure 1. Then the proteoliposomes were incubated with L-[^{3}H]leucine (0.1 mM) (specific activity, 3.7×10^2 dpm/pmol). The preincubated proteoliposomes were diluted into K^+ medium with or without 5 mM Me-AIB or L-leucine and incubated at room temperature for the times given. The [^{3}H]leucine retained in the proteoliposomes was determined by a filtration assay (see Methods). Proteoliposomes made with total asolectin (a) and Pa-free asolectin (b). (●) Control; (○) Me-AIB; (△) L-leucine.

Discussion

We have shown that two species of acidic phospholipids, PA and cardiolipin, are fully effective in restoring Na^+-dependent transport activity to inactive proteoliposomes made with Ba^{2+}-precipitated asolectin. It is interesting to note that these two phospholipids are closely related species since the latter may be regarded as a duplex PA bridged by glycerol and both PA and cardiolipin are potentially −2 charged species of phospholipid.

Our earlier observations (McCormick & Johnstone, 1988) had shown that when exogenous egg PA is used to restore activity to Ba^{2+}-precipitated asolectin proteoliposomes, the amount of exogenous PA required to regain the original activity is nearly double (20%) the measured concentration of acidic lipid (PA + cardiolipin) in asolectin. Although several possibilities may account for this observation (e.g., differences in fatty acyl moieties in PA from different sources), a likely possibility is the presence of cardiolipin as well as phosphatidic acid in asolectin (Table II) (Kagawa & Racker, 1966; Vemuri & Philipson, 1987). At low mole fractions of acidic lipids, cardiolipin is the most effective anionic lipid for restoration of amino acid transport (Table IV). The presence of significant amounts of both acidic lipids, and particularly the surprisingly high content of PA in commercial asolectin, may be a significant factor in the efficacy of asolectin in reconstitution of function for a variety of membrane proteins.

Our results also indicate that the presence of lipids with unsaturated acyl chains is of great importance in restoring activity to proteoliposomes. Fully saturated phosphatidic acids (C_{10}, C_{12}, and C_{16}) are relatively ineffective in this regard, whereas synthetic PA containing two unsaturted fatty acids produces vesicles with high activity, comparable to that seen with egg PA. In PA with a single unsaturated fatty acyl moiety, the presence of the unsaturated acyl group in the β position is more effective in restoring activity than one with the unsaturated acyl group in the γ position. Such observations further argue for a specific interaction between the Na^+-dependent amino acid transporter and specific phospholipids. Although it is well-known that natural phospholipids normally have an unsaturated fatty acid in the β position, we believe this to be the first report that a membrane protein function is influenced by the position of the unsaturated moiety in the phospholipid.

Recently, several reports have appeared which showed highly specific lipid requirements for the expression of different

protein functions in different systems (George et al., 1989; Chiba & Mohri, 1989; Philipson & Nishimoto, 1984; Vemuri & Philipson, 1987; Lee & Bell, 1989; Pierce & Panagia, 1989). All these studies, including the present one, argue for the specificity of interaction between selected proteins and their immediate lipid environment.

It is unlikely that the present observations reflect a simple modulation of membrane fluidity (Sandermann, 1978; McElhaney, 1982; Yeagle, 1989) by exogenous PA and other acidic lipids, for the following reasons. (1) Our studies were carried out at room temperature where the liquid-crystalline state is already likely to exist. (2) The changes in transport are brought about by a relatively small change (~15%) in lipid content, the bulk (80%) of the bilayer being unchanged. (3) The glucose transporter and L-system exchange do not show the anionic lipid requirement associated with the Na^+-dependent A system. This specific effect of particular anionic phospholipids on the A system argues against a generalized modulation of protein function through fluidity changes. Overall, all our evidence argues for a selective interaction of specific lipids with the Na^+-dependent (A system) amino acid transporter.

In addition, we have by chance also made an observation which may prove technically useful in reconstitution studies of transporters. Treatment of reconstituted vesicles with PEG has been found to enhance the net accumulation of AIB as well as to reduce nonspecific permeability. Proteoliposomes with high nonspecific permeability are likely to have lower peak levels of solute accumulation than those with low nonspecific permeability. Recently, Fafournoux et al. (1989) commented that PEG precipitation improved the purification of a Na^+-dependent amino acid transporter from rat liver. Trace amounts of PEG remaining after precipitation may have altered the permeability of reconstituted proteoliposomes, accounting for some of the reported increase in Na^+-dependent amino acid transport. Although the action of PEG is not well documented, it is known to be an excellent dehydrating agent. Transient removal of water associated with either the proteins or the lipids during PEG incubation may allow for better integration of the protein into the bilayer as well as the formation of a less leaky bilayer, both of which would result in better uptake and energy coupling. Further work is needed to address the universality of the effects of PEG on the efficacy of restoration of transport in reconstituted systems.

In conclusion, this paper provides evidence that the nature of the phospholipid environment influences the behavior of the amino acid A-system transporter in a highly specific way. The specificity is reflected both in the nature of the lipid requirements for optimal A-system transport function and in the lack of similar lipid requirements for the glucose transporter and L-system amino acid transporter.

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