Role of the Phospholipid Environment in Modulating the Activity of the Rat Brain Synaptic Plasma Membrane Ca^{2+}-ATPase†

Mira Hermoni-Levine and Hannah Rahamimoff*
Department of Biochemistry, Hebrew University, Hadassah Medical School, P.O. Box 1172, Jerusalem 91010, Israel
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ABSTRACT: The role of the phospholipid environment in modulating the activity of the rat brain synaptic plasma membrane (SPM) Ca^{2+}-ATPase was investigated by its reconstitution into different phospholipids. Retention of activity of the solubilized Ca^{2+}-ATPase depended on addition of exogenous phospholipids. As the cholate concentration used for solubilization of native SPM increased, a larger excess of exogeneous phospholipids, relative to membrane protein, had to be added to maintain optimal activity. Highest ATP-dependent Ca^{2+} transport activity was obtained when reconstitution was carried out in calf brain phospholipids (BPLs) followed by soybean phospholipids (SPLs) and the lowest in egg PC; reconstitution at a 40:1 weight ratio of exogenous phospholipids to native SPM protein resulted in ATP-dependent Ca^{2+} transport of 40.0 ± 4.16, 23.4 ± 8.48, and 11.54 ± 2.31 nmol of Ca^{2+} (mg of protein)$^{-1}$ (5 min)$^{-1}$, respectively. Partial substitution of egg PC with BPLs led to an increase in the activity of the reconstituted Ca^{2+} pump. The highest ATP-dependent Ca^{2+} uptake was obtained when ratios of 15:25 or 10:30 egg PC to BPLs were used. Testing the individual phospholipids participating in the BPL mixture showed that addition of PS to egg PC led to a consistent increase in Ca^{2+} pump activity. Substitution of 50% of the PC with PS resulted in a 3.8-fold higher ATP-dependent Ca^{2+} uptake than that obtained in egg PC alone. No other phospholipid tested—PE, SM, or PI—had a similar effect. Increasing the proportion of PS within the BPL mixture above its original content led to a gradual decrease in the reconstituted SPM Ca^{2+} pump activity. Enrichment of asolectin with PS led first to increased Ca^{2+} pump activity; then, as the proportion of PS increased, Ca^{2+} transport of the reconstituted pump decreased. An increased proportion of PE, SM, or PI within the BPLs or asolectin, above their original contents, resulted in decreased Ca^{2+} transport. These results indicate that optimal SPM Ca^{2+} pump activity requires the combined presence of a critical amount of PC and PS within the reconstituted membrane.

The ATP-dependent Ca^{2+} pump is one of the major Ca^{2+} transporters instrumental in the maintenance of the low resting level of Ca^{2+} in the nerve terminal (DiPolo & Beauge, 1983). In this work, we have investigated the modulating role of the phospholipid environment on the Ca^{2+} transport activity of the rat brain synaptic plasma membrane (SPM)[1] Ca^{2+}-ATPase.

There is a considerable amount of evidence that changes in the phospholipid composition of biological membranes accompany some pathological and physiological conditions such as muscular dystrophy, ischemia, and aging (Owens & Hughes, 1970; Rouser & Salomon, 1969; Rubin et al., 1973; Chien et al., 1981). In view of the importance of the Ca^{2+} pump in the regulation of intracellular calcium ion concentration in nerve terminals and its relevance to synaptic communication (Augustine et al., 1987), studying the modulating role of the phospholipid environment on its activity might be of importance to understand some of the functional changes occurring in normal physiological processes such as aging, or in some pathological processes such as ischemia and muscular dystrophy.

Our experimental approach involved solubilization of the native membrane in a detergent (cholate), in the presence of a large excess of exogeneous lipids of well-defined composition, followed by detergent removal, reconstitution into a vesicle, and studying the corresponding Ca^{2+} pump activity of the reconstituted protein.

The presence of a large excess of exogenous phospholipids served four purposes: (1) With the increase in phospholipid to protein ratio, the probability of having the Ca^{2+} pump and another unrelated membrane protein in the same vesicle is considerably reduced, and therefore, the changes in phospholipid composition of the reconstituted membrane can be related to the Ca^{2+} pump (see Table III). (2) As the added phospholipid to protein ratio increased, the average size of the resulting reconstituted vesicle decreased. In the native SPM vesicle where the phospholipid to protein ratio is 1.14:1, the intravesicular volume was 6.14 μL/mg of phospholipid (Erdreich & Rahamimoff, 1983). This value decreased to 1.1 μL/mg of phospholipid (see Table II) when a 40:1 weight ratio of phospholipid to protein was used for reconstitution. As a consequence, the surface area of the reconstituted vesicle decreased to 30% of the native preparation. (3) The high phospholipid content also permits use of relatively high detergent concentrations, which increases the probability that the Ca^{2+} pump was stripped of its native phospholipid environment. (4) In addition, with a 40-fold excess of exogenous phospholipids, the native phospholipids are diluted considerably within the exogenous mixture, and the phospholipid environment of the reconstituted pump reflects the composition of the added lipids.

Using this approach, our results indicate that the phospholipid environment has an important role in modulating the

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* To whom correspondence should be addressed.

[1] Abbreviations: ATPase, adenosinetriphosphatase; EDTA, ethylenediaminetetraacetic acid; BPL(s), calf brain phospholipid(s); βME, β-mercaptoethanol; CMC, critical micellar concentration; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PL(s), phospholipid(s); SDS, sodium dodecyl sulfate; SM, sphingomyelin; SPM(s), synaptic plasma membrane(s); SPL(s), soybean phospholipid(s); KP_i, potassium phosphate buffer.

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activity of the SPM Ca^{2+}-ATPase. Among the phospholipid head groups used individually or in various combinations for reconstitution of the SPM Ca^{2+}-ATPase, optimal pump activity required the combined presence of phosphatidylcholine (PC) and phosphatidylserine (PS) within the reconstituted membrane. This could be provided by mixing appropriate amounts of PC/PS or by using BPLs fortified with PC. No other phospholipid, such as PE, SM, or PI, had a similar role in supporting optimal activity of the reconstituted Ca^{2+} pump.

Experimental Procedures

Synaptic plasma membranes (SPMs) were prepared from 14-day-old Sabra strain rats. Each SPM preparation consisted of 80 rat brains. The experimental procedure was similar to our previously published protocol (Rahamimoff & Spanier, 1979; Erdreich & Rahamimoff, 1983), except that a three-step Ficoll gradient (2%, 8%, and 12%) instead of five-step one was used. The same SPM-containing fraction collected from the 8/12% Ficoll interphase was used. It has been characterized and found to be enriched relative to the fraction below 12% Ficoll in the following activities: Ca^{2+}-dependent ATP hydrolysis [between 15 and 30 nmol of ATP hydrolyzed (mg of SPM protein)$^{-1}$ min^{-1} in different preparations], ATP-dependent Ca^{2+} transport [8–15 nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1} in different preparations] which is insensitive to FCCP [carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone], Na^+–Ca^{2+} exchange activity [9–15 nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1}], Na^+-coupled GABA (γ-aminobutyric acid) uptake [20 pmol (mg of protein)$^{-1}$ min^{-1}], and 5′ AMP nucleotidase activity [4.8 nmol (mg of protein)$^{-1}$ min^{-1}]. The 2/8% Ficoll interphase contains usually only small amounts of material with similar activities. The pellet at the bottom of the gradient and the fuzzy material above it contain the mitochondrial enzyme marker succinate dehydrogenase [assayed as described by Bonner (1955)] and ATP-dependent Ca^{2+} uptake [1.5 nmol (mg of protein)$^{-1}$ min^{-1}] that is completely inhibited by FCCP. This fraction also contains traces of Na^+-dependent Ca^{2+} and GABA uptake, but both activities are completely lost upon exposure of the fraction to hypoosmotic lysis medium (5 mM Tris-HCl buffer, pH 7.4, and 0.1 mM EDTA) and freezing in liquid N_2, which are the commonly used protocols for the preparation and storage of the SPM-containing (8/12% Ficoll interphase) fraction (Rahamimoff & Spanier, 1979). In the SPM-enriched fraction, (8/12% Ficoll interphase), these activities can be conserved for at least several months.

Reconstitution, using Sephadex G50 mini columns for detergent removal, was performed as described in our previously published procedure (Barzilai & Rahamimoff, 1987). The reconstitution involved solubilization of SPM (0.5 mg/mL) in a mixture containing potassium cholate, pH 7.4 (between 0.5 and 2% as specified in the individual experiments), 0.15 M KP_i, pH 7.4, and the desired amount of added phospholipids (as specified). The ratio of protein to the added phospholipid in the solubilization mixture varied, according to the experiment that was done. The mixture was kept 12 min at 23 °C and then was centrifuged 20 min at 4 °C at 27000*g* to remove unsolubilized membranes. Cholate was removed by gel filtration. This was achieved by passing the solubilized membrane–phospholipid mixture through Sephadex G50 mini columns. Three consecutive mini columns (except when specified otherwise) were used each time. Tuberculin syringes were filled with the gel previously preequilibrated with 0.15 M KP_i. The syringes were centrifuged for 2.5 min at about 1000*g* at room temperature. This step led to partial drying and shrinking of the gel. Careful introduction of the solubilized protein–phospholipid mixture (up to 250 μL/1-mL syringe) into the gel led to its reswelling to the original size. Centrifugation of these syringes under conditions exactly identical with those employed before led to vesicle formation, concentration, and collection in a minimal volume that has been excluded from the gel.

Measurement of the intravesicular volume of the reconstituted SPM vesicles was carried out by determination of the amount of trapped [^{3}H]inulin which has been introduced into the vesicles during the reconstitution procedure. [^{3}H]Inulin was added to the solubilizing solution of the SPMs and the phospholipids. The mixture was passed through two consecutive Sephadex mini columns preequilibrated with an amount of [^{3}H]inulin identical with that added to the solubilization mixture, followed by two Sephadex mini columns preequilibrated with unlabeled inulin of identical concentration. The amount of [^{3}H]inulin trapped within the reconstituted vesicles was determined.

Vesicle size distribution was measured by dynamic light scattering using the Malvern 4700C submicron particle size analyzer. A laser line of 488 nm at 90° was used. The temperature was 25 °C, and the solution viscosity was maintained at 0.8904. At least two different batches of reconstituted vesicles were used for each measurement, and it was repeated 3 times.

Measurement of calcium uptake activity in the reconstituted vesicles was performed by using Dowex 50 mini columns (Gasko et al., 1976). Usually, 5–10 μL of vesicles was diluted to 100 μL of the test medium which contained 0.15 M KP_i, 5 mM $MgCl_2$, and 100 μM $^{45}CaCl_2$ (0.1 μCi). Ca^{2+} uptake was measured at 23 °C in the presence of 2 mM ATP and in its absence. Each experiment was performed at least 5 times in duplicate. Initial ATP-dependent Ca^{2+} uptake experiments were carried out in the presence and in the absence of 1 mM ouabain or 0.1 mM strophantidin. These inhibitors of Na^+–K^+-ATPase had no affect, however, on the ATP-dependent Ca^{2+} uptake, and, therefore, they were later omitted from the reaction mixtures.

The amount of Ca^{2+}-dependent ATP hydrolysis was calculated from the difference in the hydrolysis of [γ-^{32}P]ATP in the presence of 5 mM $MgCl_2$ and 100 μM $CaCl_2$ to that obtained in the presence of 5 mM $MgCl_2$ and 0.1 mM EGTA as described by Papazian et al. (1984).

Brain phospholipids (BPLs) were prepared by modification of the Bligh and Dyer extraction procedure (Sheltawy & Dawson, 1969) followed by chromatography on a potassium oxalate washed column of silicic acid. The column was washed with chloroform to remove neutral lipids followed by extraction of the phospholipids with methanol/chloroform (80:20). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from egg yolks as described by Ansell and Hawthorne (1964). The composition of phospholipid mixtures was determined by two-dimensional thin-layer chromatography as described by Yavin and Zutra (1977). Quantitative phospholipid phosphate determination (Bartlet, 1959) of each spot was done by scraping the plates. The fatty acid composition of the different phospholipids was determined by gas chromatography employing a Tracor 540 instrument and a Sillar 10C column. Protein was determined by the method of Lowry et al. (1951). High lipid containing samples were assayed in the presence of SDS (sodium dodecyl sulfate). Phospholipid phosphate was determined as described (Ames, 1974). Asolectin was purchased from Associated Concentrates (Woodside, NY). All other biochemical reagents, including bovine brain PS and SM, were purchased from Sigma, Israel.

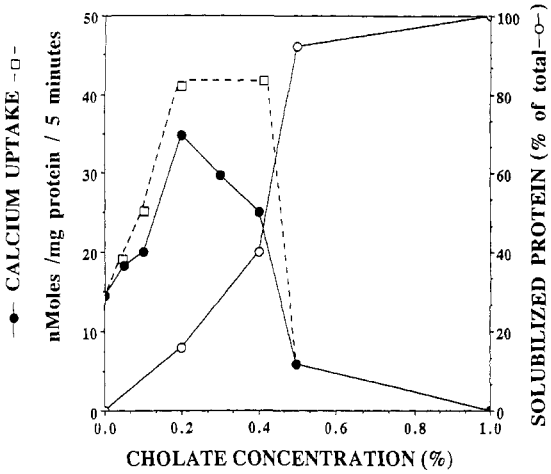

FIGURE 1: ATP-dependent Ca^{2+} transport in cholate-treated SPM vesicles. 0.25 mg of SPM protein was added to 0.5 mL of a mixture that contained 0.15 M KP_i, pH 7.4, 5 mM βME, 0.1 mM EDTA, and different cholate concentrations. Cholate removal was carried out by gel filtration as described under Experimental Procedures. ATP-dependent Ca^{2+} uptake (●) was measured as described by Gasko et al. (1976), by diluting 10 μL of the cholate-treated SPM vesicles (about 5 μg of protein) into 90 μL of a mixture that contained 0.15 M KP_i, pH 7.4, 100 μM $^{45}CaCl_2$ (0.1 μCi), 5 mM $MgCl_2$, and 2 mM ATP. The Ca^{2+} taken up in the absence of ATP was subtracted. The percent of protein solubilized at each cholate concentration (○) was determined by measuring the total protein content of the cholate-treated SPMs after detergent removal and following centrifugation at 27000*g* for 20 min in the supernatant. ATP-dependent Ca^{2+} uptake in cholate-treated SPMs after correction for the amount of protein solubilized (□).

$^{45}CaCl_2$ and [^{3}H]inulin were purchased from Amersham, England, and [γ-^{32}P]ATP was from NEN, Boston, MA.

RESULTS

Optimal Conditions for Reconstitution of the SPM Ca^{2+} Pump

Determination of the Cholate Concentration Required To Solubilize the SPM Ca^{2+} Pump. In order to investigate systematically the modulating effect that the phospholipid environment exerts on the SPM Ca^{2+} pump, optimal conditions for its reconstitution were established. This included the choice of cholate as the detergent to be used in these experiments, determination of its appropriate concentration relative to the amount of membrane protein to be used, and determination of the optimal phospholipid to protein ratio.

Figure 1 shows the activity profile of the SPM Ca^{2+} pump following exposure of the membranes to 0–1% cholate and its subsequent removal by gel filtration. Detergent treatment was carried out without addition of exogeneous phospholipids.

Cholate treatment of native SPMs exhibited two phases: up to 0.4% cholate, an increase in ATP-dependent Ca^{2+} uptake relative to that measured in the native preparation was observed. Higher detergent concentrations led to gradual loss of Ca^{2+} transport activity.

Determination of the amount of SPM protein which has been solubilized by the detergent treatment revealed that in 0.2% cholate about 16% of the membrane protein was solubilized and in 0.4% cholate 40%, and from 0.5 to 1% cholate, between 85% and 99% of the protein was in the soluble fraction.

The specific Ca^{2+} transport activity of the detergent-treated SPMs corresponding to each cholate concentration was calculated by subtracting the amount of solubilized protein from the total amount of SPM protein (dashed line connecting the open squares).

Table I: Effect of Added Phospholipids on ATP-Dependent Ca^{2+} Uptake in Cholate-Treated SPM Vesicles[a]

cholate treatment (%)	protein:exogenous lipid weight ratio	ATP-dependent Ca^{2+} uptake [nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$]
none		27.0
0.2		54.0
0.2	1:2	63.0
0.4		46.3
0.4	1:2	84.9
0.4	1:5	96.4
0.5		8.8
0.5	1:2	46.9
0.5	1:5	93.08
1.0		0.51
1.0	1:2	0
1.0	1:5	1.08
1.0	1:10	63.2

[a] 0.25 mg of SPM proteins was added to a 0.5-mL solution that contained 0.15 M KP_i, pH 7.4, 0.1 mM EDTA, 5 mM βME, between 0 and 1% potassium cholate, and between 0 and 2.5 mg of BPL. The method of cholate removal and measurement of Ca^{2+} uptake is described under Experimental Procedures.

From this experiment, it is evident that substantial solubilization of the SPM proteins leads to loss of ATP-dependent Ca^{2+} transport activity. The lost activity cannot be recovered by detergent removal.

Exogenous Phospholipids Conserve the Activity of the SPM Ca^{2+} Pump. Addition of small amounts of exogenous phospholipids to the SPMs during detergent treatment leads to conservation of the Ca^{2+} pump activity. This is especially notable as the amount of detergent increases and presumably more and more SPMs are solubilized. Table I compares the ATP-dependent Ca^{2+} uptake activity measured in SPMs treated by various cholate concentrations in the absence and in the presence of exogenous phospholipids. It can be seen that in 0.2% cholate, when only a small amount of the SPM proteins are solubilized and an overall activation of the Ca^{2+} pump activity is obtained, the addition of a 2-fold excess of exogenous phospholipids has only a minor effect on the total Ca^{2+} pump activity. As the detergent concentration increases from 0.4% to 1% and increasingly higher amounts of SPM protein are solubilized, addition of exogenous phospholipids to the solubilization mixture results in conservation of the increased ATP-dependent Ca^{2+} transport activity.

Figure 2A–C compares the ATP-dependent Ca^{2+} uptake measured in SPMs solubilized in 1%, 1.5%, and 2% cholate in the presence of different amounts of exogenously added phospholipids. In Figure 2A, the detergent concentration was 1%, in Figure 2B 1.5%, and in Figure 2C 2%.

Several general conclusions can be obtained from Figure 2: (1) Exposure to detergent in the absence of exogenous phospholipids leads to loss of ATP-dependent Ca^{2+} uptake of the solubilized SPMs which is not recovered following detergent removal. This holds for all the cholate concentrations used. (2) The higher the detergent concentration that was used to solubilize the SPM, more exogenous phospholipid had to be added in order to detect Ca^{2+} pump activity. Thus, in 1% cholate, between 5- and 10-fold excess by weight of exogenous phospholipid over SPM protein had to be added, in 1.5% between 10- and 20-fold excess, and at 2% cholate between 20- and 30-fold excess had to be added to detect ATP-dependent Ca^{2+} transport activity. (3) In all the detergent concentrations used, an increased phospholipid to protein ratio led to increased ATP-dependent Ca^{2+} uptake relative to lower phospholipid to protein ratios at the same detergent concentration. Thus, an increase in the protein to phospholipid ratio

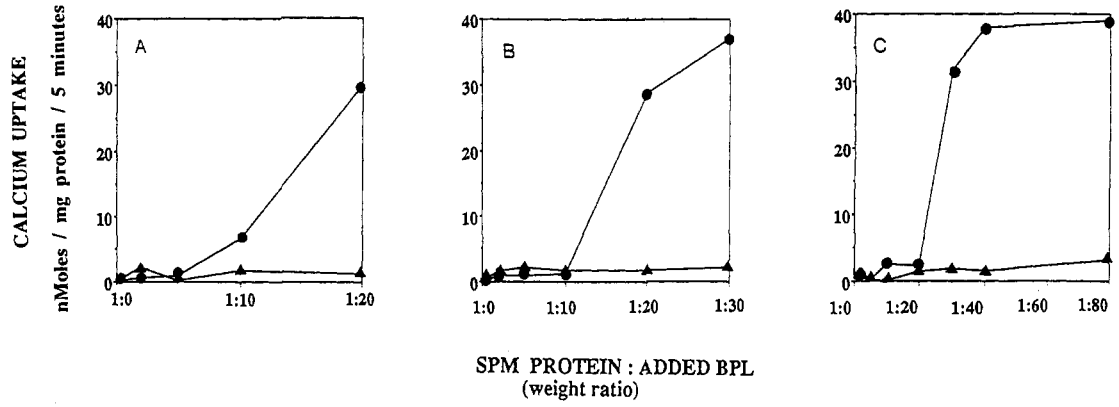

FIGURE 2: Effect of varying the protein to phospholipid ratio on the Ca^{2+} uptake in reconstituted SPM vesicles. 0.25 mg of SPM protein was added to 0.5 mL of mixtures that contained 0.15 M KP_i, pH 7.4, 5 mM βME, 0.1 mM EDTA, and varying amounts of BPLs and potassium cholate. Ca^{2+} uptake was measured by diluting 10 μL of the reconstituted SPM vesicles into 90 μL of a solution that contained 0.15 M KP_i, pH 7.4, 100 μM $^{45}CaCl_2$, and 5 mM $MgCl_2$: (●) in the presence of 2 mM ATP; (▲) in the absence of ATP. (A) The solubilization procedure was carried out in the presence of 1% potassium cholate, (B) 1.5% cholate, and (C) 2% cholate.

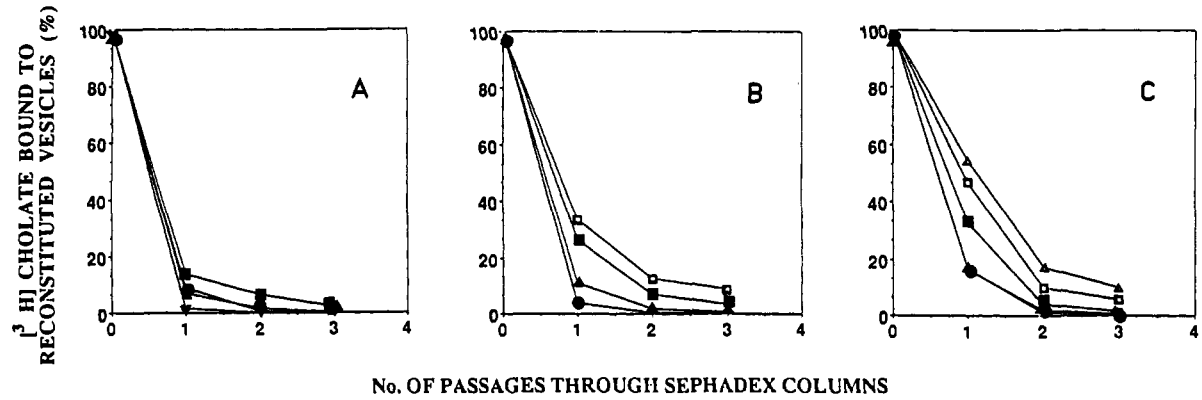

FIGURE 3: Efficiency of cholate removal from reconstituted SPM vesicles by gel filtration. 0.25 mg of SPM protein was added to 0.5 mL of a mixture that contained 0.15 M KP_i, pH 7.4, 5 mM βME, 0.1 mM EDTA, and different amounts of BPLs and [^{3}H]cholate. (A) 0.5% cholate; (B) 1% cholate; (C) 1.5% cholate. Reconstitution was carried out by consecutive passages through Sephadex G50 mini columns as described under Experimental Procedures. The amount of residual [^{3}H]cholate associated with the vesicles was determined by counting an aliquot from the membranes excluded from each column. (▼) Percent of residual cholate in the absence of added BPLs; (●) percent of residual cholate at 1:2 protein/BPLs (weight ratio); (▲) at 1:5 protein/BPLs (weight ratio); (■) 1:10 protein/BPLs; (□) 1:20 protein/BPLs; (△) 1:30 protein/BPLs.

from 1:20 to 1:30 at 1.5% cholate concentration (Figure 2B) or from 1:30 to 1:40 at 2% cholate concentration led to increasingly higher ATP-dependent Ca^{2+} uptake till a steady-state level of Ca^{2+} uptake was reached. Further increase in the phospholipid to protein ratio, even as high as 80:1 (Figure 2C), did not lead to a further increase in Ca^{2+} uptake.

Efficiency of Cholate Removal by Gel Filtration. In order to rule out that the absence of Ca^{2+} pump activity at low phospholipid to protein ratios observed in all the cholate concentrations used for solubilization of the SPMs was not the result of inefficient detergent removal by Sephadex G50 mini columns, [^{3}H]cholate was added to the membrane solubilization mixture. The amount of detergent excluded from the gel in association with the membranes was determined.

Figure 3A–C shows the residual cholate content after passage through consecutive Sephadex mini columns.

Cholate concentrations of 0.5% (Figure 3A), 1.0% (Figure 3B), and 1.5% (Figure 3C) were used. The protein to added phospholipid ratio varied between 1:2 and 1:5 in Figure 3A, 1:2 and 1:20 in Figure 3B, and 1:5 and 1:30 in Figure 3C. Native membranes solubilized in the absence of added phospholipids are also shown (Figure 3A).

It can be seen that the amount of detergent that remains associated with the reconstituted membranes depends on (1) the number of Sephadex G50 mini columns through which the membrane solubilization mixture was passed for detergent removal, (2) the concentration of the detergent used for solubilization of the membranes, and (3) the ratio of phospholipid to protein used. The higher the phospholipid content, more detergent remains associated with it, and more passages of the reconstituted membranes through the mini columns are required to remove it. This pattern is exhibited also when the cholate concentration is increased to 2% (not shown). Thus, the absence of ATP-dependent Ca^{2+} pump activity at low phospholipid to protein ratios was not a result of inefficient cholate removal.

Since after passing the solubilized membranes through three consecutive mini columns the residual amount of detergent did not exceed 10% of its original concentration and no further significant detergent removal nor an increase in ATP-dependent Ca^{2+} transport occurred by addition of more columns (not shown), in all the forthcoming experiments three passages through consecutive Sephadex mini columns were used.

Intravesicular Volume of the Reconstituted SPM Vesicles at Different Phospholipid to Protein Ratios. An increase in the phospholipid to protein ratio leads to a respective decrease in the intravesicular volume of the reconstituted preparation. In the experiments shown in Table II, SPMs were added to a solution containing different ratios of phospholipid to SPM protein. The cholate concentration was also varied. [^{3}H]Inulin was introduced into the vesicles during reconstitution. It can be seen that the intravesicular volume decreases from 6.14

Table II: Intravesicular Volume of Reconstituted SPM Vesicles at Different Phospholipid to Protein Ratios[a]

protein:phospholipid weight ratio	cholate (%)	intravesicular space (μL/mg of phospholipid)
1:10	1.5	2.7
1:20	1.5	2.0
1:20	2.0	2.0
1:30	2.0	1.5
1:20	2.8	2.1
1:30	2.8	1.49
1:40	2.8	1.11
SPM[c]	none	6.14
1:5[b]	2.0	3.07
1:2[b]	2.0	4.14

[a] Vesicles were preloaded during reconstitution with [^{3}H]inulin or [^{3}H]glucose (marked by *b*) as described under Experimental Procedures. The intravesicular space was calculated from the amount of [^{3}H]inulin or [^{3}H]glucose trapped in the vesicles. [b] ^{3}H-Labeled intravesicular glucose space from A. Barzilai, Ph.D. Thesis, Hebrew University, Jerusalem (1987). [c] From Erdreich and Rahamimoff (1983).

μL/mg of phospholipid in native SPM (measured by determination of the total ^3H_2O space corrected for intervesicular [^{14}C]inulin space) to 1.1 μL/mg of protein in the reconstituted preparation containing a 40-fold excess of phospholipids over native proteins. Lower phospholipid to protein ratios in the reconstituted preparation have intermediate intravesicular volumes. The cholate concentration used to solubilize the membranes did not affect the intravesicular volume of the reconstituted vesicles.

Reconstitution into High Phospholipid to Protein Ratio Separates Native SPM Proteins among Different Phospholipid-Containing Vesicles. As a consequence of the reconstitution process at high phospholipid to protein ratio [(30–40):1], synaptic plasma membrane proteins that were within the same native membrane structure separate among different reconstituted vesicles. This can be demonstrated by comparing the relationship between the SPM Ca^{2+}-ATPase and the Na^+–Ca^{2+} exchanger in native and reconstituted SPM vesicles. Both native and reconstituted synaptic plasma membrane vesicles can take up Ca^{2+} in at least two ways: in an ATP-dependent manner and in a Na^+ gradient dependent manner (Rahamimoff & Spanier, 1979, 1984; Barzilai et al., 1984; Papazian et al., 1984). In native SPM vesicles, Ca^{2+} efflux from Ca^{2+}-loaded vesicles can be induced by their dilution into an external high [Na^+] containing medium regardless of the manner in which they were loaded with Ca^{2+}. This can be done since the Na^+–Ca^{2+} exchanger can transport Ca^{2+} in both directions across the membrane (Rahamimoff & Spainer, 1979, 1984) and both activities are present within the same membrane vesicles. When, however, the experiment is repeated with the reconstituted SPM vesicles, dilution into high NaCl containing medium does not lead to Ca^{2+} efflux when the vesicles were preloaded with Ca^{2+} in an ATP-dependent manner. Table IIIA summarizes this experiment. It can be seen that upon dilution into high [Na^+] the Ca^{2+} content of native SPM vesicles decreases to 61% already after 30 s and after 10 min only 15.9% of the original Ca^{2+} content of the vesicles remained. In the reconstituted preparation, dilution into high [Na^+] containing medium does not lead to Ca^{2+} efflux, and the Ca^{2+} content of the vesicles remains almost constant throughout the test period of 10 min.

To rule out that upon reconstitution the Na^+ gradient dependent Ca^{2+} efflux mechanism is not conserved, a similar experiment was carried out except that Ca^{2+} loading was carried out in a Na^+ gradient dependent manner (Table IIIB). It can be seen that 57.7% of the Ca^{2+} content of the native vesicles and 48.2% of the Ca^{2+} content of the reconstituted vesicles are lost in 10 min. It should be remembered that a lesser amount of Na^+ gradient dependent Ca^{2+} efflux from these vesicles is expected than from the vesicles preloaded in an ATP-dependent manner since the intravesicular medium contains Na^+ and hence the driving force for Ca^{2+} efflux is smaller: 0.1 M $[Na^+]_{in}$ and 0.2 M $[Na^+]_{out}$ in the Na^+ gradient preloaded vesicles and 0 M $[Na^+]_{in}$/0.15 M $[Na^+]_{out}$ in the vesicles preloaded with Ca^{2+} in an ATP-dependent manner. Comparing the relative amount of Na^+ gradient driven Ca^{2+} efflux from reconstituted SPM vesicles to that of the native vesicles reveals that 83.5% of the Ca^{2+} is lost from the reconstituted vesicles. Thus, following reconstitution, the Na^+ gradient dependent efflux mechanism is conserved and functional, but no longer present in the same vesicles as the ATP-dependent Ca^{2+} uptake system.

Table III: Na^+ Gradient Dependent Ca^{2+} Efflux from Native and Reconstituted SPM Vesicles

(A) Vesicles Preloaded in an ATP-Dependent Manner[a]

	Ca^{2+} content (nmol/mg of protein) at *t* (min)			
	0	0.5	1	10
native SPM	19.8 (100%)	12.1 (61%)	7.4 (37%)	3.15 (15.9%)
reconstituted SPM	13.8 (100%)	14.5 (104%)	14.4 (104%)	12.8 (93%)

(B) Vesicles Preloaded in a Na^+ Gradient Dependent Manner[b]

	Ca^{2+} content (nmol/mg of protein) at *t* (min)			
	0	1	5	10
native SPM	21.68 (100%)	14.58 (67%)	9.68 (45%)	9.16 (42.25%)
reconstituted SPM	49.2 (100%)		29.5 (59%)	25.5 (51.8%)

[a] Native SPM vesicles were preloaded with 0.15 M KCl–0.01 M KP_i buffer, pH 7.4, by preincubation at 37 °C for 20 min as described by Rahamimoff and Spanier (1979). Reconstituted vesicles were preloaded with an identical solution during reconstitution on Sephadex G50 mini columns as described under Experimental Procedures. Both vesicles were exposed to 100 μM $^{45}CaCl_2$ (0.1 μCi/0.1 mL) in an external solution of identical composition, except that it also contained 2 mM ATP and 5 mM $MgCl_2$. After 10-min incubation at 23 °C, the vesicles were separated from the uptake medium by centrifugation. Native SPMs were centrifuged at 27000*g* for 15 min at 4 °C and the reconstituted SPMs at 1000000*g* for 4 h at 4 °C. The pelleted vesicles were suspended in a minimal volume of the uptake medium, and efflux was initiated by dilution into an isoosmotic NaCl-containing medium that did not contain ATP and Mg^{2+}. Five microliters of vesicles (about 3–7 μg of protein) was diluted into 250 μL of solution. The $^{45}Ca^{2+}$ content of the vesicles was determined at the times specified either by rapid dilution with ice-cold KCl solution and filtration through 0.45-μm Schleicher & Schuell nitrocellulose filters (native SPM) or by loading onto a Dowex 50 mimi column as described under Experimental Procedures (reconstituted SPM). Dilution with an external KCl control solution led to almost no Ca^{2+} loss during the test 10 min from both types of vesicles. [b] Native and reconstituted vesicles were preoaded with 0.1 M NaP_i–0.1 M choline chloride in a manner identical with that described in (A). They were diluted [3 μL into 250 μL of an external medium containing 0.2 M KCl, 0.01 M Tris-HCl, pH 7.4, and 50 μM $^{45}CaCl_2$ (0.1 μCi/reaction mixture)]. Internal high Na^+ gradient dependent Ca^{2+} loading of the vesicles was carried out for 20 min, after which the vesicles were separated from the uptake medium by centrifugation as described in (A). The pelleted vesicles were suspended in an aliquot of the uptake medium and diluted (3 μL into 500 μL) into an isoosmotic solution composed of buffered NaCl. The $^{45}Ca^{2+}$ content of the vesicles was determined as described in (A).

Is the Activity of the Reconstituted SPM Ca^{2+} Pump Affected by the Phospholipid Environment?

Reconstitution of the SPM Ca^{2+} Pump into BPLs, SPLs, and Egg PC. To compare the adeptness of different phospholipids in supporting the SPM Ca^{2+}-ATPase activity, synaptic plasma membranes were reconstituted into two different phospholipid mixtures: calf brain (BPL) and soybean phospholipids (SPL–asolectin) as well as into egg phosphatidylcholine (PC). In all the forthcoming experiments, the detergent concentration was 2%, and the protein to phospholipid ratio was 1:40. Saturating Ca^{2+} concentrations (Sorensen &

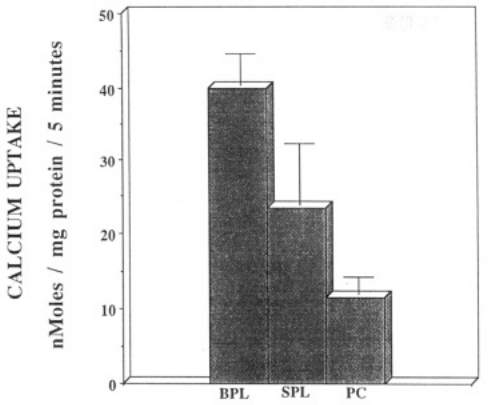

FIGURE 4: Ca^{2+} uptake in SPM vesicles reconstituted into brain phospholipids, soybean phospholipids, and egg phosphatidylcholine. 0.25 mg of SPM protein was added to 0.5 mL of a mixture that contained 0.15 M KP_i, pH 7.4, 5 mM βME, 0.1 mM EDTA, and 2% potassium cholate; each mixture contained 10 mg of the added phospholipid. Reconstitution and ATP-dependent Ca^{2+} uptake measurements were carried out as described under Experimental Procedures. (1) ATP-dependent Ca^{2+} uptake in brain phospholipids, 40.0 ± 4.16 (n = 9); (2) ATP-dependent Ca^{2+} uptake in soybean phospholipids, 23.4 ± 8.48 (n = 6); (3) ATP-dependent Ca^{2+} uptake in egg phosphatidylcholine, 11.54 ± 2.31 (n = 13). The Ca^{2+} taken up by the vesicles in the absence of ATP has been subtracted. It did not exceed 1.8% in BPLs, 1.4% in asolectin, and 9.9% in egg PC.

Mahler, 1981; Michaelis et al., 1983) (100 μM) were used. The rate of ATP-dependent Ca^{2+} uptake into the reconstituted SPM vesicles is linear up to 1 min (not shown). Thereafter, the rate gradually decreases, and it reaches steady-state values between 5 and 10 min of uptake in different phospholipid preparations. From the results presented in Figure 4, it can be seen that the highest Ca^{2+} uptake was reached when native SPMs were reconstituted into brain phospholipids, it was lower in soybean phospholipids, and the lowest ATP-dependent Ca^{2+} uptake was measured in egg phosphatidylcholine.

Similar results were obtained when the initial rates of the ATP-dependent Ca^{2+} uptake of the SPM vesicles reconstituted into the three different phospholipids were compared [16.9, 11.4, and 3.5 nmol (mg of protein)$^{-1}$ (1 min)$^{-1}$, respectively].

The Ca^{2+}-dependent fraction of the (Ca^{2+} + Mg^{2+})-dependent ATP hydrolysis of the reconstituted SPMs constitutes between 15 and 30% in different phospholipid preparations. In two different experiments, the extent of Ca^{2+}-dependent ATP hydrolysis was found to be comparable to the extent of the ATP-dependent Ca^{2+} transport. Values of 59 nmol of ATP hydrolyzed (mg of protein)$^{-1}$ (5 min)$^{-1}$ in SPMs reconstituted into BPLs, 28 nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$ in SPLs, and 13.2 nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$ in egg PC were obtained, indicating that the coupling ratio between Ca^{2+}-dependent ATP hydrolysis and ATP-dependent Ca^{2+} transport was about 1.

In order to try and understand whether the difference in the amount of the ATP-dependent Ca^{2+} uptake obtained in the different phospholipids resulted from the difference in composition of the phospholipid head groups or the corresponding fatty acyl chains, an exact analysis of the composition of the phospholipids used was performed. Table IV shows the composition of the phospholipid head groups of rat brain SPM, calf brain phospholipids, and the preparation of asolectin used. Table V shows the corresponding fatty acid content.

From Table IV, it can be seen that PC and PE (phosphatidylethanolamine) are major constituents in all the phospholipid mixtures used for reconstitution. Their combined amount reaches above 70% of the total phospholipid content. The major difference between brain phospholipids and soybean

Table IV: Phospholipid Composition of Soybean, Calf Brain, and Rat Brain Synaptic Plasma Membrane[a]

	soybean (%)	calf brain[b] (%)	rat SPM[c] (%)
PE	40.1	28–41	33.9
PC	31.9	29–48	46.9
SM	11.4	7–15	7.09
PG	8.2		
PI	3.9	7–12	5.6
PS	4.1	8–10	7.09

[a] 500 nmol from each phospholipid preparation was separated by two-dimensional thin-layer chromatography (Yavin & Zutra, 1977). Identification of the different phospholipids was carried out by comparison with known markers. Quantitative determination of the phospholipids was carried out by the phosphate assay described by Bartlett (1959). [b] Range in different preparations. [c] Average of three preparations of rat brain SPMs (80 brains each).

Table V: Fatty Acid Composition of Calf Brain, Soybean Phospholipids, and Egg Phosphatidylcholine[a]

	calf brain (%)	soybean (%)	egg PC (%)
myristic acid, 14:0	5.1		
palmitic acid, 16:0	41.0	25.1	43.3
stearic acid, 18:0	14.5	5.4	8.4
oleic acid, 18:1	25.0	9.4	34.4
linoleic acid, 18:2	2.5	53.3	11.5
linolenic acid, 18:3	1.7	7.0	
arachidic acid, 20:0	6.6		2.2
arachidonic acid, 20:4	3.8		

[a] Fatty acid identification was carried out by gas chromatography in comparison to known markers as described under Experimental Procedures.

phospholipids is manifested mainly in the negatively charged head group containing lipids: brain phospholipids contain almost 2 times more phosphatidylserine (PS) and phosphatidylinositol (PI) than soybean phospholipids. No phosphatidylglycerol (PG) was detected in the brain phospholipid mixture, while soybean phospholipids contained about 8% PG.

The two phospholipid mixtures and the egg PC used differed also in their fatty acid composition. The highest unsaturated fatty acid content was found in soybean phospholipids, and it reached 69.7%. Egg PC contained 46% unsaturated fatty acids while in brain phospholipids their total amount was only 33%. The highest content (about 70%) of long-chain fatty acids is found in asolectin. BPLs and egg PC both contain about 50% of long-chain fatty acids. Since the order of Ca^{2+} uptake activity obtained in the three different phospholipids used for reconstitution did not follow the order of the calculated ratio of unsaturated to saturated fatty acids, nor could it be linked to the chain length of the fatty acids, these parameters alone could not be the major reason for the difference in the ATP-dependent Ca^{2+} transport measured.

Importance of Diverse Phospholipid Head Groups in Supporting the Activity of the Reconstituted SPM Ca^{2+} Pump. In order to understand whether a specific proportion of the different phospholipids (such as that found in the BPL mixture) was necessary for optimal reconstitution of a functional SPM Ca^{2+} pump, or catalytic amounts of one or more of these phospholipids were sufficient in supporting ATP-dependent Ca^{2+} transport activity, an experiment was carried out in which increasing parts of the egg PC were substituted by BPLs. The total ratio of protein to lipid was kept constant at 1:40 (by weight), and its composition varied between 0 and 40 parts BPL to 40–0 parts PC. From the results shown in Figure 5, it can be seen that as increasing proportions of egg PC are exchanged with BPLs, a gradual increase in ATP-dependent Ca^{2+} transport activity occurs. It is interesting to note that when the reconstitution was carried out in the

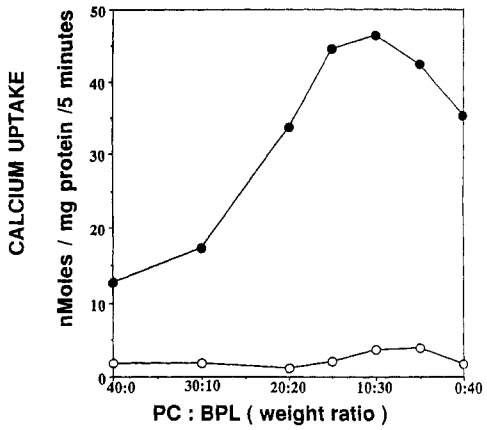

FIGURE 5: Effect of mixing egg PC with different amounts of BPLs on Ca^{2+} uptake in reconstituted SPM vesicles: 0.25 mg of SPM protein was added to 0.5 mL of a solution that contained 10 mg of phospholipids composed of different ratios of egg PC/BPLs in 0.15 M KP_i, pH 7.4, 5 mM βME, 0.1 mM EDTA, and 2% potassium cholate. The total protein to phospholipid ratio was kept constant at 1:40 by weight. The method of reconstitution and measurement of Ca^{2+} uptake is described under Experimental Procedures. Ca^{2+} uptake in the presence of 2 mM ATP (●) and in the absence of ATP (○).

presence of the BPL mixture alone, the ATP-dependent Ca^{2+} transport activity of the reconstituted pump was lower than in 15:25, 10:30, or even 5:35 mixtures of egg PC/BPLs.

From this experiment, it is clear that addition of catalytic amounts of different phospholipids to the egg PC is not sufficient to support optimal Ca^{2+} pump activity. Optimal reconstitution of the SPM Ca^{2+} pump depends on the presence of specific amounts of one or more of the phospholipids that are present in the BPL mixture. Moreover, an increase in the PC content above that which is present in the BPL mixture results in higher ATP-dependent Ca^{2+} transport activity of the reconstituted pump.

A similar experiment to that shown in Figure 5 (not shown), except that asolectin was used to enrich the egg PC in the reconstitution mixture, led to an activity profile similar to that shown in Figure 5, except, that the overall activity of the reconstituted Ca^{2+} pump was lower. Thus, fortifying egg PC either by a phospholipid mixture obtained from calf brain or from soybeans results in increased activity of the reconstituted ATP-dependent Ca^{2+} pump from SPM. The extent though of the ATP-dependent Ca^{2+} transport activity is higher when BPLs are mixed with egg PC.

Importance of PS Content in the Reconstituted SPM Ca^{2+} Pump. In order to investigate whether any phospholipid in particular, which is present in the brain phospholipid mixture, can account by itself for the increased Ca^{2+} pump activity when added to egg PC (Figure 5), these were added individually.

Among the various phospholipids, PS plays a special role in mediating Ca^{2+}-associated processes (Ekerdt & Papahadjopoulos, 1982; Zurini & Carafoli, 1982; Nir, 1984; Feigenson, 1986). Examining its effect on the activity of the reconstituted SPM Ca^{2+}-ATPase was of special interest.

To study the effect of PS in the membrane on the activity of the reconstituted SPM Ca^{2+} pump, parts of egg PC in the phospholipid mixture used for reconstitution were substituted with increasing amounts of bovine brain PS. The total phospholipid to protein weight ratio was maintained constant at 40:1. The ATP-dependent Ca^{2+} uptake corresponding to each PC/PS mixture was determined. Figure 6A shows such an experiment. It can be seen that addition of PS to PC from 0 to 50% leads to a steady increase in the ATP-dependent Ca^{2+} uptake. Higher ratios of egg PC/PS could not be reached due to the limited solubility of bovine brain PS.

Different batches of BPLs contain about 8–10% PS. In order to study the question whether increased PS content, above that found in the native BPL mixture, will lead to an increased activity of the reconstituted ATP-dependent Ca^{2+} pump, a similar experiment to that shown in Figure 6A (except that mixtures of BPLs and PS were used) was performed. This is shown in Figure 6B. Substitution of 5 parts out of 40 of the BPL mixture with PS (35:5 ratio) does not change substantially the ATP-dependent Ca^{2+} transport activity relative to BPLs alone. Since the BPL mixture which was used in these experiments contained 8% endogenous PS, substitution of 5 parts of 40 increased the amount of PS to 20.2%. Further increase in the amount of PS by substituting 10 and 20 parts of 40 (Figure 6B) in the reconstitution mixture, which increased the proportion of PS to 31.35% and 54.5%, led to a respective decrease in the ATP-dependent Ca^{2+} uptake.

Asolectin, a soybean phospholipid mixture, contains lower amounts of PS (4.1% only) than BPLs. A similar experiment to that done with BPLs was carried out using different mixtures of SPLs/PS for reconstitution. The results are presented in Figure 6C. Fortifying the soybean phospholipid mixture with added PS by increasing its amount from 4% to 16.7% increases the ATP-dependent Ca^{2+} uptake almost 2-fold. Increasing the amount of added PS to 28.5% led to a slight decrease (relative to that obtained when the PS content was 16.5%) in the ATP-dependent Ca^{2+} uptake. A further increase in the relative amount of PS in the soybean phospholipid

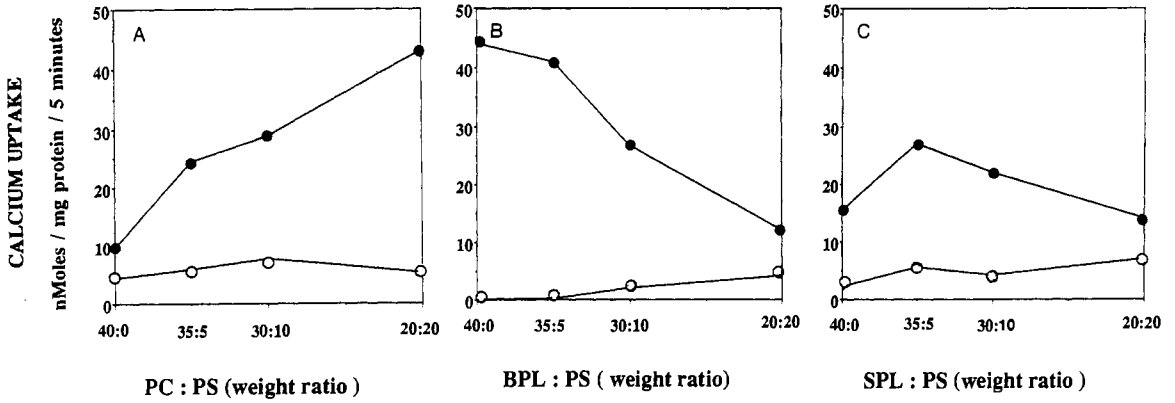

FIGURE 6: Effect of increased PS content in egg PC, BPLs, and SPL on the ATP-dependent Ca^{2+} uptake in reconstituted SPM vesicles: 0.25 mg of SPM protein was added to 0.5 mL of a mixture that contained 0.15 M KP_i, pH 7.4, 5 mM βME, 0.1 mM EDTA, 2% potassium cholate, and 10 mg of phospholipids composed of different ratios of egg PC and bovine brain PS (A), BPLs and PS (B), and SPL and PS (C). The method of reconstitution and measurement of Ca^{2+} uptake is described under Experimental Procedures. Ca^{2+} uptake in the presence of 2 mM ATP (●) and in the absence of ATP (○).

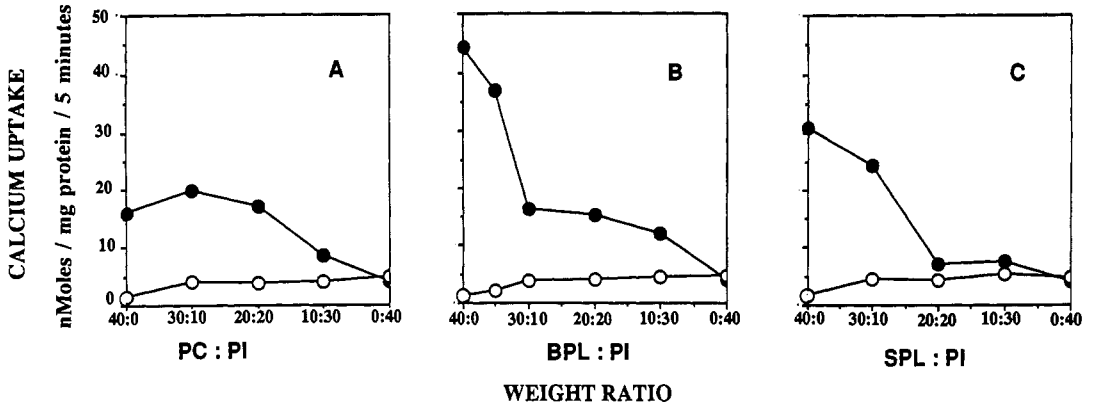

FIGURE 7: Effect of increased PI in egg PC, BPLs, and SPLs on the ATP-dependent Ca^{2+} uptake in reconstituted SPM vesicles: 0.25 mg of SPM protein was added to 0.5 mL of solution that contained 10 mg of phospholipids composed of different ratios of egg PC and bovine brain PI (A), BPLs/PI (B), and SPL/PI (C). The solution also contained 0.15 M KP_i, pH 7.4, 5 mM βME, 0.1 mM EDTA, and 2% potassium cholate. The method of reconstitution and measurement of Ca^{2+} uptake was identical with the one described under Experimental Procedures. (●) In the presence of 2 mM ATP; (○) in its absence.

mixture to 54% led to a large decrease in the ATP-dependent Ca^{2+} uptake in a similar manner to that obtained with BPLs.

Thus, while an increased PS/egg PC ratio in the phospholipid mixture used for reconstitution of the ATP-dependent Ca^{2+} pump led to a consistent increase in the Ca^{2+} transport activity, increasing the PS content in the BPL mixture up to 20% had no significant effect; a higher PS content than 20% resulted in a substantial decrease in the reconstituted Ca^{2+} pump activity. Increased PS content in the SPL mixture up to 29% resulted in increased Ca^{2+} pump activity relative to that obtained in SPLs alone, and only increased PS above 54% led to a decrease in Ca^{2+} transport.

It should be remembered that any increase in the content of a specific phospholipid, such as that obtained when parts of BPL or SPL mixtures are substituted with PS, results in a parallel decrease in all the other phospholipids that form the mixture. This decrease is especially significant in the PC and PE content of BPLs and asolectin.

Can an Increase in PE, SM, or PI Content in the Reconstituted Membrane Improve ATP-Dependent Ca^{2+} Uptake? In order to investigate whether substitution of egg PC with any other phospholipid beside PS can lead to an increase in the activity of the reconstituted SPM Ca^{2+} pump, experiments similar to that shown in Figure 6A were done.

The effect of increasing ratios of egg PE/egg PC and increased PE content in the BPL mixture on the activity of the reconstituted SPM Ca^{2+} pump is shown in Table VI.

Mixing parts of egg PC with egg PE exhibits two phases: substituting small amounts of PC with PE results in a small increase in the Ca^{2+} transport activity; when higher amounts of egg PC are substituted with egg PE, however, a decrease in ATP-dependent Ca^{2+} transport is obtained.

Although PE, along with PC, is one of the major components of the BPL mixture, unlike addition of PC, increasing the relative concentration of PE in the BPLs mixture leads to a decrease in the ATP-dependent Ca^{2+} transport (Table VI). Substitution of even a small amount of the BPLs with PE, which increases the PE content in the mixture from 36% to 44.7%, leads to a substantial decrease in the ATP-dependent Ca^{2+} uptake. Further increase in the relative concentration of PE to 53% led to a loss of 88% of the ATP-dependent Ca^{2+} transport activity in reconstituted vesicles.

When a soybean phospholipid mixture is used for reconstitution instead of the BPL mixture in a similar experiment to that presented in Table VI, substituting parts of the soybean phospholipids by egg PE resulted in a steady decreased activity

Table VI: Effect of Different Ratios of Egg PC to Egg PE and Increased PE Content in the BPL Mixture on ATP-Dependent Ca^{2+} Uptake of the Reconstituted SPM Ca^{2+} Pump[a]

pC:PE ratio (parts)	ATP-dependent Ca^{2+} uptake [nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$]	BPL:PE ratio (parts)	ATP-dependent Ca^{2+} uptake [nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$]
40:0	15.0	40:0	36.8
35:5	18.4	35:5	13.3
30:10	8.5	30:10	4.32
25:15	0.31		

[a] 0.25 mg of SPM protein was added to 0.5 mL of a solution that contained 10 mg of phospholipids composed of different ratios either of egg PC to egg PE or of BPLs and egg PE, 0.15 M KP_i, pH 7.4, 5 mM βME, 0.1 mM EDTA, and 2% potassium cholate. The method of reconstitution and measurement of Ca^{2+} uptake was identical with the one described under Experimental Procedures.

pattern relative to that observed in soybean phospholipids alone.

Sphingomyelin (SM) is another of the components that participates in the BPL mixture (11%). In the experiment presented in Table VII, the effect of substituting parts of egg PC or the BPLs used for reconstitution by SM on the ATP-dependent Ca^{2+} uptake was examined.

Increasing the proportion of SM in the egg PC used for reconstitution of the SPM Ca^{2+} pump, up to a ratio of 30:10 (PC/SM), exhibits an increased ATP-dependent Ca^{2+} transport activity. As increasing parts of the PC were exchanged by SM, a gradual decrease in transport activity was obtained.

When BPLs are substituted with SM, an increase in SM concentration in the mixture from 11% to 36% (substitution of 25% of the mixture by SM) does not affect the ATP-dependent Ca^{2+} uptake in a significant way. Further increase in the SM concentration to 61% leads to a 70% inhibition in the ATP-dependent Ca^{2+} pump. Higher SM content or reconstitution into SM-containing vesicles abolishes the ATP-dependent Ca^{2+} transport activity altogether.

Performing similar experiments with soybean phospholipids resulted in a similar activity profile curve to that obtained with BPLs (not shown).

The effects of increased phosphatidylinositol (PI) concentration in the reconstituted membrane on the activity of the SPM Ca^{2+} pump are shown in Figure 7A–C.

When PC is used as the sole phospholipid into which the SPM Ca^{2+}-ATPase is reconstituted, an increase in PI up to 50% has almost no effect on the ATP-dependent Ca^{2+} uptake.

Table VII: Effect of Different Ratios of Egg PC to Bovine Brain SM and Increased SM Content in the BPL Mixture on the ATP-Dependent Ca^{2+} Uptake of the Reconstituted SPM Ca^{2+} Pump[a]

PC:SM ratio (parts)	ATP-dependent Ca^{2+} uptake [nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$]	BPL:SM ratio (parts)	ATP-dependent Ca^{2+} uptake [nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$]
40:0	9.47	40:0	36.0
30:10	19.6	30:10	38.7
25:15	14.3		
20:20	9.65	20:20	12.8
10:30	3.52	10:30	5.13

[a] 0.25 mg of SPM protein was added to 0.5 mL of a solution that contained 10 mg of phospholipids composed of different ratios either of egg PC to bovine brain SM or of BPLs and SM, 0.15 M KP_i, pH 7.4, 5 mM βME, 0.1 mM EDTA, and 2% potassium cholate. The method of reconstitution and measurement of Ca^{2+} uptake was identical with the one described under Experimental Procedures.

Further increase in the amount of PI in the reconstituted membrane leads to a large decrease in the ATP-dependent Ca^{2+} uptake.

Increase in the amounts of PI above 12%, the amount present in the BPL mixture, and 3.9% in asolectin (Table IV) led to progressive loss of ATP-dependent Ca^{2+} transport activity.

Is the Intravesicular Space Altered by Reconstitution of SPM Vesicles into Different Phospholipids? The results presented in this work show that the activity of the SPM Ca^{2+}-ATPase is affected by the composition of the phospholipid membrane into which it is reconstituted. To rule out the possibility that the apparent activity of the reconstituted Ca^{2+} pump reflects changes in the intravesicular volume rather then altered phospholipid composition, the average [^{3}H]inulin space and the average vesicle diameter from light-scattering measurements were determined. Table VIII summarizes the results that were obtained.

Measuring the intravesicular [^{3}H]inulin space of SPM vesicles reconstituted into BPLs, SPLs, and egg PC shows that the intravesicular volume in BPLs is the largest (1.14 μL/mg of BPL), next in SPLs (0.83 μL/mg of SPLs), and in egg PC the smallest (0.64 μL/mg of PC). Measurement of the average vesicle diameter by light scattering and their distribution pattern shows that the decreasing order of the intravesicular [^{3}H]inulin space is not the sole difference between the vesicles. While vesicles composed of BPLs and SPLs are uniform homogeneous vesicles of similar size, SPM vesicles reconstituted into egg PC form large nonhomogeneous multisize distribution vesicular structures.

Addition of an equal amount of bovine brain PS to the egg PC and reconstituting the SPMs into this mixture result in a homogeneous uniform distribution profile of the vesicles. In parallel, the activity of the ATP-dependent Ca^{2+} pump increases (Figure 6A).

It can be seen that all the brain phospholipid containing mixtures [BPL alone, BPL/PE (35:5), BPL/PC (30:10), and BPL/PS (20:20)] have very close size distribution profiles from 43.4 nm (SD = 11.1) for BPL alone to 48 nm (SD = 13.8) for a BPL/PC mixture. Their respective intravesicular volumes varies between 1.14 and 1.8 μL/mg of phospholipid The ATP-dependent Ca^{2+} transport activity, however, in those phospholipid mixtures (see Figures 5 and 6 and Table VI) is different. Thus, the increased ATP-dependent Ca^{2+} transport activity observed when BPLs are enriched with egg PC and the decreased ATP-dependent Ca^{2+} transport activity obtained when BPLs are enriched with egg PE and bovine brain PS cannot be explained by alteration in vesicle size or in their intravesicular volume.

Table VIII: Intravesicular Volume of SPM Vesicles Reconstituted into Different Phospholipids[a]

PL used for reconstitution	average vesicle diameter[b] (μM) (SD)	intravesicular volume[c] (μL/mg of phospholipid)
BPL	43.4 (11.1)	1.14
SPL	33.1 (14.1)	0.83
PC[d]	99.0 (113)	0.64
PBL/PC (30:10)	48.0 (13.8)	1.71
PBL/PE (35:5)	43.5 (12.2)	1.8
BPL/PS (20:20)	43.9 (13.9)	1.75
PC/PS (20:20)	68.8 (20.5)	1.07

[a] Reconstitution of SPMs was performed as described under Experimental Procedures at a 40:1 PL:protein weight ratio, 2% cholate, and 0.5 mg of SPM protein/mL. [b] Average vesicle diameter was measured by dynamic light scattering using the Malvern 4700C submicron Particle Analyzer (Malvern, England) with a laser line of 488 nm at an angle of 90°. The temperature was kept at 25 °C. Each measurement was repeated 3 times, and 10 different samplings were done each time. All the vesicle preparations except PC were uniform homogeneous preparations composed of narrow size distribution. [c] Intravesicular volume was measured from [^{3}H]inulin space as described under Experimental Procedures. [d] Analysis of the SPM vesicles reconstituted into PC revealed a nonhomogeneous multiform size distribution. Measuring the light scattering at angles of 45°, 60°, 75°, 90°, and 105° showed that the preparation was composed of a mixture containing the following vesicle sizes: D = 86.4 nM (44%); D = 109.8 nm (48%); D = 139 nm (4.2%); D = 225.7 nm (2.55%); D = 287 nm (0.39%).

Discussion

Membrane phospholipid head group composition, membrane fluidity, and membrane thickness were all implicated as key determinants in the modulation of the activity of membrane proteins [see, for example, Johannsson et al. (1981), Caffrey and Feigenson (1981), Shinitzky (1984), Criado et al. (1984), Navarro et al. (1984), Deuticke and Haest (1988), Carruthers and Melchior (1988), Squier et al. (1988), and Yeagle (1989)]. Reconstitution of membrane proteins into homogeneous lipids of well-defined composition is the prime tool to study the correlation between the activity of a particular transport protein and defined changes in membrane physical properties. Such an approach has been adopted to study the role of membrane thickness in modulating the activity of the SR Ca^{2+}-ATPase following its reconstitution into a membrane composed of fatty acyl derivatives of PC of varying chain length and degree of unsaturation (Caffrey & Feigenson, 1981). In a similar fashion, the role of the polymorphic phase behavior of PE was found to control the coupling between Ca^{2+} transport and ATP hydrolysis in the SR Ca^{2+}-ATPase reconstituted into synthetic derivatives of PE of increasing degree of methylation or glycosylation (Navarro et al., 1984).

Synthetic homogeneous lipids were found in many cases suitable in supporting the transport activity of membrane proteins, but the activities were often lower than those obtained in complex lipid mixtures from natural sources (Bennett et al., 1980; Deuticke & Haest, 1988). Moreover, reconstitution into homogeneous lipids did not always result in the full range of activity. For example, reconstitution of the acetylcholine receptor into a membrane lacking negatively charged lipids and cholesterol resulted in agonist binding without consequent ion channel gating (Fong & McNamee, 1986). In this work, reconstitution into natural lipid mixtures was chosen to study the role of membrane lipid composition in the modulation of the SPM Ca^{2+}-ATPase. This was done to ensure that functional integrity is preserved.

The SPM Ca^{2+} pump is a high Ca^{2+} affinity enzyme with K_m values reported for Ca^{2+} in the submicromolar range (Sorensen & Mahler, 1981; Michaelis et al., 1983). It is

activated by calmodulin (Sobue et al., 1979; Sorensen & Mahler, 1981, Papazian et al., 1984), which increases even further the affinity of the enzyme for Ca^{2+}. Studies with the erythrocyte Ca^{2+} pump demonstrated (Niggli et al., 1981a,b; Carafoli & Zurini, 1982) that calmodulin activation has an inverse dependence on the presence of acidic PLs (Carafoli & Zurini, 1982). The erythrocyte Ca^{2+} pump acquires a "high affinity" state by addition of either calmodulin or acidic phospholipids; this indicates that specific lipid/protein interactions lead to conformational changes. Synaptic plasma membranes contain considerable amounts of endogeneous calmodulin even following solubilization with detergent (Papazian et al., 1984), precluding further calmodulin activation. In this work, SPMs were not depleted of endogenous calmodulin to rule out "calmodulin-like" effects of the phospholipids used for reconstitution.

Comparison of BPLs, SPLs, and egg PC as supportive membranes for the reconstituted SPM Ca^{2+} pump revealed that the BPL mixture was superior to SPLs, which was superior to egg PC. This order of activity does not correlate in a simple fashion with either the extent of unsaturation or the chain length of fatty acid constituents of the phospholipids used. This indicates that modulation of membrane physical properties such as membrane thickness or degree of unsaturation is probably not the prime cause for the differences observed in Ca^{2+} pump activity. Therefore, the possibility that the composition of the phospholipid head groups is predominant in modulating the activity of the SPM Ca^{2+} pump was examined in detail.

Among the individual PL head groups tested, substitution of increasing parts of egg PC with bovine brain PS led to a consistent increase in ATP-dependent Ca^{2+} transport. This is consistent with similar findings with the Ca^{2+} pump from erythrocytes (Carafoli & Zurini, 1982) and with the cardiac Na^{+}–Ca^{2+} exchanger in the heart (Luciani, 1984; Carroni et al., 1983; Vemuri & Philipson, 1987). One possible explanation of the effects of increasing amounts of PS in the PC-containing membrane could be linked to its well-known effects in promoting Ca^{2+} binding. This has been shown in model membranes both in the milimolar and in the micromolar range of $[Ca^{2+}]$ (Nir et al., 1978; Ekerdt & Papahadjopoulos, 1981; Tokutomi et al., 1981; Feigenson, 1986). The presence of PS on the external face of the membrane could be involved, for example, in Ca^{2+} binding to the high-affinity site of the ATPase, while its presence on the inside could facilitate Ca^{2+} release from the low-affinity site of the enzyme by its binding, lowering the intravesicular Ca^{2+} concentration which in turn would shift the Ca^{2+} equilibrium and promote Ca^{2+} influx. From comparing the diameter of the reconstituted vesicles in PC and PC/PS-containing membranes and their respective intravesicular volumes, an additional possibility to understand the activation by PS should also be considered. Reconstitution of the SPM Ca^{2+}-ATPase into PC only leads to formation of very large nonuniform vesicles of small intravesicular volume (see Table VIII). Reconstitution of the SPMs into a membrane composed of PC/PS at a 1:1 weight ratio leads to formation of a homogeneous vesicle population of much smaller diameter and with a larger intravesicular volume. It is possible that in some way the presence of PS in the membrane, which is a negatively charged phospholipid, helps to prevent the formation of the large vesicular nonhomogeneous structures of relatively small internal diameter. Were this the only explanation, addition of PI to PC should have had similar effects. However, this is not the case; therefore, a more specific function of PS in the membrane plays a role.

While addition of PS to egg PC caused an increase in the ATP-dependent Ca^{2+} transport, addition of PS to BPL caused a decreased activity. SPLs are poorer than BPLs in their PS content; here, Ca^{2+} transport was increased when they were partially enriched by PS, while further increase in the PS content of SPLs led to a decrease in the ATP-dependent Ca^{2+} transport. Since the increase in the PS content of BPLs and SPLs is accompanied by a corresponding decrease in the content of all other PLs in the reconstituted membrane, it is possible that depletion of a crucial membrane component occurred. From our results, it seems that the PC content of the membrane might play a special role in this context for several reasons: (1) A small increase in the PC content of BPLs and SPLs above their original values leads to an increase in the reconstituted Ca^{2+} pump activity. This increase in Ca^{2+} pump activity occurs although the increase in PC is accompanied by a corresponding decrease in all other PLs. (2) Any other substitution carried out in the BPL or SPL mixtures, such as increasing the PE, SM, or PI content, led to a decrease in the activity of the rerconstituted Ca^{2+} pump relative to that obtained in the native mixtures. (3) When the reconstituted membrane is composed of egg PC, it can be partially substituted by PS, and up to 50%, an increase in Ca^{2+} transport is obtained. Thus, although PS seems to play a special role in promoting ATP-dependent Ca^{2+} transport in the reconstituted SPM Ca^{2+} pump, its content can be increased only as long as a critical amount of PC is present as well. It cannot be ruled out that increased PE or SM modulates the bilayer itself and thus leads to decreased activity of the reconstituted SPM Ca^{2+} pump. Studies performed with model membranes showed that increased PE in the presence of acidic phospholipids and Ca^{2+} was linked to bilayer destabilization and liposome aggregation, especially in the physiological pH (Ellens et al., 1984; Duzgunes et al., 1981; Hope et al., 1983).

Increased SM content in biological membranes was linked to increased microviscosity (Barenholtz & Thompson, 1980). In model membranes, consisting of PC/SM/H_2O, an increase in SM and a decrease in temperature below the transition temperature led to lateral phase separation of the SM from the PC/SM liquid-crystal bilayer phase (Untrecht & Shipley, 1977).

Further studies have to be carried out to understand the specific roles of PC and PS in regulating the activity of the SPM Ca^{2+} pump. It seems clear, however, that deviations from the composition of the BPL mixture such as those occurring in some pathological or physiological conditions that result in changes in the relative amounts of these two phospholipids will modulate the activity of the Ca^{2+} pump and hence may contribute to deviations from the normal cellular Ca^{2+} homeostasis.

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Registry No. Ca, 7440-70-2; cholic acid, 81-25-4; ATPase, 9000-83-3.

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