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POLAR HEAD-GROUP AND ACYL SIDE-CHAIN REQUIREMENTS FOR PHOSPHOLIPID-DEPENDENT ($Na^+ + K^+$)-ATPase

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SUMMARY

The abilities of different phospholipids to reactivate a lipid-depleted ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) have been compared. The phospholipids contained either the same group of hydrocarbon chains with different polar groups, or different hydrocarbon groups with the same polar group; they were prepared by enzymic modification of the polar group of phosphatidylserine isolated from bovine brain and of phosphatidylcholine extracted from egg. Only the acidic phospholipids reactivated the ATPase but the amount of reactivation depended on the nature of the hydrocarbon chains as well as the polar group. These findings are discussed in relation to the compositions of the two different groups of fatty acyl chains constituting the hydrocarbon portions of the phospholipids.

INTRODUCTION

It is now well established that the ($Na^+ + K^+$)-dependent ATPase (EC 3.6.1.3) requires lipid for its activity but there has been much confusion and controversy over the exact nature of the lipid required. The various different experimental approaches and results which have led to the present situation have been admirably summarized and criticised recently by Roelofsen and Van Deenen [1]. Reviewing this literature, we were struck by the fact that until recently attention had been focussed mainly on the specificity of the polar head-group of the phospholipids under consideration for the role of the essential lipid, or lipids, the possible inportance of the nature of the fatty acyl side-chains having been comparatively neglected. This observation suggested that many of the apparently conflicting reports in the literature could have arisen from the natural variations in the acyl side-chains of the phospholipids examined, although they were pure as far as the polar head-groups were concerned. Some support for this conjecture was provided by the demonstrations of the importance of fatty acyl fluidity in the phospholipids associated with ($Na^+ + K^+$)-ATPase activity, both in the native enzyme [2, 3] and in one that had been reactivated with exogenous phospholipid after partial lipid depletion [4]. We have, therefore, tested this idea further by inactivating a ($Na^+ + K^+$)-ATPase by lipid depletion and then comparing

the reactivating abilities of various phospholipids containing identical groups of acyl side-chains but different polar head-groups, or different acyl chains with the same polar group. These phospholipids were obtained by enzymic conversion of the polar groups of two different purified natural phospholipids.

EXPERIMENTAL

ATPase preparations

A microsomal fraction was prepared from rabbit kidney homogenate and treated with NaI as described previously [5]. Lipid depletion was achieved by the following procedure [5]. A sample of the suspension obtained by the NaI treatment was mixed with a solution containing 0.2 mM EDTA (pH 7.5), 2.5 mM Na_2ATP (pH 7.5), 1 mM dithiothreitol, 35 % (v/v) glycerol and 40 mg/ml lubrol W, at 0–4 °C. The final protein concentration was 2 mg/ml. The mixture was sonicated for 2 min, the temperature being maintained at 0–4 °C, with the use of an MSE Ultrasonic Disintegrator (settings: medium power, amplitude 1) with the small probe. The mixture was then stirred on ice for 60 min before it was centrifuged at about 120 000 × *g* for 60 min. The clear supernatant solution was decanted, solid $(NH_4)_2SO_4$ added to it (0.21 g/ml) and the mixture stirred on ice until all the salt had dissolved. Centrifugation at about 40 000 × *g* for 30 min at 4 °C produced a floating disc, which was removed and resuspended by homogenisation in cold 0.2 M NaCl. The mixture was centrifuged at 120 000 × *g* for 30 min to give a sediment that was washed by resuspension and centrifugation first in cold 0.6 M NaCl and then in cold M NaCl. The final pellet wa resuspended by homogenisation in the glycerol-containing "Buffer A20" [5] and stored at −20 °C.

Phospholipid dispersions

Phospholipid solutions (in chloroform/methanol) were evaporated to dryness under a stream of nitrogen and water or Tris buffer (pH 8), previously saturated with nitrogen at 0 °C, added. The mixture was agitated by bubbling more nitrogen through it for a few minutes, until the entire vessel was filled with nitrogen. The suspension was then sonicated for 15 to 30 min in an MSE Ultrasonic Disintegrator (medium power; amplitude 1 or 2) with either the small or medium-size probe, depending on the volume. The vessel was maintained at 20 °C throughout by means of a water-jacket and circulating water; care was taken to avoid any frothing. Additional buffer was added if necessary, to keep the pH around 7.5. The resulting dispersion was briefly centrifuged at about 2000 × *g*, to remove most of the titanium dust, and then centrifuged at 120 000 × *g* for 30 min, to remove any undispersed lipid and final traces of titanium. The supernatant fluid was stored at 4 °C.

Phospholipids

All the phospholipids used in the experiments described below were derived from the same batches of either phosphatidylcholine or phosphatidylserine, which had been purchased from Lipid Products, Ltd, Surrey, U.K., and which gave single spots on thin-layer chromatograms after development in two different solvent systems that separated the compounds according to their polar groups [6]. The phospholipase D (EC 3.1.4.4) used to modify these phospholipids was "Type 1", from cabbage

leaves, obtained from both the Sigma Chemical Co. (London) and from the Boehringer Corporation, London, Ltd. Phospholipase A from Bee Venom was purchased from Sigma.

Phosphatidic acids

Samples of the phosphatidylcholine and phosphatidylserine were separately hydrolysed to phosphatidic acids with the aid of phospholipase D according to the following procedure, which is based on the methods of Davidson and Long [7] and Hübscher and Clark [8]. The phospholipid sample (2 ml of dispersion containing 15 μmol) was incubated for 90 min at 20 °C with 20 mg phospholipase D in a mixture containing 0.1 ml M $CaCl_2$, 4 ml 0.1 M acetate buffer (pH 5.6) and 1 ml diethylether. The mixture was then extracted successively with diethylether (2 samples of 20 ml), chloroform (40 ml) and chloroform/methanol (30 ml, 1 : 20, by vol.) and the pooled extracts were evaporated to dryness under a stream of nitrogen. The lipid mixture was dissolved in a small volume of chloroform and the components separated by thin-layer chromatography on silica gel [6]. The resolved components were located by brief exposure to iodine vapour and identified by comparison with pure phospholipid standards, by comparison with published chromatograms [6], and with the use of ninhydrin, Dragendorff, and acid/molybdate reagents. Analytical and preparative chromatography was carried out in parallel on the same plates and the resolved components were eluted from the gel by two successive extractions with 40 ml of a mixture containing chloroform/methanol/acetic acid/water (113 : 68 : 4 : 1, by vol.) followed by a single extraction with 30 ml chloroform. The pooled extracts were evaporated to dryness under nitrogen and then taken up in a small volume of chloroform/methanol (2 : 1, by vol.) for removal of Ca^{2+} by shaking with 0.2 vol. of a solution containing 0.05 M EGTA (pH 7.5) in 0.1 M NaCl. The upper phase and any interfacial layer were removed and the lower phase was evaporated to dryness as before. The final product was dispersed in about 2 ml of 0.1 M Tris · HCl (pH 8) by exposure to ultrasonic vibrations as described above. The concentration of phospholipid in the dispersions was determined by measurement of total phosphorus [9].

Phosphatidylglycerols

Samples of the phosphatidylcholine and phosphatidylserine were separately converted to phosphatidylglycerols by incubation with phospholipase D and glycerol, according to the procedure for conversion of phosphatidylcholine described by Papahadjopoulos et al. [10], which was based on the work of Dawson [11]. The products were extracted, separated, identified and dispersed by the methods outlined above, with the additional use of Schiff's periodate reagent to confirm identification of the phosphatidylglycerols.

Phosphatidylethanolamine

A sample of the phosphatidylserine was converted to phosphatidylethanolamine with the use of phosphatidylserine decarboxylase by the following procedure, which is based on the method described by Roelofsen and van Deenen [1]. Crude decarboxylase was isolated from *Escherichia coli* B by the method of Kanfer and Kennedy [12] and a 2-ml sample added to 2 ml phosphatidylserine dispersion (15 μmol) with 3 ml 0.1 M phosphate buffer (pH 6.9) containing 0.02 M EDTA, and 2 ml

diethylether. The mixture was incubated for 3 h at 37 °C and the products extracted and purified as described above.

Preparation of lysophosphatidylserine

A sample of phosphatidylserine was converted to its lyso-compound with the use of phospholipase A according to the following procedure, which is based on the methods of Haverkate and van Deenen [13] and Wells and Hanahan [14]. Phosphatidylserine (30 μmol) was incubated with 0.5 mg phospholipase A in 30 ml of diethylether/methanol (98 : 2, by vol.) with 3 ml 0.1 M Tris buffer containing 1.6 mg calcium acetate at pH 8.5. After a 2-h incubation at 37 °C excess solid EGTA was added to chelate Ca^{2+}. The solvent layer was decanted off and evaporated to dryness under nitrogen and reduced pressure in a rotary evaporator. The dry residue was extracted three times with chloroform/methanol (1 : 1, by vol) and the pooled extracts evaporated to dryness. The lipid mixture was redissolved in chloroform/methanol and the components separated by thin-layer chromatography as described above. Lysophosphatidylserine and phosphatidylserine were identified with the use of a standard solution of the latter and by spraying with ninhydrin reagent. The two lipids were eluted as outlined above, but the lyso-compound was not washed. The phosphatidylserine was dispersed by sonication, as described above, while the lysophosphatidylserine was simply dissolved in water.

Analysis of fatty acyl side-chains

Samples of phosphatidylcholine, phosphatidylserine and lysophosphatidylserine were separately trans-esterified by heating under a nitrogen seal for 1 h at 100 °C in a mixture of benzene/methanol/H_2SO_4 (15 : 30 : 2, by vol.). The methyl esters produced were extracted 3 times with petroleum spirit; the pooled extract was washed 3 times with 2 % (w/v) $KHCO_3$ and then with water. The organic layer was dried with Na_2SO_4 and then evaporated to dryness under nitrogen. The dried residue was dissolved in petroleum spirit and chromatographed on silica gel H. The plates were developed in benzene and the spots located under ultraviolet light after spraying with 2,7-dichlorofluorescin. The separated components were eluted from the gel and stored in diethylether. The individual methylesters were then identified and their relative concentrations measured by conventional gas chromatography in a Hewlett Packard 5750G gas chromatograph. Some identification problems with the esters from lyso-phosphatidylserine were resolved by preliminary separation on $AgNO_3$-impregnated silica gel.

Other procedures

Measurement of protein concentrations and ATPase activity, and all other procedures, were carried out exactly as described by Wheeler et al. [5].

RESULTS

Effect of lipid depletion on the ATPase

As described previously [5], the lubrol-extraction procedure removed on average about 90 % of both the total phospholipids and the ouabain-sensitive ATPase activity from the tissue preparations. The average values for the preparations used

here were 0.85 μmol phospholipid/mg protein with an activity of 0.83 μmol P_i/mg protein per min before extraction, and 0.10 μmol phospholipid/mg protein with an activity of 0.04 μmol P_i/mg protein per min after extraction. (The ouabain-insensitive ATPase activity remained at 0.07 μmol P_i/mg protein per min throughout, and that component has been substracted from the values given below.)

Effect of polar group on reactivating ability of phospholipid

The reactivating abilities of four different classes of phospholipids, each containing identical groups of hydrocarbon side-chains, are compared in Fig. 1A. Phosphatidylserine was the parent phospholipid and the phosphatidylglycerol, phosphatidic acid and phosphatidylethanolamine were each derived from it, as described above. The most obvious feature of these results was the failure of the phosphatidylethanolamine to produce significant reactivation, in marked contrast to the high restoration of activity brought about by the other three lipids. Each of the latter activated maximally when present at about 0.1 mM, although the phosphatidylglycerol always produced a lower level of activity at all concentrations than the phosphatidylserine and phosphatidic acid, which were barely distinguishable from each other. Note that the values given for phosphatidylserine are means for the original compound and for a sample recovered from the mixture of phosphatidylserine and phosphatidic acid remaining after hydrolysis with phospholipase D. The fact that those two samples produced virtually identical reactivation showed that exposure to the phospholipase and the subsequent purification procedures had not degraded the lipid, and that Ca^{2+} had been effectively removed.

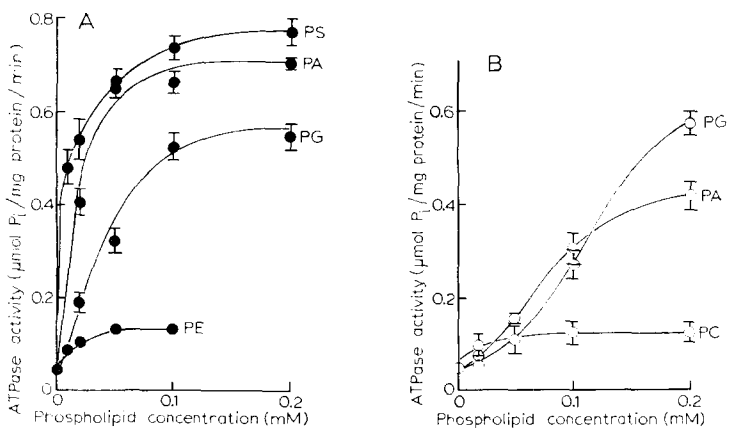

Fig. 1. Effect of polar group on ability of phospholipids to reactivate $(Na^+ + K^+)$-ATPase. An ATPase preparation from rabbit kidney was partly depleted of lipids by extraction with lubrol W and then incubated with dispersions of purified phospholipids for measurement of ATPase activity, as described in the text. The activity shown is the difference between that measured in the presence and absence of 0.1 mM ouabain. Mean values (±S.E. where possible) from at least four experiments are given. The different classes of phospholipid containing the same groups of hydrocarbon side-chains were obtained by enzymic conversions of pure samples of naturally occurring phospholipids, as described in the text. (A), Each sample of phospholipid was prepared from the phosphatidylserine (PS) as starting material. Other abbreviations are: PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine. (B) As for A, except that phosphatidylcholine (PC) was the parent phospholipid.

A similar comparison of the effect of the polar group on reactivation is shown in Fig. 1B, this time with two different classes of phospholipid derived from a parent phosphatidylcholine. Again, the striking feature is the inability of the phosphatidylcholine to reactivate, whereas both the phosphatidylglycerol and the phosphatidic acid derived from it produced considerable reactivation.

Consideration of Figs 1A and 1B together shows that the two lipids that were unable to reactivate the ATPase shared the common property of bearing no net charge, whereas the three classes of phospholipid that did reactivate were all negatively charged under the experimental conditions employed.

Effect of acyl side-chains on reactivating ability of phospholipid

The reactivating abilities of phospholipids containing the same polar groups but different hydrocarbon side-chains are compared in Fig. 2. Fig. 2A shows the relative effects of the phosphatidic acids while 2B compares the phosphatidylglycerols. Both types of phospholipid derived from the phosphatidylserine produced similar hyperbolic reactivation curves, whereas those derived from the phosphatidylcholine gave sigmoid curves. Saturating concentrations of the phosphatidic acid made from phosphatidylcholine did not produce as much activity as the phosphatidic acid derived from phosphatidylserine (Fig. 2A); but both kinds of phosphatidylglycerol eventually reactivated to the same extent (Fig. 2B). (Separate experiments, not illustrated, showed that increasing the concentration of the phosphatidylglycerol derived from phosphatidylcholine above 0.2 mM produced very little further activation.)

Fig. 2C compares the reactivating abilities of phosphatidylserine and its lysoderivative. Again, a sample of the phosphatidylserine remaining from the phospholipase A treatment was recovered by thin-layer chromatography and used to check

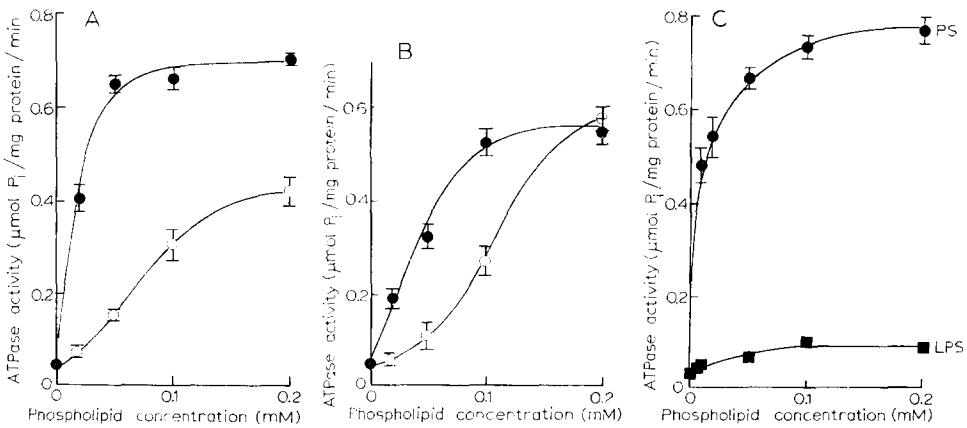

Fig. 2. Effect of hydrocarbon chains on ability of phospholipids to reactivate ($Na^+ + K^+$)-ATPase. (A), The data for the phosphatidic acids in Fig. 1A (●) are compared directly with those for the phosphatidic acids in Fig. 1B (○). (B), The data for the phosphatidylglycerols in Fig. 1A (●) are compared directly with those for the phosphatidylglycerols in Fig. 1B (○). (C) Lysophatidylserine (LPS) was prepared from phosphatidylserine (PS) as described in the text. The reactivating abilities of both phospholipids on lipid-depleted ($Na^+ + K^+$)-ATPase were measured as described in the legend to Fig. 1. The compositions of the fatty acyl chains of the two parent phospholipids (PS and PC) and the LPS are given in Table I.

that the various procedures involved had not affected its ability to reactivate. It is clear that the lysophosphatidylserine produced no reactivation, in marked contrast to the parent compound.

Nature of the fatty acyl side-chains

The compositions of the fatty acid residues from the parent phosphatidylserine and phosphatidylcholine, together with those remaining in the lysophosphatidylserine, are given in Table I. In general, the average hydrocarbon chain length was greater in the phosphatidylserine (83 % C_{18} with 15 % C_{20}) than in phosphatidylcholine (35 % C_{16} with 62 % C_{18}). Unsaturated hydrocarbons also formed a greater proportion of the chains in the phosphatidylserine (64 %) than in the phosphatidylcholine (55 %). In contrast, only 30 % of the acyl residues in the lysophosphatidylserine were unsaturated. Provided the phospholipase A was specific for the β-position of the glycerol moiety, this shows that of the 64 % unsaturated chains in the phosphatidylserine, 49 % were attached to the β-carbon of the glycerol moiety.

TABLE I

COMPOSITION OF HYDROCARBON CHAINS OF THE PHOSPHOLIPIDS

Samples of the parent phosphatidylserine and phosphatidylcholine, and lysophosphatidylserine, were separately hydrolysed and the compositions of their hydrocarbon residues determined by gas chromatography, as described in the text.

Chain length and unsaturation	Proportion present (% total) in		
	Phosphatidyl-choline	Phosphatidyl-serine	Lysophosphatidyl-serine
16 : 0	34.7	0.7	3.9
16 : 1	—	0.1	0.9
17 : 0	—	0.8	0.9
17 : 1	—	0.1	—
18 : 0	10.7	33.3	64.5
18 : 1	33.3	47.1	25.4
18 : 2	18.0	0.7	—
18 : 3	—	1.6	—
20 : 0	—	0.8	1.0
20 : 1	—	13.4	—
20 : 4	3.3	—	—
20 : 5	—	1.4	3.4

DISCUSSION

The results presented above show clearly that the natures of both the polar head-groups and the hydrocarbon side-chains were important in determining the ability of exogenous phospholipids to reactivate the lipid-depleted ATPase. Moreover, partial discrimination between the effects of these polar and non-polar moieties was achieved by independent variation of either the head-group or the side-chains. Thus it seems that a necessary condition for reactivation was that the polar group should bear a net negative charge (Fig. 1) while the way in which reactivation varied with

concentration of added phospholipid depended mainly on the nature of the side-chains (Fig. 2). The maximal level of reactivation attained was a function of both the polar groups (Fig. 1) and the hydrocarbon chains (Fig. 2A).

Although there have been exceptions, preferential or exclusive reactivation of lipid-depleted ATPase by acidic phospholipids has been reported frequently, particularly in the more recent publications on this topic (for detailed references see ref. 1) and we have never observed significant and consistent reactivation by other phospholipids. Such a requirement for acidic lipids could mean either that the net charge is necessary to bring about interaction between the added lipid and the protein, or that it is essential for the enzyme's functioning. If the latter alternative is correct, the negative charge could be involved in the interactions of Na^+ and K^+ with the enzyme. On the other hand, the former possibility could perhaps explain some of the inconsistencies in the literature, because the requirement of a net charge might reflect the physical state of the enzyme after lipid depletion, which could easily vary considerably from one preparation to another. Discrimination between these two possibilities is clearly an important matter; but some different experimental approach will be required to do so.

A related question concerns the lipid requirement of the $(Na^+ + K^+)$-ATPase system in vivo. If we tentatively assume that the net charge on the phospholipid is necessary for the enzyme's functioning, then it appears that any one of the acidic phospholipids could fulfil the role in vivo, provided only that it possesses suitable acyl chains. Hence, again, two possibilities exist: either one of the acidic phospholipids, for example phosphatidylserine, is always associated with the ATPase in situ, or else the "natural" phospholipid varies with the tissue or species in which the enzyme is situated. Resolution of these possibilities probably must await complete purification of the ATPase from a number of different tissues and species, if a pure lipid-protein complex actually exists as a unit.

The effects of the fatty acyl side-chains of the phospholipids on their ability to reactivate the ATPase provide a good explanation for some of the confusion in the literature. Thus, although both phosphatidic acid and phosphatidylglycerol derived from the phosphatidylcholine were capable of reactivating the enzyme, the concentration dependence of their reactivation was quite different from that of the phosphatidic acid and phosphatidylglycerol derived from phosphatidylserine. (Fig. 2). The result was that if only low concentrations of phosphatidic acid and phosphatidylglycerol were compared, those made from the phosphatidylserine produced considerable reactivation whereas those derived from the phosphatidylcholine had no significant effects. That difference must have arisen from some difference in the compositions of the two groups of hydrocarbon chains constituting the parent phosphatidylcholine and phosphatidylserine. Attempts to pinpoint precisely which particular acyl chains were critically important, however, were frustated by the complexity of the mixtures (Table I). On the other hand, the data do provide a basis for reasonable speculation when combined with the recent findings of Kimelberg and Papahadjopoulos [15]. These authors showed that dioleoylphosphatidylglycerol ($C_{18:1}$) reactivated a lipid-depleted $(Na^+ + K^+)$-ATPase maximally when added at low concentrations, whereas both dipalmitoyl-($C_{16:0}$) and distearoylphosphatidylglycerol ($C_{18:0}$) reactivated maximally only at much higher concentrations. Moreover, neither of the completely saturated phospholipids reactivated to the same extent as the unsaturated

phospholipid. (Since each of those compounds was tested above its phase-transition temperature, effects of fluidity seem to be excluded.) It seems feasible, therefore, that it was the proportion of di-unsaturated molecules (i.e. both acyl chains unsaturated) that determined the effectiveness of our acidic phospholipids in reactivating the ATP-ase when added at low concentrations.

The combined analyses of the phosphatidylserine and lysophosphatidylserine (Table I) enable accurate calculation to be made of the proportion of di-unsaturated phosphatidylserines. Since 64 % of the hydrocarbon chains were unsaturated, at least 28 % of the phosphatidylserines must have contained two unsaturated chains. And since 30 % of the lysophosphatidylserines contained unsaturated hydrocarbon chains, then no more than 30 % of the phosphatidylserines could have been di-unsaturated. Hence the proportion of di-unsaturated phosphatidylserines must have been 28 % to 30 %. Unfortunately we hadn't sufficient phosphatidylcholine to hydrolyse to the lyso-derivative and analyse in the same way. However, the data in Table I do show that the minimal proportion of di-unsaturated phosphatidylcholines was 10 %; and since unsaturated residues generally are preferentially attached to the β-carbon of the glycerol moiety in natural phospholipids (see ref. 16, and the data for phosphatidylserine, above) it is unlikely that many more than 10 % of the phosphatidylcholines were di-unsaturated.

This hypothesis is obviously speculative and can, at present, refer only to reactivation, not to the natural, in vivo, situation. But it does provide both an explanation for our findings and a basis for further study of the interaction between phospholipids and the ATPase protein. In addition, the idea of unsaturated hydrocarbon chains being important in the $(Na^+ + K^+)$-ATPase system is in keeping with recent work indicating the importance of fluidity of the lipids involved [2, 3, 15]. Although we had no means of measuring the phase-transition temperatures (T_c) of our phospholipids, values in the literature suggest that the T_c for the egg phosphatidylcholine would be about 20 °C [17], whilst that for bovine brain phosphatidylserine would be 10–20 °C (broad peak) [15]. Thus, we were working at temperatures above the T_c values throughout, though somewhat further above the average T_c for phosphatidylserine and its derivatives than for phosphatidylcholine and its derivatives. Whether or not that difference was important is unclear; but differences in fluidity could not explain the observed variations in concentration dependence between the two groups of acyl side-chains. In fact, the pronounced sigmoid character of the reactivation curves found with the derivatives from phosphatidylcholine suggests either that more than one molecule of phospholipid had to bind to the appropriate site on the protein in order to reactivate, or that some cooperative mechanism was involved.

It is difficult to explain the lack of reactivation by lysophosphatidylserine in terms of the nature of the acyl residues. Instead this finding suggests that either the phospholipid must have two hydrocarbon chains for correct interaction with the protein, or else the physical state of the lipid dispersion is important. Thus, it is possible that only the lamellar forms are suitable, molecular dispersions of lyso-compounds being ineffective. Tanaka et al. [18] came to the opposite conclusion because lysophosphatidylcholine did reactivate in their system and at present there is no obvious explanation for this discrepancy. Lysophospholipids, however, are unlikely to be important in vivo.

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