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Studies on (Na^+–K^+)-activated ATPase XXXIV. Phosphatidylserine not essential for (Na^+–K^+)-ATPase activity

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SUMMARY

An (Na^+–K^+)-ATPase preparation, consisting of NaI-treated microsomes from cattle brain, was incubated with a phosphatidylserine decarboxylase preparation from *Escherichia coli*. This led to a reduction in the phosphatidylserine content from 10.1% to less than 0.1%, accompanied by an equimolar formation of phosphatidylethanolamine. Since the (Na^+–K^+)-ATPase activity was not reduced, it can be concluded that phosphatidylserine is not essential for the (Na^+–K^+)-ATPase activity.

Introduction

Sodium–potassium activated ATP phosphohydrolase [(Na^+–K^+)-ATPase] is directly involved in active cation transport in nearly all animal cells[1]. This enzyme is located in the plasma membrane and is activated by ions on different sides of the membrane. Evidence has accumulated in recent years for an essential role of lipids in the functioning of the enzyme, since lipid removal by detergents[2–13], phospholipase A and C[2,8,11,14–24], as well as by organic solvents[25,26] leads to partial or complete inactivation of the enzyme. It is not yet clear whether this inactivation is due to removal of one specific phospholipid essential for the functioning of the enzyme or to disruption of the membrane structure as a result of lipid removal. In order to settle this question various investigators have studied the effect of adding various lipids to the delipidated preparations. With the exception of some studies, in which drastic extraction[18] or complete enzymatic hydrolysis[17,19] of phospholipids occurred, reactivation was observed in most studies. After treatment with desoxycholate the highest reactivation was obtained with phosphatidylserine[6,7,9,10,12,27,28], although this compound could be replaced by mono- and dialkylphosphates[29], lysophospha-

tidylcholine[27] and phosphatidylglycerol[12]. After treatment with Lubrol W, reactivation was observed with various phospholipids[13]. After partial inactivation with phospholipases some authors found the highest reactivation with phosphatidylserine[16,20] and phosphatidylinositol[20], while other authors found no clear specificity at all[22,23]. Entirely different results were obtained after treatment with organic solvents at −20 or −70 °C [25,26]. Although removal of cholesterol did not reduce enzyme activity, this compound gave the highest reactivation when the enzyme was inactivated by removal of the phospholipid with organic solvents at low temperature[25,26]. Treatment of the enzyme with organic solvents at higher temperature leads to irreversible inactivation of the enzyme[8,11,17,25].

Since in most cases phosphatidylserine is quantitatively the most effective of the reactivating phospholipids particularly after treatment with desoxycholate, some authors drew the conclusion that this phospholipid is essential for the functioning of the native enzyme[6,9,10,12,24]. Wheeler and Whittam[10] suggested that the transport system consists of a complex of phosphatidylserine and the (Na^+-K^+)-ATPase protein, whereas Kimelberg and Papahadjopoulos[12] correlated this specific reactivation for (Na^+-K^+)-ATPase with a selective discrimination by the acidic phospholipids for K^+ over Na^+ in cation permeability of phospholipid vesicles[30].

We have investigated whether phosphatidylserine is essential for the activity of the (Na^+-K^+)-ATPase system by treating a preparation of this enzyme with phosphatidylserine decarboxylase from *Escherichia coli*. We were able to convert phosphatidylserine quantitatively into its decarboxylation product, phosphatidylethanolamine, without loss in (Na^+-K^+)-ATPase activity. These experiments indicate that the carboxylic group of phosphatidylserine, and hence phosphatidylserine as such, is not essential for the functioning of the (Na^+-K^+)-ATPase system.

Materials and methods

NaI-treated microsomes were prepared from cattle brain cortex as described by Uesugi *et al.*[31]. The preparation had a specific (Na^+-K^+)-ATPase activity of 30–40 μmoles/mg protein per h, while the Mg^{2+}-ATPase activity was less than 1% of the total ATPase activity at pH 7.5. The preparation was stored at −20 °C as a suspension in 0.25 M sucrose. Phosphatidylserine was prepared from cattle brain white matter as described by Sanders[32].

Phosphatidylserine decarboxylase was prepared from *Escherichia coli* by a method adapted from that described by Kanfer and Kennedy[33], using 0.1% Triton X-100 instead of the detergent Cuscum. The activity of the phosphatidylserine decarboxylase preparation was tested in 0.5 ml of a medium containing 0.2 mM phosphatidylserine, 0.1 M Tris–HCl, pH 7.0, 0.1% (v/v) Triton X-100 and varying amounts of the enzyme preparation. The incubation mixture was incubated for 15–60 min at 37 °C. After the incubation period the mixture was evaporated *in vacuo* and the residue was extracted with three times 30 μl benzene–ethanol (4:1, v/v). The lipid extract was applied to a silicagel plate, containing 4% alkaline magnesium silicate (Woelm, Eschwege, West Germany) and developed with chloroform–methanol–conc. ammonia–water (90:54:5.5:5.5, by vol.). The spots were detected with iodine vapour, scraped off and their phosphorus content was determined

by a modified Fiske–SubbaRow method after H_2SO_4–$HClO_4$ digestion[34]. The enzyme activity was calculated from the percent conversion of phosphatidylserine to phosphatidylethanolamine.

For the treatment of the (Na^+–K^+)-ATPase preparation with phosphatidylserine decarboxylase the NaI treated microsomes were centrifuged for 30 min at 220 000 × *g* in a Ti 50 rotor in a Spinco L 50 ultracentrifuge and the sediment was taken up in 0.1 M Tris–HCl, pH 7.0, containing 0.1% (v/v) Triton X-100 at a concentration of 1 mg/ml protein. To 1 ml of this solution 300 μl of the phosphatidylserine decarboxylase preparation was added and the mixture was incubated for 3 h at room temperature.

As controls served a heat-inactivated (2 min at 100 °C) phosphatidylserine decarboxylase preparation and an incubation mixture in which the enzyme had been replaced by 300 μl 0.1 M phosphate buffer (pH 6.8) containing 20 mM EDTA.

The (Na^+–K^+)-ATPase activity of the preparation was determined in two 20 μl aliquots removed before and after the incubation period. The aliquots were mixed with 300 μl of the media A (55 mM Na^+, 5 mM K^+, 2 mM Mg^{2+}, 2 mM ATP, 0.1 mM EDTA 100 mM Tris–HCl, pH 7.5) and E (no K^+, *plus* 10^{-4} M ouabain), respectively. After 30 min incubation at 37 °C released inorganic phosphate was determined [1].

The phospholipid composition of the (Na^+–K^+)-ATPase preparation was determined after the 3 h incubation period. One ml of each solution was evaporated *in vacuo* and extracted with three times 30 μl benzene–ethanol (4:1, v/v). The lipid extract was not washed as commonly done in lipid extraction procedures[35,36], since the presence of Triton X-100 led to a loss of lipids in the aqueous phase. Phospholipid analysis of the lipid extract was carried out by two dimensional thin-layer chromatography on silicagel containing 4% alkaline magnesium silicate[34]; the results are shown in Fig. 1.

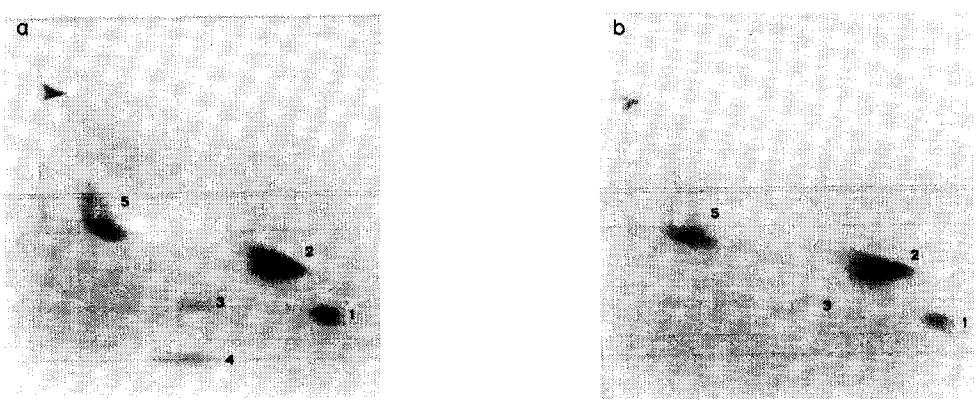

Fig. 1. Thin-layer chromatograms of the phospholipids of the (Na^+–K^+)-ATPase preparation with inactivated phosphatidylserine decarboxylase (a) and with active phosphatidylserine decarboxylase (b). The thin-layer plates were prepared and developed according to Broekhuyse[34]. The phospholipids were coloured according to Dittmer and Lester[40]. 1 = sphingomyelin, 2 = phosphatidylcholine, 3 = phosphatidylinositol, 4 = phosphatidylserine, 5 = phosphatidylethanolamine.

Results

Incubation conditions were sought, which would permit optimal functioning of phosphatidylserine decarboxylase and which would not be inhibitory to (Na^+-K^+)-ATPase under control conditions. The choice of detergent was important in this respect. In the presence of Lubrol W phosphatidylserine decarboxylase was inactive. Triton X-100 was more favorable, inasmuch as the enzyme was fully active in the presence of 1–20 μl/ml Triton X-100. Since higher concentrations of this detergent were inhibitory to (Na^+-K^+)-ATPase, we normally used a detergent concentration of 1 μl/ml. The activity of the phosphatidylserine decarboxylase preparations ranged from 2 to 5 μmoles phosphatidylserine hydrolyzed per mg protein per h. Incubation of the (Na^+-K^+)-ATPase preparation at 37 °C with or without phosphatidylserine decarboxylase led to a decrease in (Na^+-K^+)-ATPase activity, but incubation at 22 °C did not decrease activity, either in the presence or in the absence of phosphatidylserine decarboxylase (Table I). The presence in the incubation medium of 4.5 mM EDTA, originating from the phosphatidylserine decarboxylase preparation, was found to be necessary to prevent inactivation of (Na^+-K^+)-ATPase during the 3 h incubation period.

The phospholipid composition of the (Na^+-K^+)-ATPase preparation was determined after the 3 h incubation period and compared with that of a control preparation in which the phosphatidylserine decarboxylase preparation had been inactivated by boiling for 2 min.

The results in Table II (column D) show that there was no significant change in the percentages of phosphatidylcholine, sphingomyelin and phosphatidylinositol. However, the phosphatidylserine content decreased by 7.0 (S.E., 0.89)% and the phosphatidylethanolamine content increased by 11.7 (S.E., 2.58)%. These two changes are not significantly different, which indicates that an equimolar conversion of phosphatidylserine into phosphatidylethanolamine has taken place.

A comparison of the phospholipid composition of the original (Na^+-K^+)-ATPase

TABLE I

RELATIVE (Na^+-K^+)-ATPase ACTIVITIES BEFORE AND AFTER TREATMENT WITH PHOSPHATIDYLSERINE DECARBOXYLASE

Incubation time (h)	*Phosphatidylserine decarboxylase present* *	*(Na^+-K^+)-ATPase activity* **	
		%	*S.E.*
0	–	100	–
0	+	100.5	3.5
3	–	96.5	1.3
3	+	99.7	2.7

* In the control experiments (–) a phosphatidylserine decarboxylase preparation, which had been inactivated by boiling for 2 min, was present.

** Averages for 8 experiments each with standard error of the mean.

TABLE II

PHOSPHOLIPID COMPOSITION OF NaI-TREATED BRAIN MICROSOMES WITH AND WITHOUT TREATMENT WITH PHOSPHATIDYLSERINE DECARBOXYLASE

Phospholipid analysis was carried out by two dimensional thin-layer chromatography and phosphorous analysis of the spots after H_2SO_4–$HClO_4$ digestion[34]. The sum of the phosphorous contents of the spots was set at 100 percent. Averages are given with standard error of the mean. The discrepancy between the values on columns A and B is due to the presence of phosphatidylethanolamine in the phosphatidylserine decarboxylase preparation derived from *E. coli*. About 40% of the phosphatidylethanolamine (19.3% of the total lipids) given in column B originates from the *E. coli* preparation.

	A – *phosphatidylserine decarboxylase*	*B* + *phosphatidylserine decarboxylase (boiled)*	*C* + *phosphatidylserine decarboxylase*	*D* = *B*–*C*
Lysophosphatidylcholine	–	1.6 ± 0.6	0.8 ± 0.4	– 0.8 ± 0.7
Sphingomyelin	7.1 ± 0.6	6.6 ± 0.3	5.8 ± 0.5	– 0.8 ± 0.6
Phosphatidylcholine	41.4 ± 0.8	33.0 ± 1.1	30.2 ± 1.4	– 2.8 ± 1.8
Phosphatidylserine	10.1 ± 0.8	7.9 ± 0.7	0.9 ± 0.4	– 7.0 ± 0.9
Phosphatidylinositol	5.4 ± 1.4	2.6 ± 0.3	2.3 ± 0.3	– 0.3 ± 0.4
Phosphatidylethanolamine	36.0 ± 1.1	48.3 ± 1.8	60.0 ± 1.9	+ 11.7 ± 2.6
No. of determinations	6	10	11	

preparation (Table II, column A) with that of the mixture of $(Na^+–K^+)$–ATPase with boiled phosphatidylserine decarboxylase (column B) shows a difference which is mainly due to the phosphatidylethanolamine content of the phosphatidylserine decarboxylase preparation. This enzyme is prepared from membranes from *E. coli* in which phosphatidylethanolamine is by far the most important phospholipid[37].

The average residual amount of phosphatidylserine for all 11 experiments was 0.9 (S.E., 0.04)%, which is hardly significant ($P = 0.05$). In three experiments the residual amount of phosphatidylserine was undetectable (< 0.1%), suggesting that a complete conversion to phosphatidylethanolamine had occurred.

The results in Table I indicate that enzymatic conversion of phosphatidylserine to phosphatidylethanolamine caused no significant decrease in $(Na^+–K^+)$-ATPase activity. This was also true in the case of the three experiments, in which the residual amount of phosphatidylserine had become undetectable. Here the average $(Na^+–K^+)$-ATPase activity was 99.8% (S.E., 0.8)% relative to the control activity before incubation.

Discussion

The results described in this paper show that it is possible to convert virtually all phosphatidylserine present in a $(Na^+–K^+)$-ATPase preparation from cattle brain into phosphatidylethanolamine without reduction in $(Na^+–K^+)$-ATPase activity. This proves that the carboxylic group of this phospholipid is not essential for the $(Na^+–K^+)$-ATPase activity. Since this group is the only difference between phosphatidylserine and phosphatidylethanolamine, we may conclude that the presence of phosphatidylserine is not essential for $(Na^+–K^+)$-ATPase activity.

This conclusion, however, must be qualified somewhat. From the (Na^+-K^+)-ATPase activity in our preparation of 40 μmoles ATP hydrolyzed per mg protein per h, the molecular activity (turnover number) of (Na^+-K^+)-ATPase of 12 000 min^{-1} (refs 38, 39) and from the phospholipid content of 0.52 μmoles/mg protein in our preparation, we calculate a ratio of 9300 moles of phospholipid per mole (Na^+-K^+)-ATPase. Even when the total amount of phosphatidylserine is reduced to less than 0.1% of the total phospholipids, there could still be 9 molecules of phosphatidylserine present per molecule of (Na^+-K^+)-ATPase. Supposing that these molecules are located in the immediate neighbourhood of the (Na^+-K^+)-ATPase unit and that they are not broken down by the phosphatidylserine decarboxylase preparation, one might argue that this phospholipid could still be essential for the (Na^+-K^+)-ATPase activity. However, one must keep in mind that the only evidence in favour of a role of phosphatidylserine for (Na^+-K^+)-ATPase function is the finding that after delipidation with deoxycholate[6,7,9,10,12] and in some studies with phospholipases[16,20] addition of this phospholipid specifically reactivates the (Na^+-K^+)-ATPase activity. Calculations of Kimelberg and Papahadjopoulos[12] show that for optimal reactivation about 3000 moles of phosphatidylserine per mole of (Na^+-K^+)-ATPase are necessary, which is nearly three orders of magnitude higher than the amount left in our preparation.

In a recent study Goldman and Albers[24] report that after treatment with phospholipase A the (Na^+-K^+)-ATPase activity was completely inactivated, whereas after treatment with a phospholipase C preparation of *Clostridium welchii* only partial inhibition of (Na^+-K^+)-ATPase activity was observed without change in either the steady state level of the phosphoryl enzyme or the rate of the Na^+-dependent nucleotide transphosphorylation. Since the phospholipase C preparation reduced phosphatidylcholine and phosphatidylethanolamine contents but not the phosphatidylserine content, they concluded that phosphatidylserine plays an important role both in the phosphorylation and the dephosphorylation of the enzyme. The evidence for this conclusion is very indirect. It could also be that the (Na^+-K^+)-ATPase unit is kept in its proper configuration when the diglycerides are still present, while after lysophospholipid and free fatty acid formation this configuration is lost, possibly by the detergent action of the lysophospholipids.

Hence, we conclude that the evidence for a specific role of phosphatidylserine in (Na^+-K^+)-ATPase function is very indirect, while our findings together with those of several other investigators, who find no specificity for phosphatidylserine after delipidation with various agents[13,18,22,23,25,26], strongly disfavour this hypothesis.

A possible explanation of the different results may be that for optimal activity the (Na^+-K^+)-ATPase activity must be in a particular conformation, which is partly sustained by the various phospholipids. Removal of these phospholipids by means of enzymes or by extraction with detergents or organic solvents may lead to a conformational change such that the enzyme activity is reduced or completely inactivated. It depends on the resulting conformation, which may differ for the various delipidation methods, which lipid or group of lipids, when added to the inactivated enzyme, is able to return the enzyme to an active conformation. The preferential effect of addition of phosphatidylserine may be related to its net negative charge resulting from its three charged groups.

Note added in proof (Received October 2nd, 1973)

In a very recent study on the lipid dependence of (Na^+-K^+)-ATPase from human erythrocyte ghosts, Roelofsen and van Deenen[41] (kindly made available to us by the authors in manuscript form) come to different results. In their experiments phosphatidylserine decarboxylase treatment removes 88% of phosphatidylserine without change in (Na^+-K^+)-ATPase activity, but after prior removal of "loosely bound lipids" with anhydrous ether treatment with phosphatidylserine decarboxylase removes 96–98% of phosphatidylserine with 99–100% loss of (Na^+-K^+)-ATPase activity. This contrasts with our removal of up to 99% of phosphatidylserine without loss of the latter activity.

Since the specific (Na^+-K^+)-ATPase activity in erythrocyte ghosts is about 100 times lower than in our cattle brain preparation, the ratio phosphatidylserine (Na^+-K^+)-ATPase in their residual preparation is at least 200 times higher than in our preparation. Although a difference in tissue and/or species can always be invoked to explain the opposite result, it seems more likely to us that the pretreatment with anhydrous ether, though not affecting the activity by itself, may cause the loss of (Na^+-K^+)-ATPase activity during the subsequent treatment with the phosphatidylserine decarboxylase preparation.

REFERENCES

1 Bonting, S.L. (1970) *Curr. Topics Bioenergetics* 3, 257–363
2 Tatibana, M. (1963) *J. Biochem. Tokyo* 53, 260–261
3 Tanaka, R. and Abood, L.G. (1964) *Arch. Biochem. Biophys.* 108, 47–52
4 Tanaka, R. and Strickland, K.P. (1965) *Arch. Biochem. Biophys.* 111, 583–592
5 Askari, A. and Fratantoni, J.C. (1964) *Biochim. Biophys. Acta* 92, 132–141
6 Fenster, L.J. and Copenhaver, Jr, J.H. (1967) *Biochim. Biophys. Acta* 137, 406–408
7 Towle, D.W. and Copenhaver, Jr, J.H. (1970) *Biochim. Biophys. Acta* 203, 124–132
8 Zamudio, I., Cellino, M. and Canessa-Fisher (1969) *Arch. Biochem. Biophys.* 129, 336–345
9 Wheeler, K.P. and Whittam, R. (1970) *Nature* 225, 449–450
10 Wheeler, K.P. and Whittam, R. (1970) *J. Physiol. London* 207, 303–328
11 Roelofsen, B., Zwaal, R.F.A. and van Deenen, L.L.M. (1971) *Adv. Exp. Med. Biol.* 14, 209–228
12 Kimelberg, H.R. and Papahadjopoulos, D. (1972) *Biochim. Biophys. Acta* 282, 277–299
13 Palatini, P., Dabenni-Sala, F. and Bruni, A. (1972) *Biochim. Biophys. Acta* 288, 413–422
14 Skou, J.C. (1961) in *Membrane Transport and Metabolism* (Kleinzeller, A. and Kotyk, A., eds), pp. 228–236, Academic Press, London, New York
15 Schatzmann, H.J. (1962) *Nature* 196, 677
16 Ohnishi, T. and Kawamura, H. (1964) *J. Biochem. Tokyo* 56, 377–378
17 Roelofsen, B., Baadenhuysen, H. and van Deenen, L.L.M. (1966) *Nature* 212, 1379–1380
18 Emmelot, P. and Bos, C.J. (1968) *Biochim. Biophys. Acta* 150, 341–353
19 Hegyvary, C. and Post, R.L. (1969) in *Molecular Basis of Membrane Function* (Tosteson, D.C., ed.), pp. 519–528, Prentice Hall, Inc., New Jersey
20 Taniguchi, K. and Tonomura, Y. (1971) *J. Biochem Tokyo* 69, 543–557
21 Sun, A.Y., Sun, G.Y. and Somorajski, T. (1971) *J. Neurochem.* 1711–1718
22 Hokin, L.E. and Hexum, T.D. (1972) *Arch. Biochem. Biophys.* 151, 453–463
23 Stahl, W.L. (1973) *Arch. Biochem. Biophys.* 154, 56–67
24 Goldman, S.S. and Albers, R.W. (1973) *J. Biol. Chem.* 248, 867–874
25 Noguchi, T. and Fried, S. (1971) *Nat. New Biol.* 230, 148–150
26 Järnefelt, J. (1972) *Biochim. Biophys. Acta* 266, 91–96
27 Tanaka, R. (1969) *J. Neurochem.* 16, 1301–1307

28 Tanaka, R., Sakamoto, T. and Sakamoto, Y. (1971) *J. Membrane Biol.* 4, 42–51
29 Tanaka, R. and Sakamoto, T. (1969) *Biochim. Biophys. Acta* 193, 384–393
30 Papahadjopoulos, D. (1971) *Biochim. Biophys. Acta* 241, 254–259
31 Uesugi, S., Dulak, N.C., Dixon, J.F., Hexum, T.D., Dahl, J.L., Perdue, J.F. and Hokin, L.E. (1971) *J. Biol. Chem.* 246, 531–543
32 Sanders, H. (1967) *Biochim. Biophys. Acta* 144, 485–487
33 Kanfer, J. and Kennedy, E.P. (1964) *J. Biol. Chem.* 239, 1720–1726
34 Broekhuyse, R.M. (1968) *Biochim. Biophys. Acta* 152, 307–315
35 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) *J. Biol. Chem.* 226, 497–509
36 Bligh, E.G. and Dyer, W.J. (1958) *Can. J. Biochem. Physiol.* 37, 911–917
37 Haest, C.W.M., de Gier, J. and van Deenen, L.L.M. (1969) *Chem. Phys. Lipids* 3, 413–417
38 Bader, H., Post, R.L. and Bond, E. (1968) *Biochim. Biophys. Acta* 150, 41–46
39 Jörgensen, P.L. and Skou, J.C. (1971) *Biochim. Biophys. Acta* 233, 366–380
40 Dittmer, J.C. and Lester, R.L. (1964) *J. Lipid Res.* 5, 126–127
41 Roelofsen, B. and van Deenen, L.L.M. (1973) *Eur. J. Biochem.*, in the press