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PHOSPHATIDYLINOSITOL AS THE ENDOGENOUS ACTIVATOR OF THE ($Na^+ + K^+$)-ATPase IN MICROSOMES OF RABBIT KIDNEY

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Summary

Incubation of rabbit kidney microsomes with pig pancreatic phospholipase A_2 produced residual membrane preparations with very low ($Na^+ + K^+$)-ATPase activity. The activity could be restored by recombination with lipid vesicles of negatively-charged glycerophospholipids. Vesicles of pure phosphatidylcholine and phosphatidylethanolamine were virtually inactive in this respect, but could reactivate in the presence of cholate.

Incubation of the microsomes with a combination of phospholipase C (*Bacillus cereus*) and sphingomyelinase C (*Staphylococcus aureus*) resulted in 90—95% release of the phospholipids. The residual membrane contained only phosphatidylinositol and still showed 50—100% of the ($Na^+ + K^+$)-ATPase activity.

Introduction

The specificity of the phospholipid requirements for ($Na^+ + K^+$)-stimulated ATPase activity has been the subject of much debate. In many experiments (see Table 3 of ref. 1) negatively-charged phospholipids have been shown to be potent reactivators of the enzyme activity of preparations which had been delipidated with the aid of detergents or phospholipases. However, there are observations which suggest that neutral phospholipids, such as phosphatidylcholine, may also fulfil the necessary requirements for reactivation (refs. 2, 3; see also Table 3 of ref. 1). Using the enzymes phosphatidylserine decarboxylase from *Escherichia coli* and phospholipase C from *Clostridium welchii* it could be shown that in the erythrocyte membrane phosphatidylserine is the endogeneous activator of the ($Na^+ + K^+$)-ATPase [4,5]. On the other hand experiments with microsomes from cattle brain have shown that a virtually

Abbreviation: EGTA, ethyleneglycol bis(α-aminoethylether)-*N*,*N*′-tetraacetic acid.

complete decarboxylation of phosphatidylserine with the decarboxylase did not result in a reduction of the ($Na^+ + K^+$)-ATPase activity [6]. These conflicting results suggest that in different membranes different phospholipids are associated with the ($Na^+ + K^+$)-ATPase. This possibility has already been recognized by Walker and Wheeler [7]. In this communication some evidence is presented which indicates that, in a microsomal fraction prepared from rabbit kidney, phosphatidylinositol is the most likely endogeneous activator.

Experimental

After the removal of blood vessels and muscle tissue from frozen rabbit kidney, microsomes were prepared according to Tashima and Hasegawa [8]. The final precipitate was suspended in water and stored as small samples at −20°C [9]. These preparations were shown to have a Mg^{2+}-dependent and a ($Na^+ + K^+$)-stimulated ATPase activity of about 8 and 20 μmol of inorganic phosphate released/mg protein per h, respectively.

Controlled incubations with phospholipases were carried out at 37°C in the cell of an automatic titration unit (Radiometer, Copenhagen). In the experiments aliquots of the microsomes corresponding to 20 mg of protein were diluted to a final volume of 10 ml. For incubation with phospholipase C and sphingomyelinase C the medium contained 5 mM $CaCl_2$ and 0.25 mM $MgCl_2$. During the period of incubation the system was maintained at pH 7.4 using 0.02 M NaOH. At zero time 150 I.U. of phospholipase C (from *Bacillus cereus*) [10] and 25 I.U. of sphingomyelinase C (from *Staphylococcus aureus*) [10] were added, and after 30 min of incubation the addition of enzymes was repeated. After a further 30 min the incubation was terminated by cooling in ice and the addition of 5 ml ice-cold 3 mM histidine/imidazole buffer, pH 7.4, containing 15 mM ethyleneglycol bis(α-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA). The treated membranes were collected by centrifugation for 30 min at 115 000 × *g* and suspended in H_2O at a concentration of about 2 mg protein per ml.

For the incubation with phospholipase A_2 the medium contained 5 mM $CaCl_2$ and 1% (w/v) fat-free bovine serum albumin (Calbiochem). At zero time 175 I.U. of phospholipase A_2 (from pig pancreas) [11] were added and the pH was maintained at 7.4. When the titration of produced fatty acids stopped the suspension was quickly frozen in CO_2/acetone. After thawing, the titration was resumed until the production of fatty acids stopped again. In order to achieve maximal hydrolysis the whole procedure was repeated, starting with the addition of a new sample of 175 I.U. of phospholipase A_2. After a total incubation time of 1 h the phospholipase A_2 activity was inhibited by cooling in ice and diluting the suspension with 5 ml ice-cold buffer (3 mM histidine/imidazole/15 mM EGTA/1% (w/v) albumin, pH 7.4). The suspension was centrifuged for 30 min at 115 000 × *g* and the pellet washed with 0.25 M sucrose/10 mM Tris · HCl/1 mM EDTA buffer (pH 7.4), once with and once without 1% (w/v) bovine serum albumin. Finally, the pellet was resuspended in water at a concentration of about 2 mg protein per ml. Blanks were treated in the same way without addition of the enzymes.

For the recombination experiments, phospholipids were dispersed in 0.25 M

sucrose/10 mM Tris · HCl/1 mM EDTA buffer (pH 7.4) and sonicated for 5 min, whilst ice-cooled and under N_2, using a Branson sonifier-B12 (microtip, 70 W output). pH control after the sonication showed no significant deviation from pH 7.4.

Phosphatidylcholine was purified from egg yolk by acetone precipitation and subsequent chromatography over alumina and silicagel [13]. Cattle brain phosphatidylserine was isolated according to the procedure of Sanders [12]. Phosphatidylcholines with defined fatty acid chains were obtained by synthetic methods [13]. Various phosphatidylcholines were converted into the corresponding phosphatidylglycerols, phosphatidylserines and phosphatidylethanolamines with the aid of phospholipase D [14]. Phosphatidylinositol from soybean was a commercial preparation obtained from Sigma. The individual phospholipids appeared to be pure when examined by two-dimensional thin layer chromatography [19].

Reconstitution of lipid-protein interaction in the phospholipase A_2-delipidated enzyme was achieved by the addition of 200 μl of a 1 mM phospholipid dispersion to 100 μl of the treated microsome suspension (containing 0.2 mg protein). In earlier studies this phospholipid to protein ratio was found to be optimal [15,16]. The recombinate was vigorously mixed, kept in ice for 20 min and finally frozen and thawed [17]. The ATPase activity of each preparation was determined in duplicate as described by Palatini et al. [15], using the following incubation medium: 100 mM NaCl/20 mM KCl/2.5 mM $MgCl_2$/3 mM Tris-ATP/21 mM histidine/50 mM sucrose/0.2 mM EDTA, pH 7.4, and with or without 1 mM ouabain.

Phospholipids were extracted from the enzyme preparations according to Reed et al. [18], after denaturation of the protein by addition of trichloroacetic acid (final concentration 5%) *. The phospholipids were separated by two-dimensional thin layer chromatography as described by Broekhuyse [19] and determined as phosphate after destruction with 70% $HClO_4$ at 200°C by a modification of the procedure of Fiske and Subba Row [20]. Protein concentrations were determined according to Lowry et al. [21] after dissolving particulate material with sodium desoxycholate [15], which detergent was also included in the blank and standard protein solutions.

Results and Discussion

The native microsomes contained about 0.75 μmol of phospholipid per mg protein. The mean composition of the phospholipids was as follows: 30% phosphatidylcholine, 24% sphingomyelin, 22% phosphatidylethanolamine, 6.5% phosphatidylserine, 4.5% phosphatidylinositol, 6.5% lysophosphatidylcholine and 6.5% lysophosphatidylethanolamine.

Treatment of the microsomal membranes with porcine pancreatic phospholipase A_2 resulted in an efficient degradation of the glycerophospholipids together with a destruction of the ($Na^+ + K^+$)-ATPase activity (Table I, Expts.

* An additional advantage of adding trichloroacetic acid is that the pH of the system is lowered, which facilitates the complete recovery of acidic phospholipids by the extraction procedure. In the context of these studies this is of paramount importance.

TABLE I

EFFECT OF PHOSPHOLIPASE A_2 TREATMENT OF MICROSOMAL MEMBRANES ON THE ($Na^+ + K^+$)-ATPase ACTIVITY

Microsomes were treated with phospholipase A_2 from porcine pancreas as described in Experimental. Percentages of residual glycerophospholipids were calculated by taking sphingomyelin as internal standard [26].

Expt. No.	Residual phospholipids (% of control)						($Na^+ + K^+$)-ATPase (μmol P_i/h per mg protein)	
	Total phospholipid *	Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Phosphatidylinositol	Control	Treated
1	44	100	0	0	0	0	22.6	—0.7
2	51	100	0	0	0	0	21.2	2.1
3	62	100	81	16	35	0	27.8	0.4
4	74	100	48	0	100	0	20.0	1.6

* Residual total phospholipid includes minor fractions of the produced lyso-compounds which remain in the membranes.

1 and 2). An even more interesting observation is, however, that an essentially complete inactivation of this ATPase is also observed in those cases where the preparations still contained a considerable amount of residual phosphatidylserine (Table I, Expts. 3 and 4). It should be emphasized that in all these experiments virtually all of the phosphatidylinositol had been hydrolysed. These results differ significantly from those obtained with human erythrocyte ghosts which, after extensive treatment with purified *C. welchii* phospholipase C, have shown that phosphatidylserine, as the only remaining phospholipid, is able to preserve full ($Na^+ + K^+$)-ATPase activity [5].

As shown in Table II, ($Na^+ + K^+$)-ATPase activity could be restored by recombination with sonicated vesicles of phosphatidylserine, phosphatidylglycerol and phosphatidylinositol. On the other hand, vesicles of phosphatidylethanolamine and phosphatidylcholine were found to be inactive in the recombination experiments. This confirms the view, already adopted by many others (see Table 3 in ref. 1 and refs. 7 and 22), that a negatively-charged lipid is essential in order to restore the enzymatic activity.

Hilden and Hokin [3] recently found that a purified ($Na^+ + K^+$)-ATPase preparation from the dogfish shark rectal gland, in which it is claimed that the endogeneous phospholipids had been displaced by pure phosphatidylcholine, still exhibit a coupled transport of Na^+ and K^+ which, of course, is in conflict with the observations discussed above. However, the fact that this phospholipid displacement had been catalysed by cholate prompted us to test the reactivation capacity of phosphatidylcholine in the presence of this detergent. Cholate by itself appeared incapable of restoring activity and, in fact, caused some inhibition in non-enzyme-treated blank experiments (Table III). The data in Table III also show, however, that by a combination of cholate and phosphatidylcholine the ($Na^+ + K^+$)-ATPase activity was restored. A possible explanation of this observation could be that the presence of cholate induces the necessary negative charge on the lipid structure, thus making the phosphatidyl-

TABLE II

REACTIVATION OF $(Na^+ + K^+)$-ATPase IN MICROSOMAL MEMBRANES DELIPIDATED BY TREATMENT WITH PORCINE PANCREATIC PHOSPHOLIPASE A_2

Control membranes were subjected to the same series of treatments, with the exception of the presence of phospholipase.

Expt. No.	Phospholipid added		$(Na^+ + K^+)$-ATPase	
	Type	μmol/mg protein	Control membranes (μmol P_i/h per mg protein)	Delipidated membranes (μmol P_i/h per mg protein)
1	—	—	20.1	0.4
	Phosphatidylserine (pig brain)	1	26.5	13.9
	Phosphatidylserine (prepared from egg phosphatidylcholine)	1	27.0	17.1
2	—	—	21.3	0.5
	Phosphatidylglycerol (di 18 : 1)	1	19.0	10.6
	Phosphatidylglycerol (prepared from egg phosphatidylcholine)	1	25.0	14.9
3	—	—	22.6	—0.7
	Phosphatidic acid (prepared from egg phosphatidylcholine)	1	24.4	11.5
4	—	—	21.9	0.7
	Phosphatidylinositol	0.5	18.4	5.2
	Phosphatidylinositol	1	23.3	6.6
	Phosphatidylinositol	1	21.5	6.2
	Phosphatidylinositol	2	18.1	5.8
5	—	—	20.0	1.6
	Phosphatidylethanolamine (di 18 : 1)	1	19.5	1.5
6	—	—	22.1	1.4
	Phosphatidylcholine (di 18 : 1)	1	20.0	0.4
	Phosphatidylcholine (di 14 : 0)	1	21.9	1.5
	Phosphatidylcholine (egg)	1	23.0	0.5
7	—	—	21.2	2.1
	Phosphatidylcholine (di 14 : 0)/ phosphatidylinositol (1 : 1)	2	18.8	3.4

choline suitable for reconstitution of activity. Walker and Wheeler [7] have already pointed out that this requirement for a negative charge could mean either that this charge is essential for the ATPase activity of the lipid · protein complex, or that it is only necessary to bring about the proper interaction between the added lipid and the protein. In order to gain some insight into this point we tested the activity of the phosphatidylcholine/cholate reactivated enzyme after removal of the cholate by extensive dialysis (twice for 30 min against 100 vols. of 0.25 M sucrose/10 mM Tris · HCl/1 mM EDTA, pH 7.4, and once for 21 h against 300 vols. of this buffer; the whole procedure was carried out at 4°C). Activities of the dialyzed preparations were compared with those of corresponding preparations after storage for 22 h at 4°C. The dialysis procedure alone appeared not to affect the ATPase activity in preparations which were free of cholate. However, after dialysis of a phospholipase A_2-delipidated preparation in which the activity had been restored by the addition of phosphatidylcholine plus cholate (0.2%, w/v) to 63% of the control value (10.5

TABLE III

REACTIVATION OF $(Na^+ + K^+)$-ATPase IN MICROSOMAL MEMBRANES BY EGG PHOSPHATIDYLCHOLINE PLUS CHOLATE

Membranes were delipidated by treatment with porcine pancreatic phospholipase A_2 plus bovine serum albumin. Control membranes were subjected to the same treatments without the presence of phospholipase. Phosphatidylcholine (prepared from egg) was added in amounts equivalent to 1 μmol per mg protein; cholate concentrations in the final preparations were as indicated.

Expt. No.	Addition		$(Na^+ + K^+)$-ATPase	
	Cholate (%, w/v)	Phosphatidylcholine	Control membranes (μmol P_i/h per mg protein)	Delipidated membranes (μmol P_i/h per mg protein)
1	—	—	27.8	1.4
	—	+	27.2	0.4
	0.02	—	22.7	1.8
	0.02	+	24.7	2.7
	0.10	—	17.8	1.9
	0.10	+	23.8	5.0
	0.20	—	20.8	2.9
	0.20	+	18.9	9.9
2	—	—	19.4	0.5
	—	+	18.9	0.6
	0.20	+	8.3	6.7

μmol P_i/h per mg protein), the residual $(Na^+ + K^+)$-ATPase activity was reduced to less than 6% of the blank. In another series of experiments, the $(Na^+ + K^+)$-ATPase in the delipidated microsomes was reactivated by the phosphatidylcholine/cholate mixture up to 38% of the control value. Subsequent dialysis caused a complete loss of the activity, which could be restored again by the addition of phosphatidylserine to 11% of the corresponding blank. Despite this rather low level, which seemed to be a consequence of the presence of phosphatidylcholine in the dialyzed microsomal preparation, the result indicates that the loss of activity observed after dialysis of the phosphatidylcholine/cholate-reconstituted preparation is indeed due to the removal of the cholate. The results therefore support the view that the negative charge on the lipid structure is essential for the functioning of the $(Na^+ + K^+)$-ATPase.

In three independent experiments a very efficient breakdown of the phospholipids in the microsomal membranes could also be obtained by the combined action of phospholipase C (*B. cereus*) and sphingomyelinase C (*S. aureus*). About 95% of the total phospholipid fraction could be degradated. Two-dimensional thin layer chromatography of the lipids extracted from the treated microsomes resulted in a single spot. The identity of this spot was established as being phosphatidylinositol by co-chromatography with a radioactive-labelled preparation of this phospholipid. A remarkable finding was that these highly delipidated membranes still exhibited 50—100% of the $(Na^+ + K^+)$-ATPase activity when compared to the non-enzyme-treated blanks. Accepting the fact that the enzyme requires association with negatively-charged phospholipids for activity, it can be concluded that phosphatidylinositol, representing only 5% of the total phospholipid complement of the membranes, completely fulfils this requirement. Fig. 1 summarizes the results derived from 8 individual

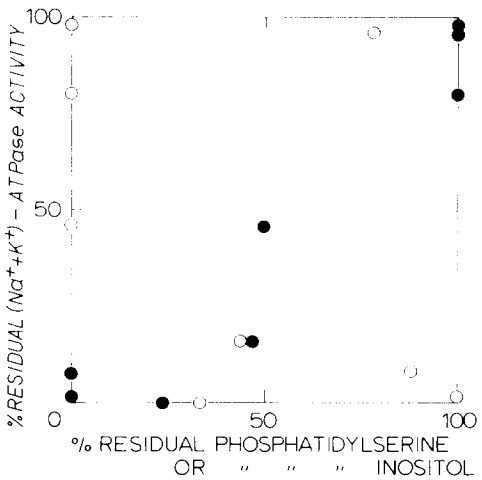

Fig. 1. Residual ($Na^+ + K^+$)-ATPase activity (% of control) related to residual phosphatidylserine (○) and phosphatidylinositol (●) (% of control) in microsomal membranes after treatment with either porcine or equine pancreatic phospholipase A_2 or *B. cereus* phospholipase C plus sphingomyelinase C. All these treatments resulted in an essentially complete degradation of phosphatidylcholine and phosphatidylethanolamine, as well as of sphingomyelin in those cases where sphingomyelinase C was included. The data represented by each pair of open and closed circles on the same horizontal line are derived from one experiment.

experiments involving the use of various phospholipases (porcine [10] and equine [23] pancreatic phospholipase A_2, and *B. cereus* phospholipase C plus sphingomyelinase C) which differ significantly in their substrate specificities, in particular with respect to the degradation of phosphatidylserine and phosphatidylinositol. This figure again clearly shows that phosphatidylserine, as the only remaining negatively-charged phospholipid, is unable to preserve the ($Na^+ + K^+$)-ATPase activity, whereas a complete degradation of this phospholipid does not significantly affect the activity, provided that phosphatidylinositol remains intact. On the other hand, however, there is an apparent relationship between residual ($Na^+ + K^+$)-ATPase activity and the remaining quantity of phosphatidylinositol, which indicates that this phospholipid is the endogeneous activator of the ($Na^+ + K^+$)-ATPase in this particular membrane. This may also explain the observations by de Pont et al. [6] that the ($Na^+ + K^+$)-ATPase activity is not affected by a conversion of phosphatidylserine into phosphatidylethanalamine, as the bovine brain microsomes contain amounts of phosphatidylinositol which are comparable to our preparations. The finding that phosphatidylinositol is the endogeneous activator of the ($Na^+ + K^+$)-ATPase in these microsomal membranes seems not to be in complete agreement with the results from the reconstitution experiments, which showed that this particular phospholipid is less effective than the other negatively-charged ones. However, the effectiveness of a given phospholipid to reactivate a lipid-requiring enzyme is determined not only by the nature of its polar head group, but also by the composition and fluidity of its acyl chains [7,16]. Furthermore, it may be argued that differences in structure and stability of the sonicated dispersions of the various phospholipids will affect the reconstitution of the lipid-protein interaction and thereby will influence the apparent reactivation capa-

city [5]. Thus, it appears that such reconstitution experiments can only give information as to whether or not a certain phospholipid class is able to reactivate the enzyme, but they do not provide quantitative data on the actual degree of effectiveness in maintaining the ATPase activity.

The concept that phosphatidylinositol is directly associated with the enzyme and determines its activity is of particular importance when we consider the dynamic metabolism in which this phospholipid is involved. A variety of cell stimuli are known to increase the turnover of phosphatidylinositol [24,25] and it may be speculated that the activity of the $(Na^+ + K^+)$-ATPase pump can be regulated by metabolic synthesis and breakdown of this activating phospholipid.

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