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POSSIBLE ROLE OF SULPHATIDE IN THE K^+-ACTIVATED PHOSPHATASE ACTIVITY

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A microsomal fraction rich in (Na^+ + K^+)-ATPase has been isolated from the outer medulla of pig kidney. (Mg^{2+} + K^+)-activated ouabain-sensitive phosphatase activity was studied in this preparation treated with arylsulphatase, an enzyme that specifically hydrolyzes ceramide galactose-3-sulphate. The activity of phosphatase was inactivated in proportion to the amount of sulphatide hydrolyzed. A maximum inactivation of ouabain-sensitive activity was obtained with 60% of the sulphatide content hydrolyzed. The inactivation caused by arylsulphatase was partially reversed by the sole addition of sulphatide. The evidence offered in this paper about sulphatide function in the sodium pump mechanism supports the idea that sulphatides are involved in the K^+-activated phosphatase, a partial reaction of the (Na^+ + K^+)-ATPase.

Introduction

Recent evidence indicates that (Na^+ + K^+)-ATPase from a wide variety of sources catalyzes the (Mg^{2+} + K^+)-activated, ouabain-sensitive hydrolysis of *p*-nitrophenyl phosphate. The close association of both enzyme activities suggests that the phosphatase is involved in the final step of the reaction catalyzed by the (Na^+ + K^+)-ATPase, hydrolyzing the phosphate ester provided by the Na^+-dependent phosphorylation of the enzyme [1–3].

Analysis of sulphatide content in tissues specialized in sodium transport suggests that sulphatide may act as a lipid requirement for (Na^+ + K^+)-ATPase activity. In a previous paper we established a correlation between (Na^+ + K^+)-ATPase activity, sulphatide content and sodium flux [4]. We also showed that ouabain-sensitive ATPase activity is fully inhibited after specific sulphatide hydrolysis. Moreover, we recently showed that most sulphatide is localized externally in the red blood cell membrane and that the active sodium efflux, in human red cells after sulphatide hydrolysis, is reduced to a value similar to that found in the absence of external potassium [5]. These results seem to indicate that sulphatide may be involved in the sodium pump mechanism.

The sulphatide cofactor model [6] postulates affinity between sulphatide and potassium ion. As the affinity site for potassium would be lacking when sulphatide is absent, inhibition of (Mg^{2+} + K^+)-activated phosphatase activity would be expected, since K^+ would be unable to reach the inside of the cell, the site of the enzyme.

In the present paper we show that the activity of K^+-phosphatase is reduced after sulphatide hydrolysis, and this reduction is not reversed even with high concentration of K^+ in the incubation medium.

Materials and Methods

(Na^+ + K^+)-ATPase preparation was isolated from the outer medulla of fresh pig kidney and purified by sodium deoxycholate treatment according to methods previously described [7], except that (Na^+ + K^+)-ATPase was recovered in

the sediment after centrifugation at 55 000 × *g* for 30 min instead of zonal centrifugations. The partially purified enzyme had an activity of 150 μmol P_i per h per mg protein, of ouabain-sensitive ATPase; 30-fold higher than the activity of outer medulla homogenate. The activity of $(Mg^{2+} + K^+)$-activated phosphatase, in the ATPase preparation, was estimated using 20 μg of protein under the assay conditions previously described [8], except that incubations were carried out at pH 5.4 and in the absence of bovine serum albumin. Hydrolysis of *p*-nitrophenyl phosphate was estimated by measuring the concentration of *p*-nitrophenol by its absorbance at 410 μm. The activity of ouabain-sensitive $(Mg^{2+} + K^+)$-activated phosphatase measured in the partially purified ATPase preparation ranged from 300 to 400 nmol *p*-nitrophenyl phosphate hydrolyzed per min per mg of protein, 30-fold higher than the value found in outer medulla homogenate.

Partially purified arylsulphatase was prepared from pig kidney cortex with some modifcations [4], showing an increase of about 875-fold over the homogenate with specific activity of 7 to 10 μmol of *p*-nitrocatechol sulfate hydrolyzed per min per mg of protein. It is necessary to point out that this preparation was free of other enzymes or inhibitors such as proteases or other hydrolases [5].

Lipids were obtained by extracting rat brain with 20 volumes of chloroform/methanol (2 : 1, v/v) at room temperature [5] and freed of nonlipid contaminants by chromatography on Sephadex G-25 [9]. Neutral lipid, cerebroside, sulphatide and phospholipid were separated from the total extract by silicic acid chromatography column (Unisil, Clarkson Chemical Co., Williamsport, PA) using as eluents chloroform, chloroform/acetone (1 : 1, v/v), acetone and methanol, respectively [9]. The acetone-eluted sulphatide fraction and methanol-eluted phospholipid fraction were evaporated under reduced pressure to a moist residue. Sulphatide and phospholipid thus obtained were microdispersed according to methods described previously [5]. Sulphatide concentration was determined [10], using bovine sulphatide (Applied Science Laboratories) as a standard, and the phospholipid concentration was measured on the basis of its phosphorus content [11]. For this purpose a mean mol. wt. of 744 was assumed.

Determination of the activity of $(Mg^{2+} + K^+)$-activated phosphatase in the presence of arylsulphatase and/or microdispersed lipids was conducted under identical ionic conditions. The tubes containing the medium were pre-incubated for 15 min at 37°C. *p*-Nitrophenyl phosphate/Tris was added to the mixture and incubation continued for 5 min. For every 20 μg of protein, 1 to 4 units (a unit is defined as the amount of enzyme which hydrolyzes 1 μmol of *p*-nitrocatechol per min at 37°C, pH 5.4) of the partially purified arylsulphatase were used.

Reactivation of arylsulphatase-treated ATPase preparation. Aliquots of 20 μg of protein were incubated with and without 4 units of arylsulphatase for 20 min at 37°C in a final volume of 0.4 ml containing 30 mM histidine, pH 5.4, 20 mM $MgCl_2$ and 8 mM KCl. The pellets of the arylsulphatase-treated preparation obtained after their centrifugation at 80 000 × *g* for 20 min were resuspended with different volumes of microdispersed sulphatide or phospholipid, and allowed to stand for 30 min at room temperature. Finally, samples were diluted with the required media for phosphatase activity measurements.

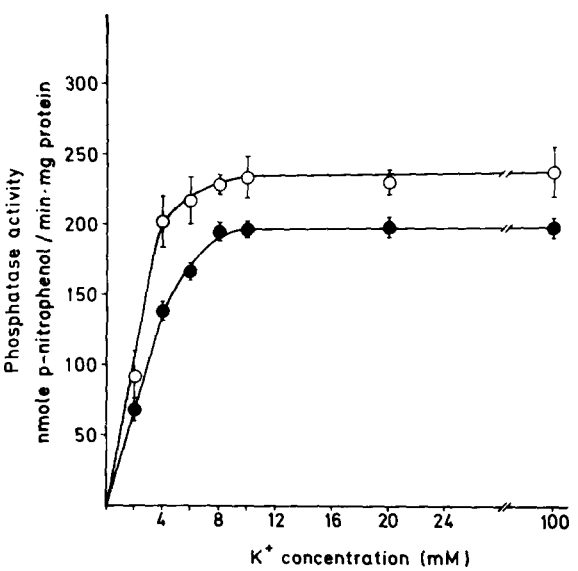

Fig. 1. Effect of varying potassium concentration on the activity of ouabain-sensitive K^+-activated phosphatase at pH 7.6 (○) and 5.4 (●). The ouabain-sensitive activity was calculated as the difference in activity with and without 1 mM ouabain present in the incubation medium. Each point represents the mean ± S.D. of five preparations.

TABLE I

EFFECT OF ARYLSULPHATASE ON PHOSPHATASE ACTIVITY

Activity is expressed as nmol *p*-nitrophenol liberated per min per mg protein. Aliquots of 200 μg of protein (ATPase preparation) were incubated with arylsulphatase for 15 min at 37°C in the ionic medium containing 8 mM K^+, pH 5.4. *p*-Nitrophenyl phosphate/Tris was added to the mixture and incubation continued for 5 min. One tenth of the final volume was used to measure the *p*-nitrophenol release, the remaining volume was extracted with chloroform/methanol (2 : 1, v/v). In the isolated glycolipid fraction, obtained by silicic acid chromotography column using as eluent acetone, sulphatide content was measured. The values represent the means ± S.D. of five preparations.

Arylsulphatase (units)	Ouabain (mM)	Specific activity	Sulphatide hydrolyzed (%)	Inhibition (%)	Inactivation (%)
None		498 ± 60			
1		357 ± 32	5.6 ± 0.8		28.4
2		248 ± 23	23.4 ± 2.1		50.3
4		115 ± 10	59.1 ± 4.9		77.0
8		114 ± 9	62.3 ± 5.7		77.1
None	1	112 ± 8		77.5	
4	1	112 ± 8		77.6	

Protein concentration was estimated in aliquots freed of histidine [12] using crystalline bovine serum albumin as standard [13].

Results

The effect of varying potassium concentration on the activity of ouabain-sensitive K^+-activated phosphatase at pH 7.6 and 5.4 is shown in Fig. 1. Maximum activity is obtained with 8 mM potassium in the assay mixture. The use of pH 5.4 instead of pH 7.6 results in a slightly lower activity of the enzyme, which is stable even after 60 min of incubation, at 37°C. The ouabain-insensitive activity of the preparation is reduced in 43% at pH 5.4 when compared with the same activity at pH 7.6 (not shown).

The effect of arylsulphatase on the ability of ($Na^+ + K^+$)-ATPase preparation to hydrolyze *p*-nitrophenyl phosphate is shown in Table I. It is

TABLE II

EFFECT OF ARYLSULPHATASE ON PHOSPHATASE ACTIVITY IN THE PRESENCE OF DIFFERENT K^+ CONCENTRATIONS

Activity is expressed as nmol *p*-nitrophenol liberated per min per mg protein. Aliquots of 20 μg of protein were treated with arysulphatase as described in Table I. The values represent the means ± S.D. of five preparations.

K^+ (mM)	Arylsulphatase (units)	Ouabain (mM)	Specific activity	Inactivation (%)	Inhibition (%)
0	None		114 ± 8		
0	None	1	112 ± 13		
0	4		115 ± 7		
8	None		498 ± 60		
8	None	1	112 ± 8		77.5
8	4		115 ± 10	77.0	
20	4		113 ± 9	77.3	
80	4		115 ± 5	77.0	
100	4		115 ± 6	77.0	
100	None	1	112 ± 5		77.5

TABLE III

PHOSPHATASE ACTIVITY IN THE PRESENCE OF MICRODISPERSED LIPID

Activity was assayed with 8 mM K^+ and is expressed as nmol *p*-nitrophenol liberated per min per mg protein. Sulphatides and phospholipids added to the incubation medium corresponds to 5, 15, 20, 30 and 5, 10, 20, 30 times the sulphatide and phospholipid content of the microsomal preparation used, respectively. The values represent the means ± S.D. of five preparations. The sulphatide and phospholipid content of the microsomal fraction were 46.4 and 725.0 μg per mg of protein, respectively. Data are the averages of four preparations.

Sulphatide (μg)	Phospholipid (μg)	Ouabain (mM)	Specific activity	Inhibition by ouabain (%)	Activation of ouabain-sensitive K^+-phosphatase (%)
None			513 ± 52		
None		1	145 ± 14	71.8	
4.5			549 ± 58		7.1
13.5			582 ± 69		13.7
18.0			571 ± 44		11.3
27.0			534 ± 51		4.1
13.5		1	154 ± 16	70.0	
	80		535 ± 51		4.4
	160		566 ± 45		10.4
	320		461 ± 56		
	480		395 ± 52		
	160	1	161 ± 26	71.6	

evident that the phosphatase activity is reduced when sulphatide is hydrolyzed. With 62% of the sulphatide content hydrolyzed, the phosphatase activity is reduced to 23% of the control. As the ouabain-insensitive activity is not affected, this represents an almost total (98%) inhibition of the ouabain-sensitive activity.

The effect of 4 units of arylsulphatase on the activity of K^+-activated phosphatase in the presence of different potassium concentrations is shown in Table II. Arylsulphatase causes a maximum inactivation of 98% on the activity of ouabain-sensitive phosphatase, even in the presence of a K^+ concentration as high as 100 mM.

TABLE IV

EFFECT OF ARYLSULPHATASE ON OUABAIN-SENSITIVE K^+-ACTIVATED PHOSPHATASE ACTIVITY IN THE PRESENCE OF MICRODISPERSED LIPIDS

Activity was assayed with 8 mM K^+ and is expressed as nmol *p*-nitrophenol liberated per min per mg protein. Sulphatides and phospholipid added to the incubation medium represent 10, 20 and 30 times the content of the microsomal fraction used. The values represent the means ± S.D. of five preparations.

Arysulphatase (units)	Sulphatide (μg)	Phospholipid (μg)	K^+-dependent activity	Inactivation (%)
None			376 ± 41	
4			78 ± 13	79.3
4	9		222 ± 39	40.9
4	18		252 ± 38	33.0
4	27		253 ± 34	32.7
4		160	66 ± 11	82.5
4		320	58 ± 10	84.6
4		480	69 ± 15	81.7

TABLE V

REACTIVATION OF K^+-DEPENDENT OUABAIN-SENSITIVE PHOSPHATASE ACTIVITY IN ARYLSULPHATASE-TREATED MICROSOMAL FRACTION

Activity was assayed with 8 mM K^+ and is expressed as nmol *p*-nitrophenol liberated per min per mg protein. The different amounts of lipid (μg) used to reactivate correspond to 6, 12, 18, 24, 30 and 2.5, 5, 10, 20 times the sulphatide and phospholipid content of the microsomal preparation used, respectively. The values represent the means ± S.D. of five preparations.

	Specific activity	Inactivation (%)	Inhibition by ouabain (%)	Reactivation of ouabain-sensitive K^+-phosphatase (%)
Untreated	393			
Treated	4 ± 2	99.0		
Treated plus sulphatide				
5.4	93 ± 12			23.8
10.8	223 ± 19			61.4
16.2	346 ± 29			88.1
21.6	351 ± 33			89.4
27.0	289 ± 37			73.6
26.6 + ouabain	11 ± 2		97.3	
Treated plus phospholipid				
40.0	17 ± 3			
80.0	20 ± 5			
160.0	42 ± 7			
320.0	68 ± 17			
320.0 + ouabain	70 ± 10			

The activity of phosphatase in the presence of microdispersed lipids is summarized in Table III. It is evident that the activity is slightly increased with the addition of sulphatide or phospholipid and both activations are related to ouabain-sensitive K^+-dependent phosphatase. A maximum activation of 10% and 14% is obtained by adding as much as 10- and 15-times the phospholipid and sulphatide content, respectively.

The effect of arylsulphatase on the activity of ouabain-sensitive K^+-activated phosphatase in the presence of microdispersed sulphatide or phospholipid is shown in Table IV. The inactivation in the presence of arylsulphatase is reduced from 79 to 33% as the sulphatide in the medium is increased from 0 to 27 μg. On the other hand, the different amounts of phospholipids added to the incubation medium do not diminish the effect of arylsulphatase. The effect of sulphatide and phospholipid added to arylsulphatase-treated microsomal fraction on the ouabain-sensitive phosphatase activity is shown in Table V. It can be observed that the different amounts of phospholipid do not reverse the effect of arylsulfatase on ouabain-sensitive phosphatase activity, and the partial reactivations observed correspond to an ouabain-insensitive K^+-dependent phosphatase activity. On the other hand, sulphatide does gradually reverse the effect of arylsulphatase on ouabain-sensitive phosphatase activity when the amount of sulphatide used is increased. A maximum reactivation of almost 90% of the ouabain-sensitive phosphatase activity is obtained with 24-times the sulphatide content of intact microsomal preparation.

Discussion

Due to previous evidence, suggesting a close association between ($Na^+ + K^+$)-ATPase and *p*-nitrophenyl phosphatase, enzymes involved on the Na^+ pump mechanism, and the hypothesis that sulphatide plays a specific function in the active sodium transport, supported by enzymatic and physiological studies [4,5], our interest has been focused on the role of sulphatide in K^+-activated phosphatase activity. The present study reports on the phosphatase activity in ($Na^+ + K^+$)-ATPase preparations when sulphatide is hydrolyzed by arylsulphatase treatment.

Our findings show that the breakdown of sulphatide of membranes rich in ATPase is accompanied by inactivation of the ouabain-sensitive phosphatase activity. Moreover, the sole addition of sulphatide in the medium is able to protect the phosphatase enzyme from arylsulphatase action. Furthermore, in arylsulphatase-treated preparations the activity of ouabain-sensitive phosphatase can be recovered by the addition of sulphatide.

Since the addition of mixed phospholipids does not protect the ouabain-sensitive phosphatase activity from the action of arylsulphatase, it is unlikely that the inactivation observed is due to the presence of traces of phospholipases in the arylsulphatase preparation.

The fact that in red blood cells the one-for-one sodium exchange is not altered on treatment with arylsulphatase [5] suggests that the conformational transition $E_1P \rightarrow E_2P$ remains unchanged. On the other hand, the fact that arylsulphatase reduces sodium efflux by about a third (that is, a reduction as high as that produced in intact red blood cells maintained in K^+-free medium) seems to indicate that ouabain-sensitive phosphatase, enzyme of the partial reaction of the $(Na^+ + K^+)$-transport system, requires sulphatide for its activity.

The ouabain-sensitive phosphatase activity needs extracellular K^+ for full activation [14], and this enzyme is largely inactive in the absence of external potassium. Thus the fact that the hydrolysis of sulphatide abolishes the enzyme activity, and that this effect can be reversed by the addition of sulphatide, seems to indicate that the sulphatide plays a role in the affinity site for potassium in the outside of the membrane. Since K^+-activated phosphatase activity in the absence of ATP and Na^+ is stimulated by K^+ at the cytoplasmic surface [2], when sulphatide is absent, inactivation of K^+-activated phosphatase activity would be expected as sulphatide has been proposed as essential for K^+ translocation.

The present results, together with the demonstration that there is straight correlation between $(Na^+ + K^+)$-ATPase-sulphatide content and sodium flux in skin [4], and our recent finding showing that the specific hydrolysis of sulphatide, localized externally in the red cell membrane, reduced the sodium efflux to the same extent of efflux in the absence of external potassium [5], strongly suggest that sulphatide is involved in the specific affinity site for K^+ on the outside of the membrane. Preliminary experiments in the same ATPase preparation treated with arylsulphatase show that the K^+-dependent dephosphorylation as well as the ouabain binding are blocked, and both are reversed by the sole readdition of sulphatide.

In spite of many studies carried out on the requirement for specific polar headgroups of phospholipid for the activity of purified $(Na^+ + K^+)$-ATPase and *p*-nitrophenylphosphatase, it has been impossible to establish the kind of phospholipid which would activate both enzymes, through a specific interaction with them [1,15–17]. This suggests that the lipid requirements could be other than phospholipidic.

Since our results in red blood cells under treatment with arylsulphatase [5] suggest that sulphatide is the affinity site for potassium ion, it would be expected that the conformation E_2PK does not take place, and a concomitant inactivation of ouabain-sensitive phosphatase activity would be also expected.

However, to envisage the particular role of sulphatide in the sodium pump enzyme, further studies such a potassium binding experiments are needed.

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