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PHOSPHATIDIC ACID PHOSPHATASE IN THE ERYTHROCYTE MEMBRANE

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SUMMARY

A magnesium-dependent α-phosphatidic acid phosphatase is present in the erythrocyte membrane which is stimulated by sodium, and to a lesser extent by lithium, ammonium, potassium and rubidium. Cesium produces little or no stimulation. No magnesium-dependent, sodium-stimulated, activity was observed with β-phosphatidic acid or lysophosphatidic acid; but both of these substrates were hydrolyzed. α-Glycerophosphate was hydrolyzed very feebly. Beryllium inhibited the magnesium-dependent phosphatidic acid phosphatase 30% at 10^{-4} M and completely at 10^{-3} M. Cesium and fluoride inhibited the sodium stimulation of the magnesium-dependent phosphatidic acid phosphatase. The initial phosphatidic acid phosphatase activity was at least 41.6 mμmoles/mg dry weight of ghosts/h, which compares favorably with the activity of the $Na^+ + K^+$-dependent, ouabain-inhibitable ATPase in the erythrocyte membrane; this is compatible with the idea that phosphatidic acid phosphatase may function as part of the $Na^+ + K^+$-dependent ATPase.

INTRODUCTION

Evidence has been presented that sodium transport is coupled to the renewal of phosphate in phosphatidic acid and that this renewal is catalyzed by the combined action of diglyceride kinase and phosphatidic acid phosphatase[1-3]. This mechanism has been termed the phosphatidic acid cycle. In the previous paper[4] it was shown that diglyceride kinase is present in the erythrocyte membrane, and its activity is sufficient to account for the rates of sodium transport in this structure (based on a ratio of 3 Na^+ per ATP). In this paper a Mg^{2+}-dependent phosphatidic acid phosphatase has been shown to be present in the erythrocyte membrane and to have an activity which is of the same order of magnitude as the diglyceride kinase. The Mg^{2+}-dependent phosphatidic acid phosphatase activity appears to be a distinct enzyme from the Mg^{2+}-independent activity. The Mg^{2+}-dependent activity is stimulated by sodium and to a lesser extent by potassium and lithium. The effects of certain inhibitors on phosphatidic acid phosphatase have also been studied. Preliminary reports of this work have been presented[1,2,5].

EXPERIMENTAL

Preparation and storage of erythrocyte membranes

Erythrocyte ghosts were prepared as described in the previous paper[4]. With the exception of the experiment shown in Table VIII the ghosts were suspended in $5 \cdot 10^{-4}$ M imidazole–histidine–Tris buffer (pH 7.1) and stored for as long as several months at −20°. In the experiment shown in Table VIII the ghosts were stored as described in the previous paper; *i.e.*, 10^{-4} M cysteine was added to the buffer, and the ghosts were stored in dry ice.

Assay for phosphatidic acid phosphatase activity

In most of the experiments reported here, ^{32}P-labelled phosphatidic acid (synthesized from diolein as described below) was used as the substrate and phosphatidic acid phosphatase activity was assayed by measurement of the amount of [^{32}P]orthophosphate liberated. Erythrocyte ghosts were incubated in an imidazole–histidine–Tris buffer (pH 7.1) which contained [^{32}P]phosphatidate and other additions for 1 h at 37.5°; the exact conditions of incubation are given in the tables and figures. After incubation, 0.05 ml of an aqueous 10% solution of albumin was added to the 1 ml of incubation mixture; 1 ml of cold 10% trichloroacetic acid was then added. The mixture was centrifuged and the supernatant fluid was transferred to a 12-ml capacity conical centrifuge tube. Wet ether (2 ml) was added and the mixture was mixed, using a rapid piston-like movement, with a glass rod which had a flange fashioned at the lower end. After thorough mixing, the two phases were allowed to separate and the ether was removed by aspiration. The extraction with ether was repeated two additional times. (The extractions with ether were carried out in order to remove the small amounts of [^{32}P]phosphatidic acid which had remained in the trichloroacetic acid supernatant solution.) A 1-ml aliquot of the aqueous phase was dried on an aluminum planchet and counted.

In order to check that this was a valid measure of the orthophosphate liberated from phosphatidic acid, radioactive orthophosphate was estimated in the initial experiments. This was carried out by an adaptation of the method of BERENBLUM AND CHAIN[6] for the estimation of orthophosphate. The method utilizes the fact that phosphomolybdate (formed from the orthophosphate) can be quantitatively extracted from aqueous solution into isobutanol. The ether-washed trichloroacetic extracts were made up to 7 ml. To these was added 0.5 ml of 10 N H_2SO_4, followed by 2.5 ml of 5% ammonium molybdate. 10 ml of isobutanol were then added and the mixtures were shaken in separatory funnels for 1 min. The isobutanol extracts were transferred to 10-ml graduated tubes and made up to 10 ml with ethanol. Aliquots were plated in 0.5-ml batches and counted. Controls without added molybdate were run to correct for the extraction of any organic ^{32}P into the isobutanol. The results from this procedure indicated that all of the acid-soluble ^{32}P released during the incubation of erythrocyte membranes with [^{32}P]phosphatidic acid was in the orthophosphate form. No [^{32}P]orthophosphate was found after incubation of boiled membrane with the substrate. The amount of acid-soluble organic phosphate in the ether-washed trichloroacetic acid extracts was very constant and was less than 30% of the smallest amount of orthophosphate liberated in these experi-

ments. Boiled controls or non-enzyme controls were routinely run to correct for this acid-soluble organic phosphate. In some experiments where an assay of only the Mg^{2+}-dependent phosphatidic acid phosphatase was required, the enzyme incubated with all components except Mg^{2+} served as the control.

Each value reported under RESULTS represents an average of triplicate or quadruplicate incubations. The reproducibility was quite good; for example, in an experiment in which 5 sets of quadruplicate incubations were carried out the average standard error of the mean for the acid-soluble radioactivity for each set of quadruplicate incubations ranged from 1.1% to 2.3% of the value for the mean.

In a few phosphatidic acid phosphatase assays, as indicated, unlabelled (dioleyl) phosphatidic acid (synthesized from diolein as described below) was used as the substrate and phosphatidic acid phosphatase activity was measured by the release of orthophosphate. After incubation, cold 50% trichloroacetic acid was added to the incubation mixture to give a final concentration of 5%; the mixtures were centrifuged. Orthophosphate in the supernatant fluid was estimated by the method of BARLETT[7].

Synthesis of ^{32}P-labelled phosphatidic acid

Phosphoric acid (85%) was dried overnight at 110° and then stored in a dessicator over P_2O_5. An aliquot of ^{32}P in dilute HCl obtained from Oak Ridge National Laboratories was dried under N_2 in a 15-ml pearshaped flask with a ground joint. Anhydrous phosphoric acid (4.3 mmoles) was then added to the ^{32}P. 12.9 mmoles of PCl_5 were added in three batches to the phosphoric acid and left at room temperature until there was no visible reaction. The mixture was then refluxed for 15 min until a clear solution resulted. In the earlier experiments the reaction product was distilled to obtain pure $^{32}POCl_3$. It was subsequently found that this was not necessary, and losses of $^{32}POCl_3$ on the walls of the glassware could be consequently reduced.

17 mmoles of diolein (batch D47-DC, kindly supplied by Distillation Products Industries) were dissolved in 10 ml of dry pyridine and this was added to the $^{32}POCl_3$ at 0°. The reaction was allowed to proceed at 0° for 1 h and then at room temperature for 15 min. The contents of the flask were transferred with 100–150 ml of ether to a 250-ml Erlenmeyer flask, and a small amount of ice was added. The ether solution was washed successively with 250, 125 and 125 ml of cold 1 N HCl, followed by 125 ml of cold 0.1 N HCl. The final ether extract was taken almost to dryness in a flash evaporator at 30°. The extract was then transferred with a small amount of ethanol to a 250-ml beaker and titrated to about pH 7 with 0.54 N ethanolic NaOH, using bromthymol blue as the pH indicator. The sodium phosphatidate separated as a slightly tannish syrupy material. The sodium phosphatidate was washed twice with cold ethanol by stirring followed by decantation and was then dried *in vacuo* over NaOH.

To prepare the Tris phosphatidate, the sodium phosphatidate was dissolved in a minimum of ether, and about 2–4 volumes of water were added to give a stable emulsion. The emulsion was dialyzed against at least 100 volumes of 0.02 M Tris buffer (pH 7.4) with three changes over three successive days. After dialysis the phosphatidate was homogenized in a Potter–Elvejhem homogenizer. The final white

emulsion was stored in the frozen state at −20°. The Tris phosphatidate had a specific activity of about 100 000 counts/min/μmole when prepared. It could be conveniently used for assaying phosphatidic acid phosphatase in erythrocyte membranes until its specific activity had fallen (by radioactive decay) to about 10 000 counts/min/μmole.

Chromatography of the [^{32}P]phosphatidate on silicic acid impregnated paper[9] revealed that 95% of the radioactivity was in phosphatidic acid and 5% was in lysophosphatidic acid. Analysis of the phosphatidic acid for α- and β-phosphatidic acid (see below) revealed that 70% was β-phosphatidic acid and 30% was α-phosphatidic acid. These percentages may be taken as those for 1,3- and 1,2-diolein, respectively, in the commercial diolein preparation.

Analysis of the phosphatidic acid for the α- and β-isomers was performed as follows. The radioactive phosphatidic acid was deacylated by the method of DAWSON[9]. The α- and β-glycerophosphates were then separated by paper chromatography on Whatman No. 1 paper with methylcellosolve–methylethylketone–3 N ammonium hydroxide (7 : 2 : 3) saturated with boric acid[10]. After chromatography, the glycerophosphate spots were either stained by the method of WADE AND MORGAN[11] or autoradiographed on Kodak No Screen X-ray film; the spots were cut out and counted.

β-[^{32}P]Phosphatidic acid was synthesized as described above from 1,3-diolein, which was prepared by recrystallization of the commercial diolein in ethanol seven times. (Oleyl)lyso [^{32}P]phosphatidic acid was synthesized from α-monoolein as described previously[10] and was provided by Dr. R. A. PIERINGER. α-Glycero-[^{32}P]-phosphate was synthesized as described previously[12].

RESULTS

Effect of magnesium ions on the hydrolysis of phosphatidic acid

Phosphatidic acid was hydrolyzed when incubated with erythrocyte ghosts. This hydrolysis was markedly stimulated by low concentrations of Mg^{2+}. This effect of Mg^{2+} was similar to that previously shown for phosphatidic acid phosphatase activity in deoxycholate extracts of brain microsomes[13]. In erythrocyte ghosts, the optimum Mg^{2+} concentration was about 0.001 M (Fig. 1). With higher concentrations of Mg^{2+} the phosphatidic acid phosphatase activity was inhibited. This coincided with visible precipitation of the phosphatidate as insoluble magnesium phosphatidate. SMITH *et al.*[13] reported that 0.001–0.003 M Mg^{2+} profoundly inhibited phosphatidic acid phosphatase. These workers suggested that the inhibition was probably due to the formation of insoluble magnesium phosphatidate, since the inhibition by magnesium was less at pH 6.3 than at pH 7.4. The inhibition of phosphatidic acid phosphatase by magnesium may be due in part to factors other than solubility of the substrate. For example in studies on solubilized kidney phosphatidic acid phosphatase magnesium does not appear to stimulate enzyme activity over a fairly wide range of concentrations and inhibits enzyme activity at the same concentration which in the erythrocyte membrane system shows maximum stimulation[14]. Washing of erythrocyte ghosts or treatment of kidney enzyme with 0.001 M EDTA did not alter the effects of magnesium on enzyme activity, but it is possible that traces of Mg^{2+} are firmly bound to the enzyme. Fig. 1 indicates that in the erythrocyte

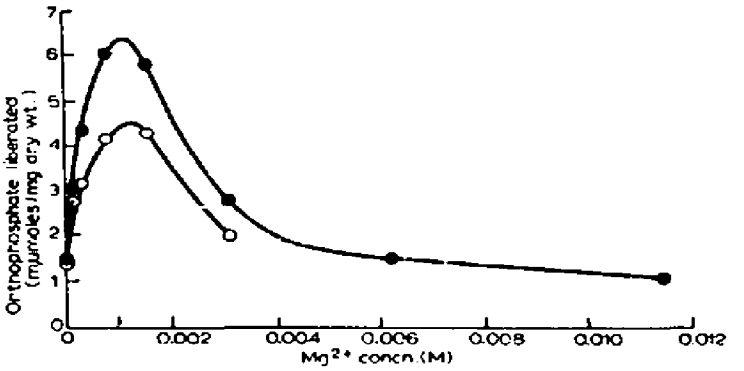

Fig. 1. Effect of Mg^{2+} on phosphatidic acid phosphatase. Erythrocyte ghosts were incubated in 1.0 ml of incubation medium at 37° for 1 h. The incubation medium contained the following substances (expressed as final concentration): 0.003 M Tris (dioleyl) [^{32}P]phosphatidate, 0.03 M imidazole–histidine–Tris buffer (pH 7.1) and 0.16 M NaCl. $MgSO_4$ was added as indicated. ●—●, ghosts prepared in the usual way; ○—○, ghosts prepared in the usual way and then washed two times with 0.001 M EDTA and finally suspended in $5 \cdot 10^{-1}$ M imidazole–histidine–Tris buffer (pH 7.1).

membrane system phosphatidic acid phosphatase activity is almost completely dependent on the presence of magnesium. Unless otherwise indicated, the data presented here will be expressed as the "Mg^{2+}-dependent phosphatidic acid phosphatase."

Effect of Na^+, K^+, and other monovalent cations on phosphatidic acid phosphatase

Na^+ markedly enhanced the Mg^{2+}-dependent phosphatidic acid phosphatase activity (Fig. 2). The optimum Na^+ concentration ranged from 0.1 to 0.15 M. Con-

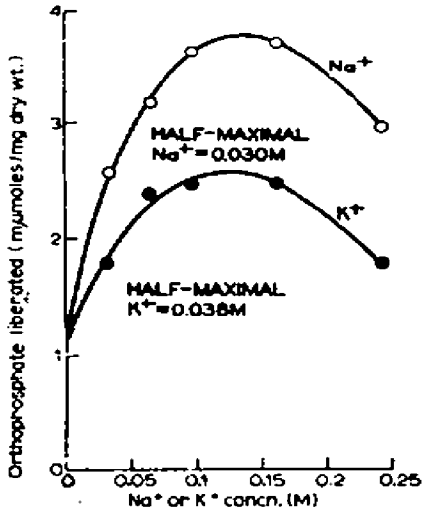

Fig. 2. Effect of Na^+ and K^+ on the Mg^{2+}-dependent phosphatidic acid phosphatase. Erythrocyte ghosts were incubated in 1.0 ml of incubation medium at 37° for 1 h. The incubation medium contained the following substances (expressed as final concentration): 0.003 M Tris (dioleyl) [^{32}P]phosphatidate, 0.03 M imidazole–histidine–Tris buffer (pH 7.1) and 0.0008 M $MgSO_4$. NaCl or KCl was added as indicated.

centrations of Na^+ from 0.15 M to 0.25 M inhibited the Mg^{2+}-dependent phosphatidic acid phosphatase. The Na^+ concentration for half-maximal stimulation was 0.030 M. Potassium also stimulated the Mg^{2+}-dependent phosphatase (Fig. 2). The effectiveness of K^+ in activating phosphatidic acid phosphatase ranged from 15 to 100% of that of Na^+, depending on the preparation of membranes. The half-maximal concentration of K^+ was very close to the half-maximal concentration of Na^+.

The effects of various monovalent cations on phosphatidic acid phosphatase activity in the presence and absence of Mg^{2+} are shown in Table I. In Expt. 1 of this Table, in the absence of a monovalent cation, Mg^{2+} had no effect on the total phosphatidic acid phosphatase activity. Expt. 2 of Table I is more representative of the

TABLE I

EFFECT OF VARIOUS CATIONS ON PHOSPHATIDIC ACID PHOSPHATASE IN ERYTHROCYTE GHOSTS

All monovalent cations were present in a concentration of 0.160 M. Other conditions were as in Fig. 2.

Experiment number	*Monovalent cation*	*Phosphatidic acid phosphatase activity (mμmoles/mg dry wt.)*	
		Total	*Mg^{2+}-dependent*
1	None	5.10	
	Mg^{2+}	5.10	
	Na^+	3.10	
	$Na^+ + Mg^{2+}$	9.10	6.00
	K^+	5.07	
	$K^+ + Mg^{2+}$	6.20	1.13
	Li^+	4.92	
	$Li^+ + Mg^{2+}$	9.20	4.28
	Rb^+	4.07	
	$Rb^+ + Mg^{2+}$	6.07	2.00
	Cs^+	5.30	
	$Cs^+ + Mg^{2+}$	5.20	0.10
	NH_4^+	4.07	
	$NH_4^+ + Mg^{2+}$	6.07	2.00
2	None	4.62	
	Mg^{2+}	9.06	4.44
	Na^+	3.98	
	$Na^+ + Mg^{2+}$	13.22	9.24

majority of experiments; in this experiment there was some Mg^{2+}-dependent phosphatidic acid phosphatase activity in the absence of monovalent cations. In the absence of Mg^{2+}, 0.16 M Na^+ usually inhibited the hydrolysis of phosphatidic acid slightly; at lower concentrations, Na^+ did not inhibit the hydrolysis in the absence of Mg^{2+} and in some cases it stimulated it somewhat. The slight stimulatory effect of lower Na^+ concentrations in the absence of added Mg^{2+} was not abolished by washing the preparations twice with 0.001 M EDTA.

Na^+ consistently stimulated the Mg^{2+}-dependent phosphatidic acid phosphatase activity. In Expt. 1, Table I, the Mg^{2+}-dependent phosphatidic acid phosphatase activity observed in the presence of Na^+ was greater than that observed in the pre-

sence of a variety of monovalent cations. Based on 100 for the Mg^{2+}-dependent phosphatidic acid phosphatase in the presence of Na^+ the other monovalent cations showed the following activities: Li^+, 72; NH_4^+, 33; Rb^+, 33; K^+, 18; Cs^+, 0. In some preparations of ghosts (see for example Fig. 2), the difference between Na^+ and K^+ was less striking; in fact, potassium was as effective as sodium in some experiments.

Effect of substrate concentration and of membrane concentration on the Mg^{2+}-dependent phosphatidic acid phosphatase activity

Fig. 3 shows the effect of increasing concentrations of phosphatidate on the Mg^{2+}-dependent phosphatidic acid phosphatase. The optimum concentration of phosphatidic acid was about 0.003 M, which would be 0.00045 M with respect to L-α-phosphatidic acid. It will be noted in Fig. 3 that concentrations of phosphatidate much higher than 0.003 M inhibited enzyme activity somewhat. The Mg^{2+}-dependent phosphatidic acid phosphatase activity was routinely assayed at the optimum substrate concentration.

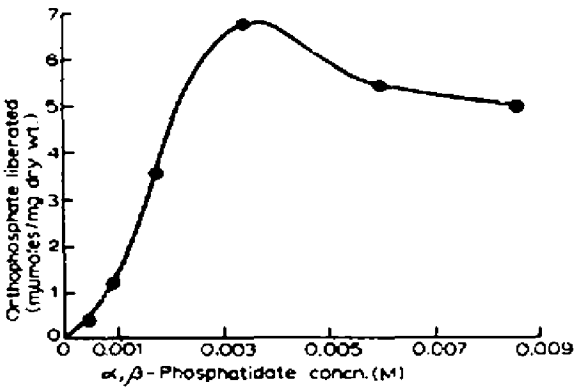

Fig. 3. Effect of substrate concentration on the Mg^{2+}-dependent phosphatidic acid phosphatase in erythrocyte ghosts. The concentration of Na^+ in the incubation mixture was 0.16 M; the concentration of Mg^{2+} was 0.0008 M. Tris [^{32}P]phosphatidate was added as indicated. Other conditions of incubation were as in Fig. 2.

When the concentration of erythrocyte ghosts in the incubation medium was varied, there was a linear increase in the Mg^{2+}-dependent phosphatidic acid phosphatase activity up to 5.5 mg (dry wt.) /ml. Above this concentration of ghosts the enzyme activity showed very little increase. In the experiments reported here the concentration of ghosts never exceeded 5.5 mg (dry wt.)/ml.

The hydrolysis of β-phosphatidate by ghosts

In experiments on phosphatidic acid phosphatase reported here, a mixture of α- and β-phosphatidic acid was used as the substrate, because of the ready availability of the commercial diolein preparation containing a mixture of 1,2- and 1,3-diolein, from which the phosphatidic acid was synthesized. Since naturally occurring phosphatidic acid is the L-α-isomer it was important to test whether the Mg^{2+}-dependent phosphatidic acid phosphatase was in fact due to the hydrolysis of only

the α-isomer. The results in Table II show that this was the case. Incubation of β-phosphatidic acid in the absence of any cations led to some release of orthophosphate. However, there was no increased liberation of orthophosphate on incubating β-phosphatidic acid in the presence of either Mg^{2+}, Na^+, or $Mg^{2+} + Na^+$. These results indicate that in the erythrocyte membrane the increased release of inorganic

TABLE II

HYDROLYSIS OF β-PHOSPHATIDIC ACID BY ERYTHROCYTE GHOSTS

The β-phosphatidate was present in a final concentration of 0.0017 M. The concentration of Na^+ was 0.16 M and that of Mg^{2+} was 0.0008 M. Other conditions of the assay were the same as in Fig. 2.

Cation added	*Phosphatidic acid phosphatase (mμmoles/mg dry wt.)*	
	Total	*Mg^{2+}-dependent*
None	0.90	
Mg^{2+}	0.91	−0.09
Na^+	0.99	
$Mg^{2+} + Na^+$	0.90	−0.09

phosphate on incubation of a mixture of α- and β-phosphatidic acids in the presence of Mg^{2+} or $Mg^{2+} + Na^+$ is due to the cleavage of the α-phosphatidic acid component. The Mg^{2+}-independent release of inorganic phosphate may be due to the action of a non-specific phosphatase.

Hydrolysis by erythrocyte membranes of compounds related to phosphatidic acid

It could be argued that the release of orthophosphate on incubation of erythrocyte membranes with phosphatidic acid occurred via a pathway other than the simple hydrolytic cleavage of phosphate from the substrate. There are two other possible pathways. One of these would be the removal of one fatty acid to form lysophosphatidic acid, which would then be hydrolyzed to monoglyceride and orthophosphate. In the absence of added metallic ions there was considerable release of acid-soluble radioactivity on incubation of 0.00057 M lyso[^{32}P]phosphatidic acid. However, there was no further increase in hydrolysis on adding either Mg^{2+} alone, Na^+ alone, or $Mg^{2+} + Na^+$ (Table III).

TABLE III

HYDROLYSIS OF LYSOPHOSPHATIDIC ACID BY ERYTHROCYTE GHOSTS

The concentration of lyso[^{32}P]phosphatidic acid was 0.00057 M; this preparation was a mixture of equal amounts of the α- and β-isomers with respect to phosphoric acid. 0.0008 M Mg^{2+} and 0.08 M Na^+ were added as indicated. Other conditions of incubation were as in Fig. 2. Acid-soluble radioactivity was assayed as described in the text.

Cation added	*Hydrolysis of lysophosphatidate (mμmoles acid-soluble phosphate/mg dry wt.)*
None	4.54
Mg^{2+}	4.04
Na^+	4.13
$Mg^{2+} + Na^+$	4.50

TABLE IV

HYDROLYSIS OF PHOSPHATIDATE AND α-GLYCEROPHOSPHATE IN ERYTHROCYTE GHOSTS

Na^+, K^+, and Mg^{2+} were added as indicated. Other conditions were as in Fig. 2 except that α-glycerophosphate phosphatase was assayed by incubating erythrocyte ghosts with α-glycero[^{32}P]-phosphate under the same conditions as those for phosphatidic acid phosphatase. The concentrations of α-glycero[^{32}P]phosphate and [^{32}P]α-phosphatidate were 0.00075 M. The α-glycerophosphate had a specific activity of $8.18 \cdot 10^6$ counts/min/μmole. After incubation 3 ml of 10% trichloroacetic acid was added, the vessels were centrifuged, and the residues were washed with another 3 ml of 10% trichloroacetic acid. [^{32}P]Orthophosphate was estimated in the pooled trichloroacetic acid extracts as described in the text.

Cation added	Phosphatidic acid phosphatase (mμmoles/mg dry wt.)		α-Glycerophosphate phosphatase (mμmoles/mg dry wt.)	
	Total	Mg^{2+}-dependent	Total	Mg^{2+}-dependent
None	4.13		0.37	
0.001 M Mg^{2+}	14.9	10.8	0.48	0.11
0.160 M K^+	2.62		0.77	
0.160 M K^+ + 0.001 M Mg^{2+}	14.4	11.8	1.26	0.49
0.160 M Na^+	2.46		0.25	
0.160 M Na^+ + 0.001 M Mg^{2+}	16.7	14.2	0.51	0.26

Another possibility for release of orthophosphate is the removal of both fatty acids from phosphatidic acid, followed by the hydrolytic cleavage of the glycerophosphate. Using the same preparation of ghosts, α-glycerophosphate was found to release far less orthophosphate than phosphatidic acid in the absence and presence of a variety of cations and cation combinations (Table IV). There appeared to be a very slight stimulation by K^+ or Mg^{2+} + K^+ of the release of orthophosphate, but the increments in activity were only a small percentage of the increments observed under comparable conditions when phosphatidic acid was used as substrate.

Inhibitors of phosphatidic acid phosphatase

The Mg^{2+}-dependent phosphatidic acid phosphatase activity was not affected by 10^{-5} M Be^{2+} (Table V); it was inhibited about 30% by 10^{-4} M Be^{2+} and was

TABLE V

EFFECT OF BERYLLIUM ON PHOSPHATIDIC ACID PHOSPHATASE ACTIVITY IN ERYTHROCYTE GHOSTS

Beryllium sulfate was added as indicated. The concentration of Mg^{2+} was 0.001 M. 0.080 M NaCl was present in all vessels. Phosphatidic acid phosphatase activity was assayed with non-radioactive phosphatidic acid, as described under EXPERIMENTAL. Other conditions of incubation were as in Fig. 2.

Beryllium concentration	Phosphatidic acid phosphatase activity (mμmoles/mg dry wt.)		
	Control	+ Mg^{2+}	Mg^{2+}-dependent
0	2.5	5.2	2.7
10^{-5} M	2.7	5.4	2.7
10^{-4} M	2.6	4.5	1.9
10^{-3} M	2.2	2.4	0.2

completely inhibited by 10^{-3} *M* Be^{2+}. The Mg^{2+}-independent activity was not affected by Be^{2+} at these concentrations. These results indicate that Be^{2+} is a fairly specific inhibitor of the Mg^{2+}-dependent phosphatidic acid phosphatase.

In the absence of Na^+, Cs^+ (0.03–0.16 M) did not inhibit the Mg^{2+}-dependent phosphatidic acid phosphatase (Table VI); the higher concentration of Cs^+ increased this activity, possibly by partial replacement of Na^+ by Cs^+. Both 0.03 M and 0.16 M Cs^+ lowered the increment in phosphatidic acid phosphatase activity due to addition of Na^+.

TABLE VI

EFFECT OF CESIUM ON THE Na^+-STIMULATED, Mg^{2+}-DEPENDENT PHOSPHATIDIC ACID PHOSPHATASE IN ERYTHROCYTE GHOSTS

Cesium chloride was added as indicated. Other conditions of incubation were as in Fig. 3. The values in each case are the means of values from three vessels.

Cesium concentration	*Mg^{2+}-dependent phosphatidic acid phosphatase (mμmoles/mg dry wt.)*		
	Control	*+ Na*	*Δ*
0	4.66	7.18	2.52
0.03 M	4.71	6.50	1.79
0.16 M	5.39	5.55	0.16

Fluoride (0.0125–0.05 M) did not inhibit the Mg^{2+}-dependent phosphatidic acid phosphatase observed in the absence of Na^+ (Table VII); the highest concentration (0.05 M) increased this activity. At concentrations of 0.0125 M and 0.025 M, fluoride did not greatly affect the increment in the Mg^{2+}-dependent phosphatidic acid phosphatase activity due to additions of Na^+ but 0.05 M fluoride markedly inhibited this increment.

TABLE VII

EFFECT OF FLUORIDE ON THE Na^+-STIMULATED Mg^{2+}-DEPENDENT PHOSPHATIDIC ACID PHOSPHATASE IN ERYTHROCYTE GHOSTS

Tris fluoride was added as indicated. Other conditions of incubation were as in Fig. 3.

Fluoride concentration	*Mg^{2+}-dependent phosphatidic acid phosphatase (mμmoles/mg dry wt.)*		
	Control	*+ Na^+*	*Δ*
0	2.21	3.48	1.27
0.0125	2.72	3.70	0.98
0.025	2.88	4.08	1.20
0.05	3.42	3.70	0.28

Rate of breakdown of exogeneous phosphatidic acid by the erythrocyte membrane

If erythrocyte membranes were suspended in 10^{-4} M cysteine, stored in dry ice, and incubated immediately after thawing in the presence of Tris phosphatidate emulsified by ultrasonic radiation the phosphatidic acid phosphatase activity was

as shown in Table VIII. It will be noted that the activity fell off somewhat during the 1-h incubation period. Using the Mg^{2+}-dependent activity observed at 15 min, the calculated phosphatidic acid phosphatase activity would be 41.6 mμmoles/mg dry wt./h. The activity for the $Na^+ + K^+$-dependent ATPase reported by POST *et al.*[15] for erythrocyte ghosts prepared and incubated under similar conditions was 20 mμmoles/mg dry wt./h. The Mg^{2+}-dependent phosphatidic acid phosphatase activity is thus sufficient for it to be a component of the $Na^+ + K^+$-dependent ATPase.

TABLE VIII

PHOSPHATIDIC ACID PHOSPHATASE ACTIVITY IN ERYTHROCYTE GHOSTS

Erythrocyte ghosts were stored in 10^{-4} M cysteine in dry ice and thawed just before use. They were incubated in triplicate in the presence of "sonicated" 0.003 M Tris phosphatidate, 0.080 M NaCl and 0.03 M imidazole-histidine–Tris buffer (pH 7.1) at 40°.

Time (min)	Phosphatidic acid phosphatase activity (mμmoles/mg dry wt.)		
	Mg^{2+}	$+Mg^{2+}$	Mg^{2+}-dependent
15	2.7	13.1	10.4
30	3.7	20.6	16.9
60	6.5	28.0	21.5

This phosphatidic acid phosphatase activity is likely to be a minimal value. The phosphatidic acid formed within the membrane is presumably in a more favored position for attack by phosphatidic acid phosphatase than is exogeneous substrate which has a very limited solubility and probably has poor access to the enzyme.

DISCUSSION

The effects of Mg^{2+} on phosphatidic acid phosphatase

The data presented here clearly show that phosphatidic acid phosphatase in human erythrocyte membranes is stimulated by Mg^{2+}. This is similar to what was reported previously for a deoxycholate extract of brain microsomes[12]. The effects of Mg^{2+} on phosphatidic acid phosphatase are, however, quite variable. SMITH *et al.*[13] reported an inhibition of phosphatidic acid phosphatase by Mg^{2+} in a chicken-liver preparation. A similar inhibition was found by COLEMAN AND HÜBSCHER[16]. At concentrations of Mg^{2+} comparable to those used here we have observed either no stimulations or inhibitions of enzyme activity in partially purified soluble preparations of kidney phosphatidic acid phosphatase and in salt gland preparations[14]. Treatment of these preparations with EDTA did not alter the effects of Mg^{2+}. It is possible that traces of Mg^{2+} still remain so firmly bound to the enzyme in some of these preparations that no Mg^{2+} stimulation can be observed.

Significance of phosphatidic acid phosphatase in erythrocyte ghosts

The presence of an active phosphatidic acid phosphatase in ghosts provides a further basis for the existence of a phosphatidic acid cycle in the erythrocyte mem-

brane; to this extent the results are compatible with the possibility that Na^+ transport in the erythrocyte may involve the phosphatidic acid cycle.

Although phosphatidic acid phosphatase activity is stimulated by Na^+, the data do not support the view that phosphatidic acid phosphatase determines the specificity of the sodium pump for sodium as against potassium. On the basis of the concentrations of Na^+ and K^+ for half-maximal stimulation of the Mg^{2+}-dependent phosphatidic acid phosphatase (Fig. 2) Na^+ does not seem to have a significantly greater affinity for phosphatidic acid phosphatase than does K^+. The sodium pump in the erythrocyte membrane discriminates highly in favor of Na^+, even though the intracellular concentration of K^+ is six times higher. Previous studies have shown that phosphatidic acid is bound to lipoproteins within the membrane[3], and a direct participation of these lipoproteins in the transport process has been suggested[1,2]. It seems more probable that the selectivity of the pump would reside on the sodium carrier site of this lipoprotein.

The sum of the activities of the two enzymes of the phosphatidic acid cycle is an ATPase:

$$\text{D-1,2-diglyceride} + \text{ATP} \xrightarrow[\text{kinase}]{\text{diglyceride}} \text{L-}\alpha\text{-phosphatidic acid} + \text{ADP}$$

$$\text{L-}\alpha\text{-phosphatidic acid} + H_2O \xrightarrow[\text{phosphatase}]{\text{phosphatidic acid}} \text{D-1,2-diglyceride} + H_3PO_4$$

$$\overline{ATP + H_2O \longrightarrow H_3PO_4 + ADP}$$

There is no direct proof as yet that the phosphatidic acid cycle enzymes are components of the $Na^+ + K^+$-dependent ATPase. However, there are several indications that this may be the case. The phosphatidic acid cycle[1,2] and the $Na^+ + K^+$-dependent ATPase[15,17] are affected by ouabain. Both activities are present in the same preparations, and the data presented here and in the preceding paper demonstrate that the rates of diglyceride kinase and phosphatidic acid phosphatase are sufficient to account for the $Na^+ + K^+$-dependent ATPase. Inasmuch as strong evidence has been accumulated that both the phosphatidic acid cycle and the $Na^+ + K^+$-dependent ATPase are related to sodium transport, both must eventually be fitted into the overall sodium transport mechanism. Although it is conceivable that both activities are concerned in sodium transport in different and unrelated ways it seems more reasonable that they are in fact closely related. It is hoped that studies now in progress will throw further light on this problem.

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