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DIGLYCERIDE KINASE AND OTHER PATHWAYS FOR PHOSPHATIDIC ACID SYNTHESIS IN THE ERYTHROCYTE MEMBRANE

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SUMMARY

Various pathways for the synthesis of phosphatidic acid in ghosts from human erythrocytes have been studied. The synthesis of phosphatidic acid by the diglyceride kinase reaction is 10–40 times more active than the synthesis of phosphatidic acid by phosphorylation of monoglyceride followed by acylation, and 2500 times more active than the synthesis by acylation of α-glycerophosphate. Diglyceride kinase activity is as great or greater than the $Na^+ + K^+$-dependent, ouabain-inhibitable, ATPase in the erythrocyte membrane; this is compatible with its being a component of the ATPase. Various factors influencing diglyceride kinase activity have been studied, such as detergents, the state of dispersion of the diglyceride substrate, freezing of the ghosts, Na^+ and K^+, and ouabain. The kinetic curve at 37° for phosphatidic acid synthesis from diglyceride shows an initial rapid component, followed after about 1 min by a slower component.

INTRODUCTION

A hypothesis has been presented which states that sodium transport is coupled to the renewal of phosphate in phosphatidic acid, and this renewal is catalyzed by the combined action of diglyceride kinase and phosphatidic acid phosphatase[1,2]. This hypothesis has been termed the phosphatidic acid cycle. If diglyceride kinase catalyzes the synthetic step in the phosphatidic acid cycle it should be present in all membranes where this cycle may function in sodium transport. One of the chief functions of the erythrocyte membrane is to pump sodium outwardly and potassium inwardly against concentration gradients. The main purpose of this paper has been to study the mechanisms by which phosphatidic acid can be formed in the erythrocyte membrane and to compare their relative activities. The formation of phosphatidic acid via the diglyceride kinase reaction[3] has been found to be by far the most active pathway. The formation of phosphatidic acid from monoglyceride via lysophosphatidic acid[4,5] has also been demonstrated, but this pathway is only one-fortieth to one-tenth as active in forming phosphatidic acid as is the diglyceride kinase reaction. The formation of phosphatidic acid by acylation of α-glycerophosphate also occurs in the erythrocyte membrane, but this pathway is only 1/2500th as active as the diglyceride kinase reaction.

Various factors affecting diglyceride kinase activity have been studied. Under optimum conditions there is sufficient diglyceride kinase activity in the erythrocyte membrane to account for the $Na^+ + K^+$-dependent ATPase which is believed to be related to Na^+ and K^+ transport in this structure. Preliminary reports of this work have been presented[1,2,6].

EXPERIMENTAL

Preparation of erythrocyte ghosts for incubation

Blood-bank blood which was from 10 30 days old was used. The membranes were prepared by the method of POST *et al.*[7], but on a larger scale. As starting material, 500–1000 ml of blood was used. A yield of about 75–100 ml of a suspension of ghosts/500 ml of blood was usually obtained. The dry weight of the ghosts averaged about 18 mg/ml of suspension. Tubes containing 4.5 ml of ghosts suspended in $5 \cdot 10^{-4}$ M imidazole–histidine–Tris buffer (pH 7.1) containing 10^{-4} M cysteine were stored at −70°. They were thawed slowly at 2° the night before use. After thawing they were homogenized briefly at slow speed in an all-glass homogenizer in order to obtain an even suspension.

Assay for diglyceride kinase activity

The standard procedure for assaying diglyceride kinase was as follows. The incubation vessels contained the following basic medium: $MgCl_2$, 0.002 M; imidazole–histidine–Tris buffer (pH 7.1), 0.05 M; sodium adenosine diphosphate, 0.002 M; Tris fluoride, 0.01 M; carbamate kinase[3], 0.03 units/ml and ammonium carbamyl [^{32}P]-phosphate. This basic medium is similar to that previously described for generating [γ-^{32}P]ATP in diglyceride kinase assays[3]. Other additions were made as indicated in the tables and figures. The above basic medium plus indicated additions was incubated for 3 min at 40°. Freshly thawed ghosts (0.05 ml) were then added, and the vessels incubated for an additional 30 sec, except in those experiments where the effect of incubation time was studied. The final incubation volume was 0.25 ml, and all concentrations refer to this final volume.

In previous studies[3] no diglyceride kinase activity was found in the *Streptococcus faecalis* extract containing the carbamate kinase, which is used in the [γ-^{32}P]ATP generating system. In these earlier studies diglyceride kinase was assayed in the presence of 0.25% deoxycholate. In the present study it was found that in the absence of deoxycholate the *S. faecalis* extract, which had been stored for 5 years in the deep freeze, contained measurable diglyceride kinase activity. However, by diluting the enzyme to a tenth of the concentration previously used the diglyceride kinase activity in the *S. faecalis* extract became sufficiently small so that only a small correction (10% or less) for this blank activity was required. The carbamate kinase activity, on the other hand, was still sufficient to support optimal synthesis of ATP under the conditions of the experiments. To correct the diglyceride kinase activity in the *S. faecalis* extract, the extract was incubated without ghosts for the full 3.5 min and then the tube was chilled and 0.05 ml of ghosts in 0.001 M *p*-chloromercuribenzoate was added, followed by 5 ml of 5% trichloroacetic acid.

The activities of diglyceride kinase, assayed as described above are reported as

mμmoles/mg dry weight of ghosts/h. In those experiments in which the diglyceride kinase assay was carried out in a somewhat different fashion, and in which the effects of various substances were compared, the activity is expressed as a per cent of the control radioactivity in phosphatidic acid.

Emulsions of diglycerides and monoglycerides were prepared as follows. The glycerides were added to water in polypropylene tubes, and these were immersed in water in the chamber of a Raytheon ultrasonic apparatus. The tubes were exposed to 10 000 vibrations/sec at maximum amperage for 1–2 min. The emulsions were prepared immediately before use.

At the end of the incubation period, 5 ml of cold 5% trichloroacetic acid were added to the vessels. The lipids were extracted and washed as described previously[9]. The final volume of the chloroform extract was 0.4 ml. Aliquots (0.05 ml) were directly plated and counted for total radioactivity, and other aliquots (0.2 ml) were applied to paper and chromatographed by the method of BEISS AND ARMBRUSTER[8]. In some experiments 0.18 μmole of Tris phosphatidate was added as carrier to the trichloroacetic acid precipitate and the phosphatidic acid spot on the chromatogram was detected by staining with Rhodamine G (see ref. 9). In other experiments no carrier was added, and the phosphatidic acid spot was detected by autoradiography. The phosphatidic acid spots on the paper were cut out and counted.

Other assays

The formation of phosphatidic acid and lysophosphatidic acid from monoglyceride, ATP, CoA, and palmitate was measured in a manner similar to that for the diglyceride kinase assay. The detailed conditions are shown in the appropriate tables and figures. Since the α-monoolein emulsion was not stable at low temperatures it was kept at room temperature and added to the 3-min preincubated stock medium at the same time as the ghosts were added. The lysophosphatidic acid spot on the chromatogram was detected by autoradiography. In some samples where insufficient radioactivity was present to show on the autoradiogram (when monoolein was omitted from the incubation mixture) the area corresponding to the adjacent radioactive lysophosphatidic acid spots on the chromatogram was cut out and counted. The method of MARINETTI *et al.*[9] was used to detect lysophosphatidic acid.

The formation of phosphatidic acid from α-glycerophosphate, ATP, CoA, and fatty acids was also measured in a manner similar to the diglyceride kinase assay, except the preliminary pre-incubation of the stock medium without ghosts was not carried out. The detailed conditions are shown in the appropriate tables and figures.

Materials

Commercial diolein was a kind gift of Distillation Products Industries, Rochester, N.Y. The percentages of 1,3-diolein and 1,2-diolein were determined as described elsewhere[10]. The preparation was found to contain 70% 1,3-diolein and 30% 1,2-diolein. All concentrations of diolein are expressed in terms of the D-1,2-diolein present in the diolein preparation (15%).

D-1,2-Diglyceride was prepared as follows. An "Alcohol Soluble Fraction of Lecithin" derived from soybeans was kindly provided by Associated Concentrates,

Inc., Woodside, L.I., N.Y. 3.48 g were dissolved in 75 ml of chloroform and applied to a 120-g alumina column. The column was washed with 200 ml of chloroform. The lecithin fraction was then eluted with 375 ml chloroform–methanol (1 : 1) according to the method of RHODES AND LEA[11]. 1.45 g of the lecithin fraction was recovered. Chromatography on silicic acid paper by the method of MARINETTI *et al.*[9] revealed lecithin as the major component with a smaller amount of lysolecithin and two unidentified spots near the solvent front. 1 g of the lecithin fraction was incubated with *Clostridium perfringens* toxin (kindly provided by Lederle Laboratories, Pearl River, N.Y.) and the resulting diglyceride isolated according to the method of HANAHAN AND VERCAMER[12]. The diglyceride was finally purified over a silica gel column by the method of QUINLIN AND WEISER[13]. The final product was a faintly yellowish oil, weighing 529 mg. It gave a single spot on thin-layer chromatography[14] with the same R_F as authentic 1,2-diolein. On the basis of the fatty acid composition of soybean lecithin[15] the diglyceride would contain 63% linoleic acid, 16% palmitic acid, 13% oleic acid, 6.3% stearic acid and 2.0% linoleic acid.

Commercial monoolein was a kind gift of Distillation Products Industries, Rochester, N.Y. It was further purified over a column of silica gel by the method of QUINLIN AND WEISER[13]. Previous studies have shown it to be better than 95% α-monoglyceride.

α-Glycero [^{32}P]phosphate was synthesized as described previously[4], the only modification being that NH_4^+ and Mg^{2+} were removed by passing the product over the Tris form of Dowex-50 rather than the H^+ form. Ammonium carbamyl [^{32}P]-phosphate was synthesized from 1 mmole of $NH_4H_2PO_4$ containing 5 mC of ^{32}P by the method of METZENBERG *et al.*[16]. A yield of 30% of carbamyl phosphate with no more than 5% orthophosphate was obtained. Tris fluoride was prepared by passing the sodium salt over a column of Dowex-50 (Tris form). *S. faecalis* extracts containing carbamate kinase were a kind gift of Drs. R. METZENBERG and M. MARSHALL. The units given are those defined by METZENBERG *et al.*[17]. CoA was obtained from Pabst Laboratories, Milwaukee, Wisc. (U.S.A.).

RESULTS

Synthesis of phosphatidic acid from ATP and diglyceride

When erythrocyte ghosts were incubated with an [γ-^{32}P]ATP-generating system and diglyceride, ^{32}P was incorporated into the total lipid extract. Paper chromatography of the lipid extracts in two solvent systems[8,9] revealed a radioactive spot with the same R_F as synthetic dioleyl phosphatidic acid. This spot accounted on average for 61% of the radioactivity in the total chloroform extracts. When the radioactive spot was deacylated by the method of DAWSON[18] it yielded only α-glycerophosphate. This was shown by mixing the deacylated product with authentic α-glycerophosphate and co-chromatographing the mixture. The radioactive spot revealed by autoradiography coincided exactly with the stained spot revealed by the method of WADE AND MORGAN[18]. These studies indicated that the product formed from ATP and diglyceride in ghosts was phosphatidic acid.

When the ghosts were heated at 100° for 10 min before incubation the amount of radioactivity found in phosphatidic acid was less than 4% of that found with unheated ghosts.

Dependence of phosphatidic acid synthesis on diglyceride

Fig. 1 shows the dependence of phosphatidic acid synthesis on the presence of diglyceride. Maximum phosphatidic acid synthesis was reached with a concentration of about 0.002 M D-1,2-diglyceride. At this concentration of diglyceride the formation of phosphatidic acid was 15 times greater than that observed in the absence of added diglyceride. These results are very similar to those previously observed with brain microsomes[3].

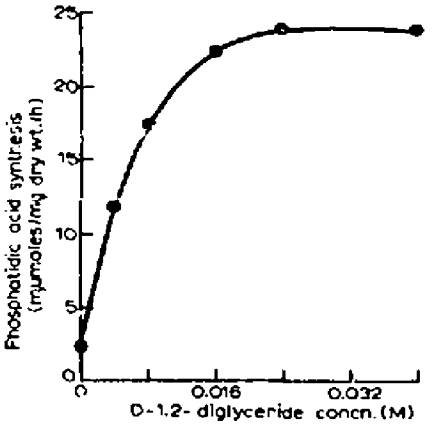

Fig. 1. The effect of diglyceride concentration on the synthesis of phosphatidic acid. Emulsified D-1,2-diglyceride was used.

The fact that phosphatidic acid was formed from ATP and that this formation was highly dependent on the presence of diglyceride indicates the presence of diglyceride kinase in the erythrocyte membrane.

Effect of various detergents on diglyceride kinase activity in erythrocyte ghosts

In brain microsomes it was necessary to add a detergent such as deoxycholate

TABLE I

EFFECTS OF VARIOUS DETERGENTS AND SOLVENTS ON DIGLYCERIDE KINASE ACTIVITY

All vessels contained 10 μl of diglyceride, which had not been emulsified by ultrasonic radiation.

Agent added	*Radioactivity in phosphatidic acid (per cent of control)*
0.1% Deoxycholate	27
0.2% Deoxycholate	7
0.3% Deoxycholate	5
1.0% Dimethylformamide	125
2.0% Dimethylformamide	112
4.0% Dimethylformamide	106
6.0% Dimethylformamide	75
8.0% Dimethylformamide	33
1.0% Octanol	22
0.125% Triton X-100	3

in order to obtain a stimulation of phosphatidic acid synthesis with added diglyceride; the optimum deoxycholate concentration was 0.25% (see ref. 3). Addition of detergents to ghosts incubated with unemulsified diglyceride did not stimulate phosphatidic acid synthesis; in fact, the detergents were markedly inhibitory (Table I). KENNEDY[20] found octanol to stimulate phosphatide synthesis in some systems. This agent markedly inhibited phosphatidic acid synthesis from diglyceride and ATP in ghosts (Table I). Dimethylformamide in concentrations ranging from 1 to 4% possibly stimulated phosphatidic acid synthesis somewhat, but higher concentrations were inhibitory.

Effect of emulsification of diglycerides on diglyceride kinase activity

Unlike brain microsomes no detergent was needed in the ghost system to obtain a stimulation of phosphatidic acid synthesis with added diglycerides (Table II).

TABLE II

EFFECTS OF VARIOUS DIGLYCERIDE PREPARATIONS ON PHOSPHATIDIC ACID SYNTHESIS IN ERYTHROCYTE GHOSTS

Diglyceride added	*Radioactivity in phosphatidic acid (per cent of control)*
None	(100)
2.2 μmole unemulsified diolein	440
0.10 μmole emulsified diolein	1010
0.25 μmole emulsified diolein	1940
0.50 μmole emulsified diolein	2400
0.25 μmole emulsified D-1,2-diglyceride	2640

Addition of diolein as an oil to the incubation medium enhanced phosphatidic acid synthesis about 5-fold. If the diolein was emulsified by ultrasonic treatment the stimulation could be increased to 25-fold. Emulsified D-1,2-diglyceride was usually somewhat more effective than emulsified diolein. These observations indicate that the particle size of the diglyceride substrate is extremely important in diglyceride kinase assays. The effectiveness of the diglycerides diminished with time after ultrasonic radiation, so it was necessary to emulsify them immediately before use. The replication in diglyceride kinase activity in triplicate incubation vessels was very poor when diglycerides were added directly to the vessels as oils; however, the replication was quite good when the diglyceride emulsions were added. This must be due to variable degrees of dispersion of the oils when they are added directly.

Why the erythrocyte ghost system should differ from the brain microsome system with respect to the effects of detergents on phosphatidic acid synthesis from diglycerides is not clear. However, these observations point to the necessity of studying the exact conditions for optimum diglyceride kinase activity in each system which is to be investigated.

Effect of freezing of ghosts on diglyceride kinase activity

If the ghosts were frozen and then thawed overnight at 2° the diglyceride kinase activity was about three times greater than when ghosts were used which were not frozen but were stored at 2° for the same period of time (Table III). This greater activity of the frozen and thawed ghosts was not due to a reduced rate of inactivation of the enzyme during storage, because if ghosts which had been stored for the

TABLE III

EFFECT OF FREEZING OF GHOSTS ON DIGLYCERIDE KINASE ACTIVITY

0.004 M emulsified D-1,2-diglyceride was used as substrate.

Condition	Phosphatidic acid synthesis (mμmoles/mg/h)
Unfrozen	12.0
Frozen and thawed	32.8
Frozen 24 h later and thawed	24.6

same amount of time in the unfrozen state were frozen and then thawed, they also showed a large increase in activity.

This effect of freezing must be due to some change in the structure of the membrane, possibly an increased permeability, making the diglyceride substrate more accessible to the diglyceride kinase.

Effect of membrane concentration on diglyceride kinase activity

The diglyceride kinase activity increased linearly with the concentration of ghosts up to about 1.5 mg dry weight of ghosts per incubation vessel (Fig. 2). In

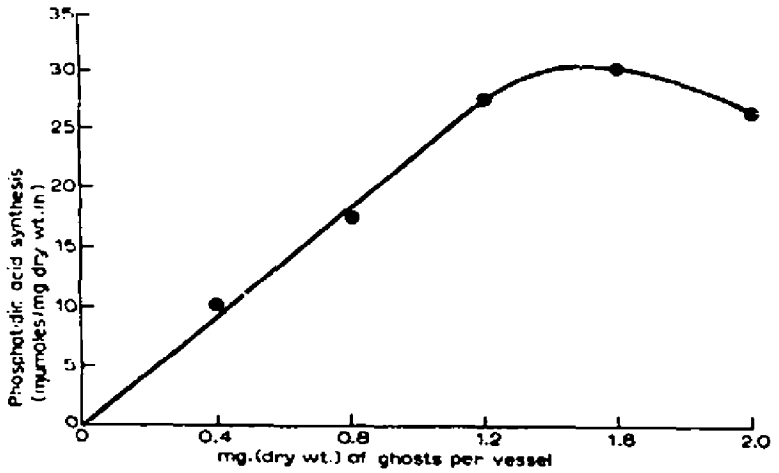

Fig. 2. Effect of increasing concentrations of ghosts on diglyceride kinase activity. 0.004 M emulsified D-1,2-diglyceride was used as substrate.

routine assays the concentration of ghosts did not exceed 1 mg dry weight of ghosts per incubation vessel.

Effects of Na^+ and K^+ on diglyceride kinase

Either Na^+ or K^+ increased by several fold the formation of phosphatidic acid from ATP and added diglyceride (Table IV). This effect of Na^+ or K^+ was not observed in the absence of added diglyceride.

TABLE IV

EFFECTS OF SODIUM AND POTASSIUM ON DIGLYCERIDE KINASE

0.004 M emulsified D-1,2-diglyceride was used as substrate.

Additions (moles/l)		Phosphatidic acid synthesis (mμmoles/mg/h)
Na^+	K^+	
0	0	5
0.05	0	14
0.10	0	19
0	0.05	20
0	0.10	20

Effects of ouabain on diglyceride kinase

Ouabain inhibits the sodium–potassium pumps[21] and elevates the level of ^{32}P-labelled phosphatidic acid in salt gland slices [2]. Ouabain was without significant effect on the formation of phosphatidic acid from [γ-^{32}P] ATP and added diolein (Table V). This suggests that the inhibition of the sodium–potassium pumps by ouabain is unlikely to be by inhibition of diglyceride kinase.

TABLE V

LACK OF EFFECT OF OUABAIN ON DIGLYCERIDE KINASE

5 μl of diolein was added as the oil to all vessels. The values are averages from three experiments.

Ouabain concentration (moles/l)	Radioactivity in phosphatidic (per cent of control)
0	(100)
$1.4 \cdot 10^{-5}$	105

Kinetics of phosphatidic acid synthesis

Fig. 3 shows the kinetic curve for the synthesis of phosphatidic acid. The curve appears to be made up of two components: an initial linear component with a rate

of about 35 mμmoles/m_o dry weight of ghosts/h, and a second linear component with a rate of about 3 mμmoles/mg/h.

The falling off in the initial rapid component was not due to exhaustion of carbamyl [^{32}P]phosphate since incubation in the presence of ten times the concentration of carbamyl phosphate did not alter the amount of phosphatidic acid synthesized or the pattern of labelling of phosphatidic acid with time.

A possible explanation of the rapid falling off of the initial rapid component is that due to the fact that phosphatidic acid is fixed in lipoproteins in the membrane[22],

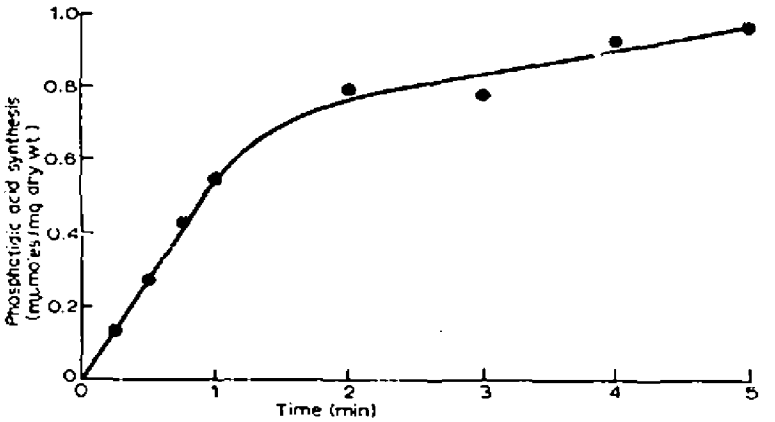

Fig. 3. Kinetic curve for the synthesis of phosphatidic acid from ATP and diglyceride. 0.004 M emulsified D-1,2-diglyceride was used as the substrate.

only a finite amount can be formed before all of the sites for newly synthesized phosphatidic acid are filled on the lipoprotein. Once these sites are filled with newly formed phosphatidic acid additional phosphatidic acid is not formed from ATP and diglyceride at these sites. The newly formed phosphatidic acid may or may not exchange its phosphate according to the reactions of the phosphatidic acid cycle; but this would not in either case lead to increased labelling in phosphatidic acid, since the phosphate is already equilibrated with the ATP. This explanation receives some support from an experiment in which the rates of synthesis of phosphatidic acid were compared when D-1,2-diglyceride was added as an oil and when it was added as an emulsion. With diglyceride added as an oil the initial rate of synthesis of phosphatidic acid was considerably less, but this rate was linear for about 10 min. The second component of the kinetic curve began when the same amount of phosphatidic acid had been synthesized as was observed with emulsified diglyceride. A more detailed analysis of the factors underlying the kinetics of phosphatidic acid synthesis is underway.

Formation of phosphatidic acid from α-glycerophosphate, fatty acids, CoA, and ATP

KORNBERG AND PRICER[23] demonstrated the formation of phosphatidic acid from α-glycerophosphate and fatty acid thioesters of CoA. In the present study a small but significant formation of phosphatidic acid occurred in ghosts incubated

TABLE VI

SYNTHESIS OF PHOSPHATIDIC ACID BY ACYLATION OF α-GLYCEROPHOSPHATE

Erythrocyte ghosts were incubated in 0.25 ml of medium containing the following additions in the indicated final concentrations: 0.002 M $MgCl_2$, 0.02 M imidazole–histidine–Tris buffer (pH 7.1), 0.047 M NaCl, 0.06 M KCl, 0.01 M NaF, 0.003 M sodium adenosine diphosphate, 0.4 units/ml carbamate kinase, 0.004 M ammonium carbamyl phosphate and 0.0005 M α-glycero [^{32}P]phosphate. The specific activity of the α-glycero[^{32}P] phosphate on the day of counting was $2.84 \cdot 10^7$ counts/min/μmole. Other additions are as indicated. 10 μl of the glycerides were added as the oils. The fatty acids were present in a concentration of 0.00005 M, and the CoA in a concentration of 0.0001 M. The vessels were incubated for 1 h at 40°.

Additions	*Phosphatidic acid synthesis (mμmoles/mg/h)*
None	0.0007
Tris palmitate, Tris oleate and CoA	0.010
Palmitate and oleate	0.0015
CoA	0.0069
Diolein	0.0004
D-1,2-Diglyceride	0.0001
Monoolein	0.0024

with α-glycero [^{32}P]phosphate, CoA, palmitate, oleate, and non-radioactive ATP (Table VI). Omission of CoA or fatty acids decreased phosphatidic acid synthesis. In this system with α-glycero [^{32}P]phosphate as the radioactive precursor, diglycerides or monoolein did not lead to any increase in the formation of radioactive phosphatidic acid above the control.

Fig. 4 shows the kinetic curve for the synthesis of phosphatidic acid from α-

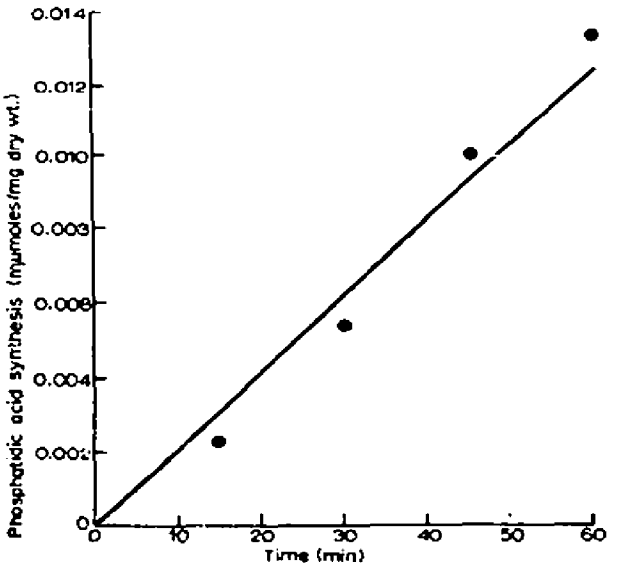

Fig. 4. The kinetic curve for phosphatidic acid synthesis from α-glycerophosphate, CoA, fatty acids and ATP. The conditions of incubation were similar to those shown in Table VI, except the reaction was stopped at the indicated times.

glycerophosphate, fatty acids, CoA and ATP. The rate was essentially constant over the 1-h incubation period.

The synthesis of phosphatidic acid from monoglyceride and ATP

PIERINGER AND HOKIN[4,5] demonstrated the formation of α-lysophosphatidic acid from ATP and monoglyceride in brain microsome preparations. The lysophosphatidic acid was in turn acylated by palmityl-CoA to form α-phosphatidic acid. The data in Table VII indicate that this pathway for formation of phosphatidic acid is present in the erythrocyte membrane. Maximum synthesis of phosphatidic acid occurred in the presence of monoolein, palmitate and CoA. Omission of any

TABLE VII

SYNTHESIS OF PHOSPHATIDIC ACID AND LYSOPHOSPHATIDIC ACID FROM MONOGLYCERIDE, ATP, CoA AND FATTY ACIDS

The basic medium was the same as described under EXPERIMENTAL for the diglyceride kinase assays. The complete system also contained 0.004 M emulsified monoolein, 0.0001 M Tris palmitate, and 0.0001 M CoA. The vessels were incubated for 30 min at 40°.

Additions	Synthesis of lysophosphatidic acid (mμmoles/mg/h)	Synthesis of phosphatidic acid (mμmoles/mg/h)
Complete	0.96	3.06
− Monoolein	0.08	0.70
− Palmitate	0.81	2.10
− CoA	1.36	1.90
− Monoolein, palmitate and CoA	0.10	0.74

one of these components reduced synthesis. Monoolein alone stimulated phosphatidic acid synthesis to some extent, but neither palmitate nor CoA was able to do so.

When monoolein was present there was some accumulation of lysophosphatidic acid, as has been previously observed for brain microsomes. In the absence of CoA there was more accumulation of lysophosphatidic acid than in its presence. This supports the idea that in the synthesis of phosphatidic acid from monoglyceride lysophosphatidic acid is formed initially, followed by its acylation. However, it cannot be ruled out hat a second pathway, involving acylation of monoglyceride, followed by phosphorylation, was also operative .HUBSCHER[24] has shown that monoglycerides can be acylated to form diglycerides.

The formation of phosphatidic acid from monoglyceride, ATP, CoA, and fatty acids was linear for 1 h (Fig. 5).

Relative rates of synthesis of phosphatidic acid in ghosts by the three known pathways

The above experiments show that all three of the known pathways[3-5,23] for synthesis of phosphatidic acid are present in ghosts; however, their activities differ considerably. The relative rates of synthesis of phosphatidic acid can be ascertained

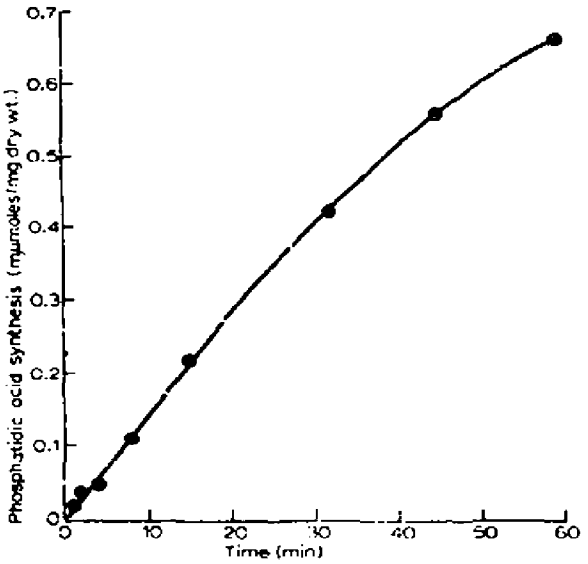

Fig. 5. Kinetic curve for the synthesis of phosphatidic acid from monoglyceride, ATP, CoA and fatty acids. The conditions for incubation were the same as for the complete system in Table VII.

from the data in Figs. 3–5, which were from experiments using the same batch of ghosts. Using the initial linear rates in all cases the relative rates of phosphatidic acid synthesis are shown in Table VIII. It can be seen that the rate of synthesis of

TABLE VIII

RELATIVE RATES OF SYNTHESIS OF PHOSPHATIDIC ACID IN GHOSTS BY THE THREE KNOWN PATHWAYS

The rates of phosphatidic acid synthesis via the different pathways were calculated from the initial linear rates shown in Figs. 3–5.

Pathway	*Substrate*	*Phosphatidic acid synthesis (mμmole/mg/h)*
Phosphorylation of diglyceride	[γ-^{32}P]ATP, diglyceride	33.6
Acylation of α-glycerophosphate	α-glycero [^{32}P]phosphate, CoA, oleate, palmitate, ATP	0.013
Phosphorylation of monoglyceride followed by acylation of lyso-phosphatidic acid	[γ-^{32}P]ATP, monoglyceride, CoA, palmitate	0.84

phosphatidic acid by the diglyceride kinase reaction was forty times greater than the rate of synthesis from monoglyceride, ATP, CoA, and fatty acids and about 2500 times greater than the rate of synthesis from α-glycerophosphate, ATP, CoA and fatty acids.

DISCUSSION

Significance of the various pathways for synthesis of phosphatidic acid in erythrocyte membranes

Of the three pathways previously demonstrated for the synthesis of phosphatidic acid[3-5,23] diglyceride kinase is the most active in ghosts from human erythrocytes. The second most active pathway, that involving phosphorylation of monoglyceride followed by acylation is about one-fortieth as active. The third pathway that involving acylation of glycerophosphate is less than one-thousandth as active as diglyceride kinase. Previous studies with brain microsomes[25] have shown that the pathway involving acylation of α-glycerophosphate does not participate in the acetylcholine-stimulated renewal of phosphate in phosphatidic acid, a process which is believed to be concerned in sodium transport. The pathway involving phosphorylation of monoglyceride followed by acylation cannot be rigorously ruled out as the mechanism involved in the acetylcholine-stimulated renewal of phosphate in phosphatidic acid. But since the diglyceride kinase reaction is at least ten times more active and is the only pathway which shows sufficient activity to account for the $Na^+ + K^+$-dependent ATPase (see below) it is the more likely candidate. Furthermore, radioactive lysophosphatidic acid is never seen on chromatograms of the total lipid extracts of tissues such as the salt gland after stimulation with acetylcholine, which elevates the radioactivity in phosphatidic acid 15-fold[28].

It could be argued that if fatty acid thioesters of CoA had been used as substrates instead of fatty acids, CoA and ATP, the activities of the pathways involving acylation of either lysophosphatidic acid or α-glycerophosphate might have compared more favorably with the diglyceride kinase pathway. However, it should be pointed out that sodium transport in ghosts is initiated on adding ATP as the only substrate[26,27], so that in comparing the various pathways for their possible role in sodium transport one should consider the overall rates starting with ATP.

Comparison of the activities of the diglyceride kinase reaction and the $Na^+ + K^+$-dependent ATPase in ghosts

The phosphatidic acid cycle is an ATPase[1,2]. Following the earlier studies of SKOU[28] on crab nerve microsomes, POST *et al.*[7] and DUNHAM AND GLYNN[29] found a $Na^+ + K^+$-dependent, ouabain-inhibitable ATPase in ghosts. The activity of this enzyme reported by POST *et al.*[7] averaged 20 mμmoles/mg dry weight of ghosts/h. An important point to establish is whether the diglyceride kinase activity in a membrane which pumps sodium is sufficient to account for the $Na^+ + K^+$-dependent, ouabain-inhibitable, ATPase (SEN AND POST[30] have found that 3 Na^+ are transported per ATP hydrolyzed). In the earlier studies using brain microsomes, in which the diglyceride kinase reaction was first demonstrated, conditions were not worked out for maximum activity of this enzyme. Various factors involved in the assay of diglyceride kinase have been studied here, and it has been possible to demonstrate a diglyceride kinase activity in ghosts which is as high as the $Na^+ + K^+$-dependent ATPase activity reported by POST *et al.*[7]. In ghosts, at any rate, the activity of the diglyceride kinase reaction is thus sufficient to be a component of the $Na^+ + K^+$-dependent ATPase.

It should be emphasized that under the conditions of assay for diglyceride kinase the $Na^+ + K^+$-dependent ATPase was probably not functioning. Fluoride was used in the diglyceride kinase assay, and this ion profoundly inhibits the $Na^+ + K^+$-dependent ATPase in erythrocyte membranes[31]. However, the conclusion that diglyceride kinase is present in the erythrocyte membrane in sufficient quantities to account for the reported activities of the $Na^+ + K^+$-dependent ATPase is still valid. The ATPase activity need not be functioning under the conditions in which diglyceride kinase is assayed, since the ATPase activity is presumably a resultant of several individual component reactions. If any component other than diglyceride kinase were blocked the diglyceride kinase would still be demonstrable but the ATPase would not be. Experiments are now in progress to determine the relationship between the $Na^+ + K^+$-dependent ATPase activity and phosphatidic acid cycle activity under conditions in which the ATPase is functioning. It should also be pointed out that to assay maximum diglyceride kinase activity, diglycerides were added exogeneously. If diglyceride kinase is a component of the $Na^+ + K^+$-dependent ATPase it would normally act on the endogenous diglyceride within the membrane structure.

It is probable that the diglyceride kinase activities reported here are minimal values. In the first place, as has been clearly demonstrated here, the accessibility of the exogeneous diglyceride to the membrane is of considerable importance in determining the activity of diglyceride kinase. It is likely that the endogenous diglyceride fixed within the membrane would be more accessible to diglyceride kinase during operation of the phosphatidic acid cycle. Secondly, the endogenous diglyceride may be more active as a substrate because of a more favorable fatty acid composition. Thirdly, we have so far been unsuccessful in assaying diglyceride kinase under conditions in which phosphatidic acid phosphatase is completely inhibited. For these reasons, it seems quite probable that the total diglyceride kinase activity in the membrane may very well exceed the $Na^+ + K^+$-dependent ATPase activity by a considerable factor.

Effects of sodium and potassium on diglyceride kinase activity

The stimulating effects of sodium and potassium on diglyceride kinase activity are difficult to interpret. It is doubtful if the diglyceride kinase component of the phosphatidic acid cycle is dependent on these ions, since sodium and potassium were without effect on the labelling of phosphatidic acid from [γ-^{32}P]-ATP when diglyceride was omitted. It is also doubtful that the specificity of the sodium pump for sodium could be determined by diglyceride kinase, since potassium seemed to be equally effective in stimulating diglyceride kinase activity. A possible role of the phosphatidic acid-containing lipoprotein in determining the specificity of the pump for Na^+ has been discussed elsewhere[1,2].

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