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PROTEOLIPID INVOLVEMENT IN HUMAN ERYTHROCYTE MEMBRANE FUNCTION

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SUMMARY

1. The incorporation of [γ-^{32}P]ATP into the phospholipids and protein of "resealed" and "leaky" human erythrocyte membranes was measured. Leaky membranes incorporated more radioactivity into polyphosphoinositides and phosphatidic acid than did intact membranes. There was no difference in the radioactivity incorporated into serine phosphate isolated from an acid hydrolysis of lipid-free protein from resealed and leaky membranes.

2. The radioactive membranes from leaky and resealed erythrocytes were fractionated into four major protein fractions. Most of the increment in radioactivity due to "opening" the membrane resided in a fraction which was soluble in chloroform–methanol. A proteolipid was extracted from this fraction by either column chromatography in Sephadex LH-20 or by dialysis of this fraction against chloroform–methanol.

3. The isolated proteolipid contained all of the phospholipid radioactivity, less than 10% of the total membrane phospholipid and 42–48% of the protein soluble in the organic phase. The proteolipid was composed of protein and the following phospholipids: phosphatidylserine and phosphatidylinositol (68%), phosphatidylethanolamine (25%), an unidentified lipid (7%) and trace amounts of the polyphosphoinositides which contained all of the phospholipid radioactivity.

4. The evidence that this proteolipid is involved in the opening and closing of pores in the erythrocyte membrane, is discussed.

INTRODUCTION

A previous study[1] had shown that the polyphosphoinositides and phosphatidic acid of the erythrocyte membrane incorporated labeled phosphate from [γ-^{32}P]ATP and that this incorporation was greatly enhanced in "leaky" membranes as opposed to membranes which were "resealed", insofar as they had regained their semipermeable properties. It was postulated that the increased metabolism of these phospholipids may be involved in the opening and resealing of the membrane. In this study the erythrocyte membranes are fractionated into various protein and lipid fractions to determine whether other membrane components are affected by opening and resealing of the membrane and also to isolate the pertinent membrane fractions.

Lysis of the membrane was found not to affect the phosphorylation of the

membrane proteins but to specifically enhance the phosphorylation of the polyphosphoinositides which are part of a membrane proteolipid. This proteolipid which represents less than 5 % of the total membrane protein and 5–10 % of the membrane phospholipids is composed in part of a select type of phospholipids, mostly acidic ones, and it contains 100 % of the phospholipid radioactivity.

MATERIALS AND METHODS

Preparation and incubation of leaky and resealed erythrocyte membranes

Fresh heparinized blood was washed 3 times with 0.15 M NaCl in 0.01 M Tris buffer (pH 7.4) care being taken to remove all of the buffy coat. The washed cells were lysed in 10 vol. of 1 mM $MgCl_2$ in 0.01 M Tris buffer (pH 7.4) and resealed by the addition of 3 M KCl/NaCl (3:1, v/v) to a final concentration of 0.17 M. This mixture was incubated at 37° C for 40 min. These reconstituted membranes are termed resealed membranes and they were either used as such or recentrifuged and resuspended in 310 mosM sodium phosphate buffer (pH 7.4). Leaky membranes were obtained by incubating these membrane preparations in hypotonic medium, either 0.01 M Tris buffer (pH 7.4) or 15.5 mosM phosphate buffer (pH 7.4). If the membranes were not to be resealed then they were prepared, free of hemoglobin, by the method described by Dodge *et al.*[3]. Incubations were carried out in either 15.5 mosM phosphate buffer (pH 7.4) or in 0.01 M Tris–HCl (pH 7.4) with 2.5 mM $MgCl_2$ containing 0.1 mM [γ-^{32}P]ATP, with a specific activity of about $2 \cdot 10^7$ cpm/μmole. All incubations were at 37 °C and the reactions were terminated by the addition of 5 % trichloroacetic acid.

Preparation of lipid-free protein and isolation of serine phosphate from a protein hydrolysate

The 5 % trichloroacetic acid precipitate obtained from the incubated membranes was washed 3 times with cold 5 % trichloroacetic acid. The washed pellet was extracted with 2 ml chloroform–methanol–HCl (200:100:1, by vol.) and then 5 ml of 0.1 M HCl was added to the mixture. The lower chloroform phase contains the lipids and the lipid-free protein residue remains in the interphase between the chloroform and the aqueous layer[39]. The protein was removed, washed twice with methanol and then digested in 2 M HCl at 100 °C for 6 h. Serine phosphate was isolated from this hydrolysate by paper electrophoresis as described by Jones and Rodnight[4]. Authentic serine phosphate was used as a marker and the compound was stained by the method of Wade and Morgan[5].

Fractionation of the erythrocyte membrane proteins

Protein from hemoglobin-free erythrocyte membranes was fractionated by a scheme described by Rosenberg and Guidotti[6] with a modification in the lipid extraction step to ensure the preservation of membrane proteolipids. The membranes from 45 ml of blood were first dialysed at 4 °C against 2 l of 1 mM EDTA and 50 mM mercaptoethanol for 48 h. Using the same nomenclature as Rosenberg and Guidotti[6], the supernatant solution was called Fraction I. The resulting pellet was then resuspended in 100 ml of 0.8 M NaCl and stirred overnight at 4 °C. It was then centrifuged at 4200 × *g* for 40 min. The supernatant from the 0.8 M NaCl treatment was termed Fraction II. The resulting pellet was then extracted with 100 ml of chloroform–

methanol (2:1, by vol.) as described by Folch *et al.*[7], and a solution of 0.75 % NaCl was added to obtain an upper aqueous phase. The upper layer was removed and then the interphase was rinsed 3 times with small amounts of upper phase solvent. The lower organic phase was called Fraction III lower and the upper layer *plus* the washes are referred to as Fraction III upper. The final protein residue was dissolved in 3 % sodium dodecyl sulfate and is referred to as Fraction IV (Table III).

Further fractionation of the proteins in the organic extract

Fraction III lower was concentrated in a flash evaporator at room temperature and was redissolved in 1/10 to 1/20 of its original volume in lower phase solvent. This was applied to a Sephadex LH-20 column (2.5 cm × 30 cm) which had been previously equilibrated for at least 1 day with chloroform. Elution was performed with 50 ml of chloroform and then with the following chloroform–methanol mixtures (v/v): 16 ml of 15:1; 22 ml of 10:1; 20 ml of 6:1; 20 ml of 4:1 and 75 ml of 2:1 (see Fig. 2).

The proteolipids in the original chloroform extract from the membranes were also obtained by exhaustive dialysis against chloroform–methanol (2:1, v/v)[8]. The chloroform extract was dialysed against 20 vol. of chloroform–methanol (2:1, v/v) at room temperature for 6 days with daily changes of the dialysis medium. The proteolipids were retained in the dialysis bag and the other lipids were removed (Table V).

Lipid chromatography

The phospholipids were separated and identified by thin-layer chromatography or by paper chromatography. Two thin-layer chromatographic systems were used, both described by Skipski *et al.*[9,10]. The phospholipids were detected by exposure to iodine vapors and were scraped from the thin-layer plates and eluted from the silica gel in order to determine the amounts of phospholipid present.

Paper chromatography was performed on silica gel loaded paper either as described by Marinetti[11] or, if the separation of the radioactive polyphosphoinositides was required, as previously described[1,12]. The radioactivity on the chromatograms was detected by autoradiography using Kodak no-screen X-ray film.

Other methods and materials

Lipids were extracted as previously described from the 5 % trichloroacetic acid precipitates[1]. Total phosphorus was measured by the method of Bartlett[13] and this value was multiplied by 25 to give the amount of phospholipid. Protein was determined by the method of Lowry *et al.*[14], using crystalline bovine serum albumin as standard. [γ-^{32}P]ATP was prepared as described by Glynn and Chappel[15].

Phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, distearyl phosphatidic acid, sphingomyelin and cholesterol were obtained from Applied Science Laboratories. Beef heart cardiolipin was obtained from General Biochemicals and a preparation of phosphatidyl inositides from beef brain (Folch Fraction I) was obtained from both Applied Sciences Laboratories and from Sigma Chemical Co.

RESULTS

Incorporation of [γ-^{32}P]ATP into phospholipids and protein from resealed and leaky erythrocyte membranes

Resealed erythrocyte membranes were incubated with [γ-^{32}P]ATP under conditions in which the membranes were either leaky or remained resealed. Leakiness was produced by incubating the membranes in a hypotonic medium. Incorporation of radioactivity from [γ-^{32}P]ATP was measured in the phospholipids and in a 2 M HCl hydrolysate (6 h at 100 °C) of lipid-free protein. A time course of incorporation of [γ-^{32}P]ATP into phospholipids showed linear incorporation for 15 min (Fig. 1). There was more incorporation into the phospholipids of lysed than of resealed membranes, but there was little or no difference in the incorporation of [γ-^{32}P]ATP into the protein part of the membrane. Thus lysis only affects the metabolism of the phospholipids and has little effect on the incorporation of phosphate into protein (Fig. 1).

To be sure that the incorporation of inorganic phosphate was occurring into proteins and not into some other membrane component, a measurement was made of the radioactivity incorporated into serine phosphate isolated from a lipid-free protein hydrolysate. Two radioactive components were obtained on paper electro-

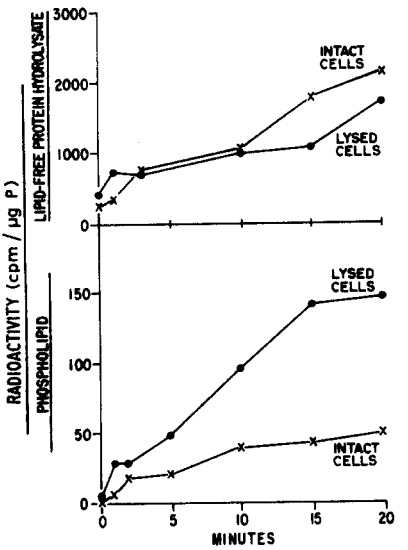

Fig. 1. Time course of incorporation of [γ-^{32}P]ATP into phospholipid and serine phosphate of resealed and leaky erythrocyte membranes. Fresh blood was washed 3 times with saline to remove all of the white cells. The red cells were lysed in 10 vol. of 1 mM $MgCl_2$ with 0.01 M Tris buffer (pH 7.4). A solution of 3 M KCl/NaCl (3:1, v/v) was added to a final concentration of 0.17 M and the cells were resealed at 37 °C for 40 min. The cells were recovered by centrifugation and were resuspended in 310 mosM phosphate buffer (pH 7.4). Incubation was in 2.5 mM $MgCl_2$, 15.5 mosM phosphate buffer (pH 7.4) and 0.1 mM [γ-^{32}P]ATP ($2 \cdot 10^7$ cpm/μmole) at 37 °C in a final volume of 2 ml. Each tube contained about 1.25 mg of membrane phospholipid. The resealed cells contained 0.18 M NaCl in the incubation media while the leaky cells had no salt added. The phospholipids were extracted with chloroform–methanol–HCl (200:100:1, by vol.) and the radioactivity determined as previously described. The lipid-free protein residue was hydrolysed in 2 M HCl in sealed ampules for 6 h at 100 °C. The amounts of phosphorus were determined in the chloroform extract and from the soluble acid hydrolysate.

phoresis of the protein hydrolysate. One component had an identical mobility with serine phosphate and the other with inorganic phosphorus. The radioactivity in these two components was the same if lysed and resealed membranes were compared. In Table I is shown the radioactivity incorporated into phospholipids and serine from lipid-free protein from resealed and leaky membranes. Only the phospholipids showed an increased metabolism due to opening the membrane with no change in the radioactivity found in protein, or in serine phosphate.

TABLE I

INCORPORATION OF ^{32}P FROM [γ-^{32}P]ATP INTO MEMBRANE PHOSPHOLIPID AND SERINE PHOSPHATE OBTAINED FROM A PROTEIN HYDROLYSATE FROM INTACT AND LEAKY ERYTHROCYTES

Resealed ghosts were prepared and incubated as in Fig. 1. Incubations were for 20 min at 37 °C. The radioactivity in the phospholipid and lipid-free protein was obtained as previously described (Fig. 1). Serine phosphate was separated from the 2 M HCl protein hydrolysate by paper electrophoresis as described by Jones and Rodnight[4]. Authentic serine phosphate was added as carrier to some of the samples and was detected on paper electrophoresis by staining as described by Wade and Morgan[5].

Membrane fraction	*Radioactivity (cpm/μg P)*		
	Intact membranes	*Leaky membranes*	*Increase (%)*
Phospholipids	22	64	190
Lipid-free protein	688	706	3
Serine phosphate from lipid-free protein	384	435	13

The membrane protein kinases and the phospholipid kinases were affected by the presence or absence of divalent cations. Magnesium, cobalt and manganese stimulated the incorporation of [γ-^{32}P]ATP into protein while calcium had a small inhibitory effect. The phospholipid kinases, measured as incorporation of [γ-^{32}P]ATP into either the polyphosphoinositides or phosphatidic acid, were completely dependent on the presence of divalent cations. Magnesium stimulated the incorporation into the polyphosphoinositides and in the presence of calcium, also into phosphatidic acid. Manganese enhanced the incorporation of [γ-^{32}P]ATP into both the polyphosphoinositides and phosphatidic acid while calcium stimulated the incorporation only into phosphatidic acid. The effect of divalent cations on phosphoinositide metabolism in other tissues has been shown by other laboratories[17,40,41]. Dibutyryl cyclic AMP which is known to affect divalent cation-dependent protein kinases, had no effect on this system under these incubation conditions (Table II).

Isolation of the actively labeled components from leaky membranes

Leakiness increases the incorporation of [γ-^{32}P]ATP into the membrane polyphosphoinositides and phosphatidic acid[1] but has no effect on the incorporation into membrane protein as assayed by measuring the incorporation into the total protein residue or into the serine phosphate obtained from a total protein hydrolysate (Fig. 1, Table II). There may, however, be differences in the phosphorylation of specific proteins, but this difference could be masked by a large radioactive incorporation into some membrane proteins which are not affected by lysis and resealing. The

TABLE II

EFFECT OF DIVALENT CATIONS ON THE INCORPORATION OF [γ-^{32}P]ATP INTO PHOSPHOLIPIDS AND ON SERINE PHOSPHATE OBTAINED FROM AN ACID HYDROLYSATE OF LIPID-FREE PROTEIN

Hemoglobin-free membranes were prepared by the method of Dodge *et al.*[3] and were incubated in 0.01 M Tris buffer (pH 7.4) for 20 min at 37 °C in the presence or absence of the above cations. The optimal concentration of the divalent cations for maximal stimulation of incorporation of [γ-^{32}P]ATP into protein was 2.5 mM and this was the concentration used in this experiment. [γ-^{32}P]ATP (10^7 cpm/μmole) 0.1 mM, was also in the incubation medium. Dibutyryl cyclic AMP at various concentrations from $1.75 \cdot 10^{-6}$ to 10^{-4} M had no effect on the protein or phospholipid kinases under the above incubation conditions. The phospholipids were separated by paper chromatography and detected by radioautography[12].

Cations added to incubation	*Radioactivity*		
	Phospholipids (cpm/mg)		*Serine phosphate (cpm/mg protein)*
	Polyphosphoinositides	*Phosphatidic acid*	
None	0	0	1 060
Magnesium	27 788	0	11 464
Magnesium and calcium	0	6476	8 208
Magnesium and cobalt	14 488	0	13 672
Magnesium and manganese	5 754	2390	5 172
Calcium	0	2410	488
Cobalt	7 846	0	9 520
Manganese	3 064	8544	8 544

chances of detecting any differences in incorporation into specific protein could be heightened by fractionating the membrane protein and by measuring the incorporation of [γ-^{32}P]ATP into the separated and purified membrane protein fractions.

The previous studies which had shown that the phospholipids were the membrane fractions that were affected by opening the membranes had used acidified chloroform–methanol to extract the phospholipids from the membrane but these conditions are known to strip the phospholipids from the proteolipids[8]. The increased incorporation of [γ-^{32}P]ATP into phospholipids may be associated with a membrane proteolipid and may not, in fact, be in phospholipids which are free, or which are not associated with protein in the membrane.

To determine if the metabolism of specific membrane proteins were affected by lysis, the erythrocyte membrane was fractionated into various protein fractions, some of which could be removed by chelating agents, or with 0.8 M NaCl and others which were soluble in an organic phase or with sodium dodecyl sulfate[6].

Membranes, free of hemoglobin, were prepared from 450 ml of blood by the method of Dodge *et al.*[3] and these membranes were divided into two equal parts. To one part was added membranes labeled with [γ-^{32}P]ATP, under conditions in which the membrane was resealed, and to the other part was added labeled membranes from "leaky" ghosts. These incubation conditions are described in Fig. 1. The membranes were then fractionated into four major fractions and the protein, phospholipid and radioactivity determined in each of the fractions. Fraction III, the lipid fraction, was not obtained by alcohol–diethyl ether extraction but rather by the method described by Folch *et al.*[7,16] which employs chloroform–methanol to extract

TABLE III

DISTRIBUTION OF RADIOACTIVITY FROM [γ-^{32}P]ATP INTO VARIOUS MEMBRANE FRACTIONS OBTAINED FROM LEAKY AND RESEALED ERYTHROCYTES

Erythrocyte membranes were incubated with [γ-^{32}P]ATP under conditions in which they were either resealed or were leaky. (See Fig. 1, for details.) The membranes were then fractionated as described in Materials and Methods. The values given are the average of two experiments. The percent change in radioactivity compares the radioactivity of the lysed membrane fractions as compared to those from intact membranes. Fraction I is protein removed from the membrane by dialysis against 1 mM EDTA and 50 mM mercaptoethanol. Fraction II represents protein removed by 0.8 M NaCl. Fraction III is material extracted from the membrane by chloroform–methanol. Fraction III lower contains lipids and proteolipids and Fraction III upper is an aqueous wash of the chloroform–methanol extract. Fraction IV represents the residual protein which is soluble in 3% sodium dodecyl sulfate.

	Membrane fraction	*Protein (mg)*	*Phospholipid (mg)*	*Radioactivity (cpm/fraction)*	*Change in radioactivity (%)*
I.	Resealed	7	—	91 505	70
	Leaky			155 185	
II.	Resealed	24	4	22 320	4
	Leaky			32 210	
III.	Upper				
	Resealed	2	0.4	8 140	52
	Leaky			12 348	
III.	Lower				
	Resealed	9	76	9 635	710
	Leaky			77 606	
IV.	Resealed	110	—	16 940	—
	Leaky			16 800	

the phospholipids and which is known to leave phospholipids and intact proteolipids in the lower organic phase.

On fractionation of the erythrocyte membrane it was found that there was a 700% increment in radioactivity due to opening the membrane in Fraction III lower, which represents the lipids and proteolipid extract (Table III). There was little or no change in the radioactivity of the other protein fractions. The majority of radioactivity was in Fraction I, the dialyzable fraction, but there was little difference between the intact and the lysed membranes (Table III). The radioactivity in this fraction was found to be in a mixture of inorganic phosphorus not incorporated into protein and protein released by dialysis against EDTA. Fraction II which represents protein removed from the membrane by 0.8 M NaCl, and the residual membrane proteins, Fraction IV, also had considerable radioactivity, but the radioactivity was not markedly different in leaky and resealed membranes.

Since the major increment in radioactivity, due to opening the membrane, was located in Fraction III lower, which contains lipids and proteolipids from the membrane, this fraction was further fractionated by chromatography on Sephadex LH-20 (Fig. 2). A proteolipid was isolated which eluted from the column with chloroform and which contained the majority of the radioactivity in Fraction III lower. In some experiments it contained a 100% of the radioactivity found in Fraction III lower.

This proteolipid contained variable amounts of phospholipid; 5–10 % of the total phospholipid content and it also contained 30–50 % of the organic phase protein. The remainder of the membrane phospholipids were eluted from the column with increasing amounts of methanol. These phospholipids, which make up the large majority of the membrane phospholipids, did not contain any radioactivity and were not associated with protein.

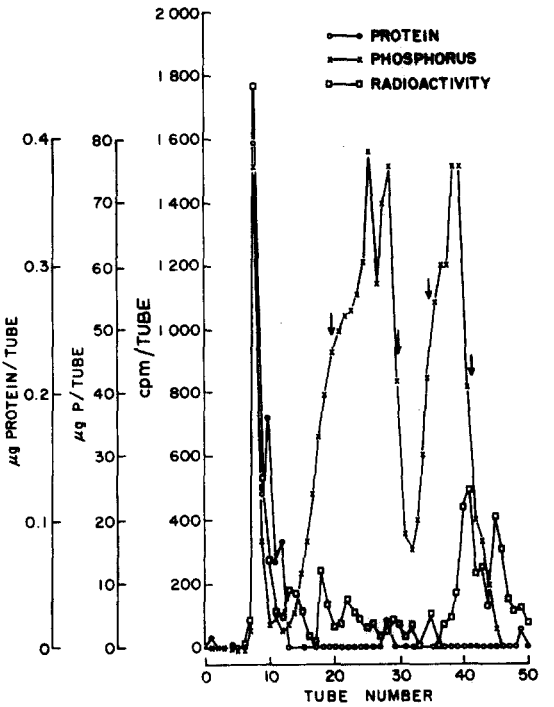

Fig. 2. Chromatography on Sephadex LH-20 of total lipid extracts (III lower) obtained from radioactive leaky membranes. Fraction III lower (see Table III) was chromatographed on a Sephadex LH-20 column (2.5 cm × 30 cm) equilibrated with chloroform. The sample was loaded on to the column dissolved in 4 ml of Folch's lower phase solvent. Elution was performed with 50 ml of chloroform; 16 ml of 15:1; 22 ml of 10:1; 20 ml of 6:1; 20 ml of 4:1 and 75 ml of 2:1, v/v chloroform–methanol. Each tube contained 3 ml of eluant. Radioactivity, phospholipid phosphorus and protein was determined on each fraction. ○—○, protein; ×—×, phosphorus; □—□, radioactivity.

Analysis of the radioactive proteolipid showed it to have a protein to phospholipid ratio of 0.17–0.5 (with an average of 0.217 from 5 experiments) and also showed that the phospholipids bound to the protein were mostly acidic phospholipids such as phosphatidylserine and phosphatidylinositol, although the presence of phosphatidylethanolamine (25 %) was also noted (Table IV). The proteolipid also contained trace amounts of polyphosphoinositides which could not be quantitatively detected by these methods but their presence is assured since it is known that the majority of the radioactivity is incorporated into the polyphosphoinositides[1,39]. Also, chromatography of the phospholipids from this proteolipid fraction on silica-gel loaded paper, with phenol–ammonia as solvent showed that polyphosphoinositides are part of the

TABLE IV

PHOSPHOLIPID ANALYSIS OF THE MEMBRANE PROTEOLIPID ISOLATED BY CHROMATOGRAPHY ON SEPHADEX LH-20

The phospholipids were separated by thin-layer chromatography by the method of Skipski *et al.*[9]. The proteolipid was obtained by Sephadex LH-20 chromatography as described in Fig. 2. The lipids were detected by exposure to iodine vapors. The radioactivity as determined by autoradiography, did not coincide with any of the above separated lipids but rather appeared as a streak. Previous studies have shown that the radioactivity resides mainly in the polyphosphoinositides[1,39]. The phospholipids were identified by cochromatography with reference phospholipids and by staining with ninhydrin for amino phosphatides or with Dragendorff reagent for choline containing lipids[36].

Membrane fraction	*Lipids*	R_F	% *of total phosphorus*
Isolated proteolipid	Not identified	0.33	7
	Phosphatidylserine and phosphatidylinositol	0.50	68
	Phosphatidylethanolamine	0.69	25
	Other phospholipids and neutral lipids	0.99	0
III. Lower	Sphingomyelin and lysophosphatidylcholine	0.14	24
	Phosphatidylcholine	0.26	29
	Phosphatidylserine	0.50	18
	Phosphatidylethanolamine	0.69	27
	Other phospholipids and neutral lipids	0.99	0

TABLE V

DIALYSIS OF THE RADIOACTIVE MEMBRANE FRACTION III LOWER

A lipid extract, from leaky radioactive membranes, was prepared by extraction of the membranes with chloroform–methanol as described by Folch *et al.*[7]. In these experiments the membranes were not pretreated with EDTA and NaCl as in Table III. The chloroform fraction thus obtained was dialyzed for 6 days at room temperature against 20 vol. of chloroform–methanol (2:1, v/v) with daily changes of the dialysate. The radioactivity which was retained in the dialysis bag was shown to be in the polyphosphoinositides. This was determined by chromatography of the phospholipids, before and after dialysis, on silica gel papers with phenol–ammonia as solvent[12].

	Expt No.	*Before dialysis*	*After dialysis*	*Recovered* (%)
Phospholipid (mg)	1	75.0	2.3	3.1
	2	57.5	5.1	8.8
Protein (mg)	1	0.750	0.36	48.0
	2	0.644	0.27	42.0
Radioactivity (cpm)	1	$1.37 \cdot 10^6$	$1.17 \cdot 10^6$	85.0
	2	$8.1 \cdot 10^5$	$8.2 \cdot 10^5$	100.0

proteolipids and that they contain the radioactivity. This latter chromatographic method does not, however, allow a quantitative measure of these lipids.

A proteolipid fraction was obtained from the chloroform extract of leaky membranes by dialysis of the extract against chloroform–methanol (2:1, v/v). This method had been used by Tenenbaum and Folch-Pi[8] to purify a proteolipid from bovine brain. The proteolipid obtained by dialysis contained 3–8 % of the total

phospholipids, 42–48 % of the organic phase protein and 85–100 % of the radioactivity (Table V). The proteolipid prepared by this method, in contrast to that obtained by chromatography on Sephadex LH-20, contained more lipids per protein giving variable protein to phospholipid ratios of 0.156–0.053. All of the detectable phospholipids and the radioactivity could be removed from the proteolipid by dialysis for 5 days against chloroform–methanol–12 M HCl (200:100:1, by vol.). This method has been used by others to obtain a water-soluble lipid-free proteolipid from brain[8].

DISCUSSION

A previous study had shown that the polyphosphoinositides and phosphatidic acid of "leaky" erythrocyte membranes were more actively labeled by [γ-^{32}P]ATP than were those lipids from "resealed" membranes and that the increased incorporation of radioactivity into these lipids was not due to an increased intracellular level of the [γ-^{32}P]ATP pool. The increased metabolism was also not due to merely changing the salt concentration since the pertinent enzymes in EDTA-treated cells, which always remain leaky, were not affected by changes in salt concentration. This is of interest since it has been shown that brain diphosphoinositide kinase is inhibited by 0.15 M NaCl (ref. 17). In the previous study it was suggested that the increased phospholipid metabolism may be associated with a repair mechanism of the membrane and as a working hypothesis it was postulated that lipoproteins, which contain polyphosphoinositides and phosphatidic acid, could be situated at certain regions of the membrane and that the phosphorylation and dephosphorylation of the lipid moieties of these lipoproteins may affect the structure of the membrane, and thus influence the "opening" and "resealing" of the membrane[1]. The studies presented in this article lend support to this scheme. [γ-^{32}P]ATP is shown to phosphorylate a number of membrane proteins, but only one type of protein, which makes up less than 5 % of the membrane protein, is affected by "opening" the membrane. The radioactivity in this protein is not in the protein *per se* but is rather into the polyphosphoinositides and phosphatidic acid which are attached to this protein. This protein is a special type of protein which is soluble in chloroform–methanol and which is similar to the proteolipids which are found in nervous tissue and which have been extensively investigated by Folch *et al.*[16,21]. The proteolipid from the erythrocyte membrane contains all of the phospholipid radioactivity obtained from [γ-^{32}P]ATP, but has less than 10 % of the total membrane phospholipids. The type of phospholipid attached to the proteolipid are mainly the acidic ones such as phosphatidylserine and phosphatidylinositol, which make up 68 % of the lipids on the proteolipid and polyphosphoinositides and phosphatidic acid which contain the radioactivity. At present it is not known whether the proteolipid contains all of the membrane polyphosphoinositides or only contains the radioactive ones, but the proteolipid does carry a selected number of lipids attached to it, and these lipids may give it a special environment which distinguishes it from other parts of the membrane. The phosphorylation and dephosphorylation of these lipids may change the polarity of the membrane proteolipid thus causing it to change its conformational shape and in so doing perhaps influencing the opening and closing of the membrane pore.

Recently De Robertis and co-workers[22,23] have isolated a proteolipid from nerve ending membranes and from the electroplax membranes from electric tissue of fishes.

These proteolipids have receptor abilities, in that the proteolipid from nerve endings binds (+)-tubocurarine, serotonin and atropine, while that from electroplax membrane binds acetylcholine and other cholinergic drugs. A model to describe the cholinergic receptor based on the properties of the proteolipid, has been suggested by these workers[23,24]. This model suggests, in part, that the proteolipid may constitute a wall of the membrane pore which is closed at the resting condition. Binding of the transmitter molecule to the proteolipid causes a change in conformational shape which allows the opening of the pore and the translocation of ions. Thus, this model, like the one that I have proposed, also envisions a membrane proteolipid as playing an important part in opening the membrane pores, and it is noteworthy that the proteolipids obtained from nervous tissues, like those seen in the erythrocyte membrane, are rich in phosphoinositides. Phosphatidylinositol has long been thought to be involved in the mode of action of acetylcholine in synaptic transmission since an increased metabolism of phosphatidylinositol accompanies this process[25,29]. The mechanism by which it is involved has not been elucidated, but Durell and co-workers[37,38] have suggested that acetylcholine may stimulate the enzymatic hydrolysis of phosphatidylinositol to phosphatidic acid and diglyceride. The diglyceride thus obtained then becomes available as substrate for the resynthesis of phosphatidylinositol.

There are numerous examples in other cells of increased turnover of phosphatidic acid or the phosphoinositides in response to various stimuli of the membrane. In all of these examples there is the strong possibility that the membranes are being "opened" or are "fusing" with another membrane. Some examples are the increased phospholipid turnover in actively secreting cells[30,31], the increased phospholipid metabolism of leukocytes during phagocytosis[32,33] and also the reactions of leukocytes to phytohemagglutinin[34,35]. Perhaps the similar response in these varied situations is due to the increased metabolism of phospholipids which are part of a membrane proteolipid which is involved in the mechanism of "opening" and "resealing" membranes. This would explain the similar phospholipid responses in these tissues with the phospholipid response which I am studying in the erythrocyte membrane and in which the "opening" and "resealing" of the membrane can be demonstrated.

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REFERENCES

1 C. M. Redman, *J. Cell Biol.*, 49 (1971) 35.
2 R. Whittam and M. E. Ager, *Biochem. J.*, 93 (1964) 337.
3 J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, 100 (1963) 119.
4 D. A. Jones and R. Rodnight, *Biochem. J.*, 121 (1971) 597.
5 H. E. Wade and D. M. Morgan, *Nature*, 171 (1953) 529.
6 S. A. Rosenberg and G. Guidotti, *J. Biol. Chem.*, 244 (1969) 5118.
7 J. Folch, M. Lees and G. H. Sloane-Stanley, *J. Biol. Chem.*, 224 (1957) 497.
8 D. Tenenbaum and J. Folch-Pi, *Biochim. Biophys. Acta*, 115 (1966) 141.

9 V. P. Skipski, R. F. Peterson and M. Barclay, *Biochem. J.*, 90 (1964) 374.
10 V. P. Skipski, M. Barclay, E. S. Reichman and J. Good, *Biochim. Biophys. Acta*, 137 (1967) 80.
11 G. V. Marinetti, *J. Lipid. Res.*, 3 (1962) 1.
12 E. Santiago-Calvo, S. Mulé, C. M. Redman, M. R. Hokin and L. E. Hokin, *Biochim. Biophys. Acta*, 84 (1964) 550.
13 C. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 466.
14 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
15 I. M. Glynn and J. B. Chappell, *Biochem. J.*, 90 (1961) 147.
16 J. Folch and M. Lees, *J. Biol. Chem.*, 191 (1951) 807.
17 M. Kai, J. G. Salway and J. N. Hawthorne, *Biochem. J.*, 106 (1968) 791.
18 E. DeRobertis, S. Fizer and E. F. Soto, *Science*, 158 (1967) 928.
19 E. F. Soto, J. M. Pasquini, R. Placido and J. LaTorre, *J. Chromatogr.*, 41 (1969) 400.
20 J. Folch-Pi, in H. Peeters, *Protides of the Biological Fluids*, Elsevier, Amsterdam, 1960, p. 21.
21 P. Stoffyn and J. Folch-Pi, *Biochem. Biophys. Res. Commun.*, 44 (1971) 157.
22 J. L. LaTorre, G. S. Lunt and E. De Robertis, *Proc. Natl. Acad. Sci. U.S.*, 65 (1970) 716.
23 E. DeRobertis, *Science*, 171 (1971) 963.
24 G. G. Lunt, O. M. Canessa and E. DeRobertis, *Nature*, 230 (1971) 187.
25 L. E. Hokin and M. R. Hokin, *Biochim. Biophys. Acta*, 18 (1955) 102.
26 L. E. Hokin and M. R. Hokin, *J. Biol. Chem.*, 233 (1958) 818.
27 M. R. Hokin, L. E. Hokin and W. D. Shelp, *J. Gen. Physiol.*, 44 (1960) 217.
28 M. G. Larrabee, J. D. Klingman and W. S. Leicht, *J. Neurochem.*, 10 (1963) 549.
29 M. R. Hokin, *J. Neurochem.*, 16 (1969) 127.
30 L. E. Hokin and M. R. Hokin, *2nd Konf. Funkt. Morphol. Org. Zelle, Wiss., 1964*, p. 50.
31 M. R. Hokin, *Arch. Biochem. Biophys.*, 124 (1968) 271.
32 M. L. Karnovsky and D. F. H. Wallach, *J. Biol. Chem.*, 236 (1961) 1895.
33 R. S. Sastry and L. E. Hokin, *J. Biol. Chem.*, 241 (1966) 3354.
34 D. B. Fisher and G. C. Mueller, *Proc. Natl. Acad. Sci. U.S.*, 60 (1968) 1396.
35 D. B. Fisher and G. C. Mueller, *Biochim. Biophys. Acta*, 248 (1971) 434.
36 H. K. Mangold, *J. Am. Oil Chem. Soc.*, 28 (1961) 708.
37 J. Durrell and M. A. Sodd, *J. Neurochem.*, 13 (1966) 487.
38 J. Durrell, J. T. Garland and R. O. Friedel, *Science*, 165 (1969) 862.
39 L. E. Hokin and M. R. Hokin, *Biochim. Biophys. Acta*, 84 (1964) 563.
40 J. Tou, M. W. Hurst, C. G. Huggins and W. E. Foor, *Arch. Biochem. Biophys.*, 140 (1960) 492.
41 R. C. Schneider and L. B. Kirschner, *Biochim. Biophys. Acta*, 202 (1970) 283.