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THE PROPERTIES AND SUBCELLULAR DISTRIBUTION OF PHOSPHATIDYLINOSITOL KINASE IN MAMMALIAN TISSUES

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SUMMARY

1. Studies of subcellular fractions from rat liver indicate the presence of more than one phosphatidylinositol kinase.

2. Some properties of two kinases in liver are reported.

3. The distribution of phosphatidylinositol kinase in subcellular fractions from rat brain, kidney, heart, skeletal muscle and testis indicates that the enzyme is most active in fractions likely to contain the plasma membrane of these tissues.

4. The enzyme is also present in human erythrocyte membranes and in the surface membrane fraction of rabbit polymorphonuclear leucocytes.

5. Rat kidney, like rat liver, has a separate Cutscum-stimulated kinase associated with the endoplasmic reticulum.

6. The kinase has been found in all tissues studied, but is most active in brain.

7. A subcellular fractionation procedure for testis is presented and the purity of the fractions discussed.

INTRODUCTION

Phosphatidylinositol kinase, which catalyses the synthesis of diphosphoinositide by the reaction below, has been described in liver and brain[1-3] and reported to occur in several other tissues.

$$\text{Phosphatidylinositol} + \text{ATP} \xrightarrow{Mg^{2+}} \text{diphosphoinositide} + \text{ADP}$$

MICHELL *et al.*[4] have shown that the kinase is found in a purified plasma membrane fraction from rat liver.

The apparent association of phosphatidylinositol kinase with cell surface membranes[3,4] has led to a more detailed study of different tissues, especially liver, where the non-ionic detergent Cutscum causes a marked difference in the subcellular distribution pattern. This work is now reported, together with evidence that there is more than one distinct phosphatidylinositol kinase in rat liver.

MATERIALS AND METHODS

Labelled ATP

Terminally labelled [^{32}P]ATP was prepared by the method of GLYNN AND CHAPPELL[5]. The 0.25 M HCl eluate was neutralised by adding solid Tris until the pH was 7.4. The ATP had a specific activity of about 10^6 counts per 100 sec per μmole in a liquid Geiger–Müller tube of 6% efficiency.

Phosphatidylinositol

This lipid was prepared from Inosithin, a phospholipid fraction from soya beans (American Lecithin Company, New York, N.Y., U.S.A.) by the method previously described[3]. Before use in the incubation systems, phosphatidylinositol solutions in chloroform were dried under N_2 at room temperature and the residual lipid dissolved in sufficient water to give an emulsion of the required concentration.

Diphosphoinositide

As a reference material for chromatography, the diphosphoinositide fraction of FOLCH[6] was prepared.

Preparation of subcellular fractions

The procedures used for the preparation of subcellular fractions from brain, liver, heart, skeletal muscle, testis and kidney are described below.

Liver. The method was that of SEDGWICK AND HUBSCHER[7] modified by the use of a loose-fitting homogeniser (radial clearance 0.20 mm; speed 500 rev./min; 6 complete strokes used) which made it possible to prepare the purified plasma membrane fraction of COLEMAN *et al.*[8] at the same time. This method is also discussed by MICHELL *et al.*[4].

Brain. The method of NYMAN AND WHITTAKER[9] was used. This yields nuclear, synaptosomal, mitochondrial, microsomal, myelin and supernatant fractions.

Testis. The centrifuge speeds used were the same as those for liver[7] and the same loose-fitting homogeniser was taken.

Heart and skeletal muscle. Centrifugation was as for testis but the initial homogenisation was with a tightly-fitting pestle for three min. Even after this vigorous treatment some material was not broken up and about one-third of the starting tissue remained on the muslin cloth used to filter the homogenate.

Kidney. SHIBKO AND TAPPEL[10] have reported a method for kidney fractionation and the initial stages of this were used. The pellet which came down at the mitochondrial speed did not have the expected three layers and from this point the method for liver was followed.

Other tissue preparations

Homogenates of rat lung, pancreas and spleen were made in 0.32 M sucrose at 10% (w/v) concentration.

Leucocytes. Fractions from rabbit polymorphonuclear leucocytes were prepared by Dr. A. M. WOODIN of Oxford. Light and heavy membrane fractions were compared with the mixed-membrane pellet and the supernatant fraction. WOODIN AND WIENEKE[11] have described the method of preparation.

Erythrocytes. Whole human blood containing a citrate anti-coagulant was obtained from the local Transfusion Centre. A volume of blood was centrifuged at 20 000 × *g* for 20 min and the packed cells were then washed three times with 4 vol. of 0.155 M sodium bicarbonate buffer (pH 7.4) and similarly centrifuged each time. The "buffy coat" was removed from the surface after each centrifugation. The final erythrocyte sediment was suspended in an equal volume of bicarbonate buffer of the same concentration and pH. This was the isotonic (310 mosM) suspension. Other suspensions prepared in this way were treated as follows with different hypotonic buffers (see Fig. 4). A 10-ml volume of cell suspension was added to 60 ml of hypotonic buffer (pH 7.4) and after 30 min at 4° the mixture was centrifuged as above. To the sediment fresh hypotonic buffer was added to give a total volume of 70 ml and the centrifuging repeated. The process was repeated until the supernatant was no longer pink in colour (3–4 washes) and the ghosts were finally suspended in hypotonic buffer to give the original total volume of 10 ml.

Assay of phosphatidylinositol kinase

The optimum conditions for the kinase reaction in liver and brain, respectively have been described by MICHELL *et al.*[4] and KAI, WHITE AND HAWTHORNE[3]. The conditions for optimum synthesis in other tissues were determined and found to be as follows. The basic incubation mixture consisted of 50 mM Tris–HCl buffer (pH 7.4), 40 mM $MgCl_2$, 5 mM labelled ATP, 5 mM phosphatidylinositol and tissue sample (less than 5 mg protein) in 0.32 M sucrose, the total volume being 1.0 ml. These modifications were made for individual tissues: kidney, 1.0 mM phosphatidylinositol; brain, 1.0 mM phosphatidylinositol and 20 mM $MgCl_2$; leucocytes, 10 mM $MgCl_2$.

Tubes were incubated at 30° for 5 min and the reaction then stopped by the addition of 3.75 ml chloroform–methanol (1:2, by vol.). The extraction of labelled diphosphoinositide followed Method A of KAI, WHITE AND HAWTHORNE[3]. This extraction method, which involves the addition of 2 M KCl–0.5 M potassium phosphate buffer, gave recoveries of labelled diphosphoinositide as great as those obtained with acidified chloroform–methanol (see Discussion, KAI, WHITE AND HAWTHORNE[3]). The final lipid extract was concentrated to a small volume under a stream of N_2 and applied to formaldehyde-impregnated papers for chromatographic determination of diphosphoinositide radioactivity as described previously[3]. Better separations were obtained if the formaldehyde treatment of the papers was continued for 8 h (instead of 6) at 110°.

Phosphatidylinositol kinase activity was measured as ^{32}P incorporated into diphosphoinositide. The number of moles of the latter which were synthesised could be calculated from the specific activity of the ATP, assuming that only its terminal phosphate was incorporated into the lipid. The absence of labelled phosphatidylinositol from the chromatograms in all cases indicates that this assumption is reasonable. Labelling of the 1-phosphate of diphosphoinositide could only arise from ^{32}P-labelled phosphatidylinositol.

The assay is not likely to give maximum kinase activity in fractions containing the phosphomonoesterase attacking diphosphoinositide, particularly the supernatant. The effect of the phosphomonoesterase was not great, however. The kinase activity of a liver microsomal fraction (1.2 mg protein) was reduced 12% by addition of supernatant fraction (0.41 mg protein). A similar effect was noted by COLODZIN AND

KENNEDY[1]. Comparison of activities in the subcellular fractions is not likely to be invalidated by this effect, though values for the kinase in the supernatant fraction will be low. Kinase activity was proportional to protein concentration with all fractions over the range 0–5 mg protein per ml.

Assay of other enzymes

Succinate dehydrogenase (EC 1.3.99.1) was determined as succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase[3]. Methods for acid phosphatase (EC 3.1.3.1), glucose-6-phosphatase (EC 3.1.3.9) and 5'-nucleotidase (EC 3.1.3.5) have been described[8]. The same applies to (Na^+-K^+)-stimulated ATPase[3]. Lactate dehydrogenase (EC 1.1.1.27) was determined by a modification of the method of JOHNSON[12], the reaction mixture being made up as follows: 0.05 ml NADH solution (10 mg/ml), 0.02 ml 0.3 M sodium pyruvate, 1.0 ml 0.27 M potassium phosphate buffer (pH 7.6), 0.02 ml satd. ethanolic solution of rotenone, approx. 0.05 ml of enzyme, and water to give a total volume of 3.0 ml. The reaction was started by adding the pyruvate and the change in absorbance at 340 mμ was measured over the 30–90-sec time interval.

Analytical methods

Protein was estimated by the method of WEICHSELBAUM[13] or of LOWRY *et al.*[14] and DNA as described previously[4]. P_i was measured by the method of KING[15] or that of BAGINSKI, FOA AND ZAK[16].

RESULTS

Liver phosphatidylinositol kinase activity

The distribution of phosphatidylinositol kinase in subcellular fractions of rat liver, including the plasma membrane fraction, is given in Fig. 1. Addition of the detergent Cutscum (iso-octylphenoxy-polyethoxyethanol) markedly stimulated the

TABLE I

EFFECTS OF IONS AND INHIBITORS ON PHOSPHATIDYLINOSITOL KINASE

The basic assay system at pH 7.4 was used with the addition of 2% Cutscum in all cases. Figures are means with the number of experiments in parentheses.

Addition	*Kinase activity (%)*	
	Plasma membrane	*Microsomal*
Control (no addition)	100	100
10 mM NaF	28 (2)	52 (2)
100 mM KCl	55 (3)	128 (3)
100 mM NaCl	93 (2)	124 (2)
10 mM $CaCl_2$	9 (4)	31 (5)
10 mM $CaCl_2$ (pH 8.3)	—	29 (1)
120 mM NaCl, 30 mM KCl	—	100 (1)
120 mM NaCl, 30 mM KCl, 0.05 mM ouabain	—	85 (1)
0.1 mM iodoacetamide	94 (2)	58 (2)
0.1 mM *N*-ethylmaleimide	26 (2)	58 (2)

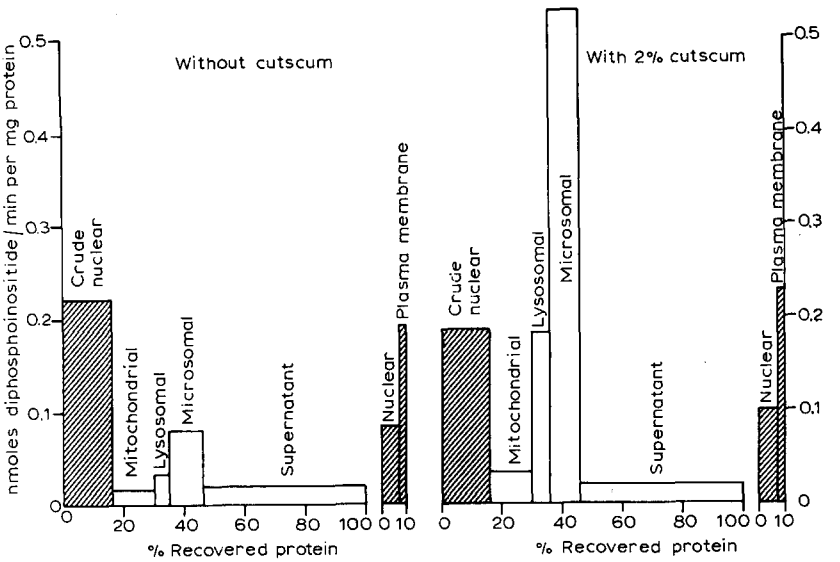

Fig. 1. Effect of Cutscum on subcellular distribution of rat-liver phosphatidylinositol kinase. The basic assay conditions were used and the reaction was started by the addition of the subcellular fraction. The ordinates give the protein content of each fraction as % recovered protein. Shaded areas represent the crude nuclear fraction and certain sub-fractions obtained from it.

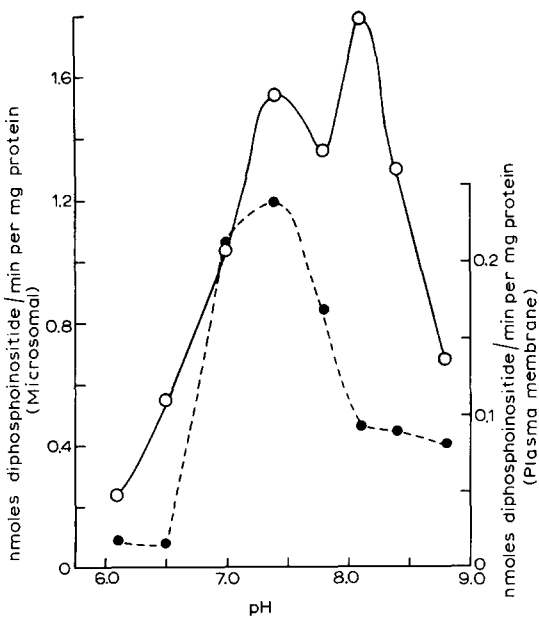

Fig. 2. Effect of pH on phosphatidylinositol kinase activity in rat-liver plasma membrane and microsomal fractions. The basic assay system was used with Tris–HCl mixtures and 2% Cutscum at each pH. The pH of the mixture was measured before and after each reaction and the average recorded on the graph. ●— — —●, plasma membrane fraction.

kinase of the microsomal fraction while the other fractions were unaffected. This suggested that there might be more than one phosphatidylinositol kinase in liver and so the properties of the enzyme in plasma membrane and microsomal fractions were investigated. Fig. 2 shows the effect of pH on the kinase activity in the two fractions. The pH optimum for the plasma membrane enzyme was 7.4, while the microsomal fraction gave two peaks of activity, one at pH 7.4 and the other at pH 8.1. The double peak was obtained in three separate experiments.

The effects of a number of ions and inhibitors are shown in Table I. The plasma membrane enzyme was more readily inhibited by Ca^{2+} than the microsomal enzyme. K^+ activated the microsomal kinase but inhibited the plasma membrane enzyme. Both iodoacetamide and *N*-ethylmaleimide differed in their effects on the two enzymes. Ouabain did not significantly inhibit the microsomal phosphatidylinositol kinase.

The requirements of the two enzymes for Mg^{2+}, ATP and phosphatidylinositol were similar, as were the graphs of reaction rate against time. The rates were proportional to enzyme concentration in the range 0–5 mg enzyme protein.

Solubilisation. The microsomal suspension was treated in several ways in an attempt to make the kinase soluble. Ultrasonic treatment had little effect on total activity whether done in the presence or absence of 2% Cutscum. The microsomal

TABLE II

EFFECTS OF CUTSCUM AND SONICATION ON PHOSPHATIDYLINOSITOL KINASE SOLUBILITY

Rat-liver microsomal and plasma membrane preparations were each suspended in 2 ml 0.32 M sucrose. The total protein content is given in the table. Details of the ultrasonic treatment are given in RESULTS. For detergent treatment Cutscum was added to give a final concentration of 2%. Suspensions treated in either of these two ways were then centrifuged at 105 000 × *g* for 180 min to give pellet and supernatant fractions as below. The basic assay system was used with 2% Cutscum and Tris buffers at pH 7.4 or 8.3. The detergent-treated supernatants contained additional Cutscum but control experiments showed that this did not affect the kinase assay.

Preparation	*Fraction*	*Quantity (mg)*	*Protein recovery (%)*	*Phosphatidylinositol kinase*			
				pH 7.4		*pH 8.3*	
				*activity**	*recovery (%)*	*activity**	*recovery (%)*
Microsomal (sonicated)	total	26.0		5.55		5.43	
	pellet	26.4		3.31		2.44	
	supernatant	4.2	117	0.99	78	0.74	59
Microsomal (Cutscum)	total	15.6		4.74		4.48	
	pellet	9.6		2.69		3.12	
	supernatant	4.0	87	0.57	69	1.65	106
Plasma membrane (sonicated)	total	2.8		0.27		0.12	
	pellet	2.8		0.27		0.11	
	supernatant	—	100	—	99	—	91
Plasma membrane (Cutscum)	total	2.4		0.18		0.05	
	pellet	2.2		0.18		0.05	
	supernatant	0.2	100	—	103	—	96

* Activity in nmoles product per min.

suspension in 0.32 M sucrose at pH 7.4 was treated at 0° and 19 000 cycles/sec for 10 sec in every 30 sec over a period of 10 min.

An acetone powder was also prepared at 0° and subsequently extracted with 20 mM Tris–HCl buffer at pH 7.4. The extract was inactive, as was a similar extract prepared from a powder produced by treatment of the microsomal suspension with 20% *n*-butanol at 0° followed by acetone at − 15°.

Table II shows the amounts of the plasma membrane and microsomal kinases solubilised by sonication or treatment with Cutscum. Neither of these methods brought the plasma membrane enzyme into solution. The microsomal fraction behaved differently. Detergent action released more protein than sonication. The enzyme solubilised by Cutscum was about three times as active at pH 8.3 as at pH 7.4. The untreated suspension and the soluble protein after ultrasonic treatment had about equal activities at the two pH values.

Further purification of the microsomal enzyme. Attempts to purify the microsomal phosphatidylinositol kinase by fractionation with $(NH_4)_2SO_4$ were not successful. Better results were obtained with columns of CM-Sephadex C50. An $(NH_4)_2SO_4$ fractionation was first made on a microsomal suspension in 0.32 M sucrose which had been sonicated as described above. The 17.6 mg protein obtained by increasing the $(NH_4)_2SO_4$ concentration from 36% saturated to 40% saturated was dissolved in 1.5 ml 20 mM Tris–HCl buffer (pH 7.4) and applied to a column 15 cm high and 2 cm in diameter. Gradient elution was arranged with 100 ml of the same buffer in the mixing chamber and 100 ml of buffer containing 1 M NaCl in the reservoir. Protein in the 10-ml fractions collected was estimated by the method of LOWRY *et al.*[14]. The column was run at 4° and a 99% recovery of protein was obtained. In three separate experiments most of the protein was eluted in the first six fractions and this protein had little kinase activity. The kinase was consistently eluted at a concentration of 0.75 M NaCl with a specific activity about 100 times that of the original homogenate.

TABLE III

PHOSPHATIDYLINOSITOL KINASE ACTIVITY IN MAMMALIAN TISSUES

The basic assay system was used in the absence of Cutscum. Tissues were homogenised in 0.32 M sucrose to give a 10% homogenate. The leucocyte suspension was made in 0.34 M sucrose[11] and the erythrocyte suspension in isotonic bicarbonate buffer (see MATERIALS AND METHODS). Where the figures are means the number of preparations is given in parentheses.

Tissue (rat unless otherwise stated)	*Kinase activity (nmoles diphosphoinositide per min per mg protein)*
Brain	0.122 (9)
Testis	0.072 (4)
Liver	0.071 (5)
Lung	0.059 (1)
Kidney	0.045 (4)
Spleen	0.023 (1)
Heart	0.022 (3)
Skeletal muscle	0.005 (3)
Pancreas	0.004 (1)
Leucocyte (rabbit)	0.056 (2)
Erythrocyte (human)	0.022 (2)

Unfortunately this purified kinase lost most of its activity when stored at −20° for one week. In one experiment the earlier fractions of the kinase peak were only active at pH 8.3, while the later fractions were only active at pH 7.4, suggesting that two enzymes were present.

Phosphatidylinositol kinase in other tissues

Since the use of the detergent Cutscum had revealed more than one phosphatidylinositol kinase in liver, a survey of other tissues was undertaken. Table III gives the total phosphatidylinositol kinase activity of the tissue homogenates measured in the absence of Cutscum under the conditions given in MATERIALS AND METHODS. Cutscum had little effect on the kinase activity in homogenates. The most active tissue was brain, followed by testis and liver. Heart muscle was four times as active as skeletal (femoral) muscle.

TABLE IV

PHOSPHATIDYLINOSITOL KINASE AND MARKER ENZYME ACTIVITY IN SUBCELLULAR FRACTIONS FROM KIDNEY AND MUSCLE

Subcellular fractions were prepared as described under MATERIALS AND METHODS. Enzyme activities are expressed as nmoles substrate converted per min per mg protein, and protein in mg. Phosphatidylinositol kinase was assayed by the basic method with Cutscum unless otherwise stated. N.D. indicates that no activity was detectable.

Enzyme	*Enzyme activity*					
	Homogenate	*Nuclear*	*Mitochondrial*	*Lysosomal*	*Microsomal*	*Supernatant*
Kidney						
Succinate dehydrogenase	184	151	665	348	48	10
Acid phosphatase	35	25	23	106	40	32
Glucose-6-phosphatase	57	50	34	70	85	14
Lactate dehydrogenase	748	270	116	111	53	2285
(Na^+-K^+)-ATPase	290	499	197	234	231	45
Phosphatidylinositol kinase	0.0585	0.0377	0.0130	0.0449	0.1374	0.0441
Phosphatidylinositol kinase (no Cutscum)	0.0146	0.0227	0.0006	0.0128	0.0498	0.0341
Protein (mg)	276	116	42	21.5	23.5	60.5
Skeletal muscle						
Succinate dehydrogenase	93	104	236	316	39	7
Acid phosphatase	14	10	18	90	0	26
Glucose-6-phosphatase	1.04	1.77	0	2.62	3.78	0.77
Lactate dehydrogenase	197	62	0	10	169	1420
5′-Nucleotidase	9.6	5.2	1.2	25.1	33.4	22.3
Phosphatidylinositol kinase	0.0024	N.D.	N.D.	N.D.	0.0250	N.D.
Protein (mg)	388	103	65	16.5	17.5	91.5
Heart						
Succinate dehydrogenase	270	128	826	382	264	64
Acid phosphatase	7.85	3.23	0	21.5	0	7.52
Glucose-6-phosphatase	1.99	0.37	0.73	1.09	4.56	2.38
Lactate dehydrogenase	266	133	93	0	42	1135
5′-Nucleotidase	30.0	19.1	18.1	22.4	51.0	23.6
Phosphatidylinositol kinase	0.010	0.0150	0.0138	0.0185	0.0750	N.D.
Protein (mg)	339	155	52.5	28.3	10.0	85.0

Table IV shows the activity of phosphatidylinositol kinase in subcellular fractions prepared from kidney, heart and skeletal muscle. Marker enzyme activities indicated that the fractionation was satisfactory. Succinate dehydrogenase was most active in the mitochondrial fraction in each case. The same applied to the following markers in the fractions indicated: DNA (nuclear), acid phosphatase (lysosomal), glucose 6-phosphate (microsomal) and lactate dehydrogenase (supernatant). The distribution of phosphatidylinositol kinase activity among the kidney subcellular fractions resembled that for liver (Fig. 1) with most of the activity in the nuclear and microsomal fractions. Because of the high phosphatase level in kidney, 5'-nucleotidase cannot be used as a marker. Distribution of phosphatidylinositol kinase was bimodal, like that of the (Na^+-K^+)-stimulated ATPase, which suggests an association with plasma membrane. As with liver, the kidney microsomal kinase was stimulated by the addition of Cutscum. Kinase activity in the rat heart and skeletal muscle was confined to the microsomal fractions, presumably as a direct result of the very vigorous homogenisation required for these tissues. Such homogenisation would cause plasma membrane fragments to appear in the microsomal rather than the nuclear fraction. The distribution of the kinase resembled that of 5'-nucleotidase and was not affected by Cutscum.

Fig. 3 shows the subcellular distribution pattern of the kinase and various

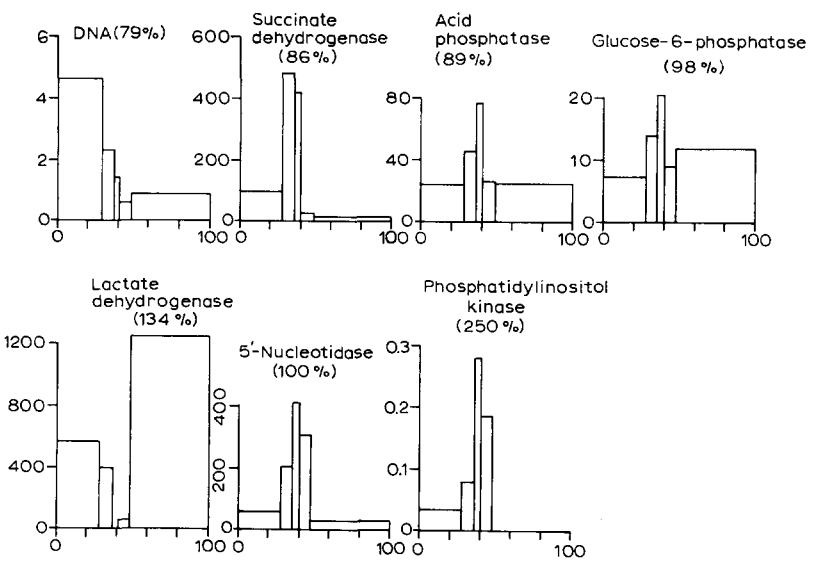

Fig. 3. Subcellular fractionation of rat testis. The ordinates give the protein content of each fraction as % recovered protein. Figures in parentheses represent the recoveries of DNA and the marker enzymes. DNA content is expressed as μg DNA-P per mg protein and the enzyme activities as nmoles substrate converted per min per mg protein. In each case the blocks represent, reading from left to right, nuclear, mitochondrial, lysosomal, microsomal and supernatant fractions.

marker enzymes in rat testis. As a result of the very gentle homogenisation employed and the delicate nature of the tissue, the fractions were not so pure as those from liver. However, the specific activity of each marker enzyme was highest in the appropriate fraction apart from glucose-6-phosphatase, which was higher in the

TABLE V

PHOSPHATIDYLINOSITOL KINASE IN SUBCELLULAR FRACTIONS FROM RABBIT POLYMORPHONUCLEAR LEUCOCYTES

The fractions were obtained from Dr. A. M. WOODIN and had been prepared by the method of WOODIN AND WIENEKE[11]. The light membrane fraction corresponds to the surface membrane. The basic assay system was used with 2% Cutscum at pH 7.4. N.D. indicates that no activity was detectable.

Leucocyte preparation Fraction	*Kinase activity (nmoles diphosphoinositide per min per mg protein) × 10³*		
Pellet	0.5	14	N.D.
Light membrane	114	642	186
Heavy membrane	26	105	72
Supernatant	11.9	17	N.D.

lysosomal than microsomal fraction. Again the distribution of phosphatidylinositol kinase resembled that of 5′-nucleotidase. Cutscum did not stimulate the activity of the kinase. Electron microscopy showed that the nuclear fraction contained spermatozoa and cells of Sertoli in addition to nuclei and clumps of interstitial cells. The mitochondrial fraction contained cell debris in addition to mitochondria and the lysosomal fraction contained plasma membrane fragments and mitochondria in addition to the dense-staining lysosomes. The latter finding accounts for the succinate dehydrogenase and 5′-nucleotidase activities in the lysosomal fraction.

Table V gives the activities of phosphatidylinositol kinase in subfractions of rabbit polymorphonuclear leucocytes. The light membrane fraction, which is rich in surface membrane, was much the most active.

Fig. 4 shows the variation of phosphatidylinositol kinase activity in human

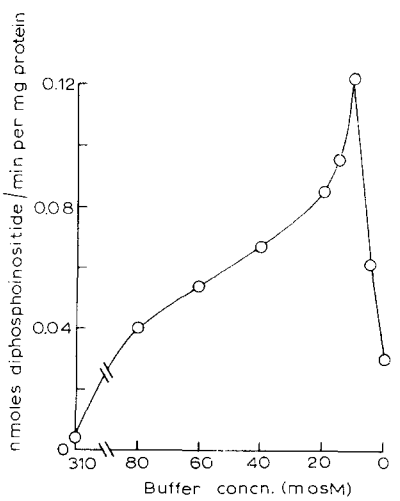

Fig. 4. Phosphatidylinositol kinase activity in erythrocyte preparations treated with hypotonic bicarbonate buffer. Details are given in MATERIALS AND METHODS.

erythrocytes treated with bicarbonate buffers of different osmolarities. The enzyme activity rises as soluble proteins are removed, suggesting that the kinase is a membrane constituent (see DISCUSSION).

Formation of triphosphoinositide

Traces of labelled triphosphoinositide were seen on the chromatograms prepared from brain fractions in the assay of phosphatidylinositol kinase, but not in those from any other tissue studied. This suggests that diphosphoinositide kinase was relatively inactive outside the nervous system, but confirmatory work is necessary since the assay conditions were not designed for this enzyme.

DISCUSSION

Erythrocyte membrane preparations

Several workers have studied the haemolysis of human erythrocytes under carefully controlled conditions. DODGE, MITCHELL AND HANAHAN[17] showed that erythrocyte membranes prepared in 15–20 mosM buffers contained least haemoglobin. Such membranes also contained minimum amounts of non-haem nitrogen and of certain enzymes[18,19]. MITCHELL, MITCHELL AND HANAHAN[19] suggested that acetylcholinesterase (EC 3.1.1.7) was tightly bound to the erythrocyte membrane since treatment with hypotonic buffers did not remove it. Fig. 4 shows that phosphatidylinositol kinase behaves in a similar way, indicating that this enzyme may also be an integral part of the membrane. The drop in specific activity after treatment with buffers of osmolarity below 10 may be due to deposition on the membrane of other proteins such as haemoglobin or to denaturation of the kinase as a result of damage to the membrane. Under these conditions the erythrocyte membrane is damaged*.

Phosphatidylinositol kinase and the plasma membrane

Previous work from this laboratory[4] indicated that phosphatidylinositol kinase is associated with the plasma membrane of rat-liver cells. Several observations in the present study suggest that such an association with the cell surface applies generally to mammalian tissues. (1) In kidney the kinase distribution among subcellular fractions followed that of the (Na^+-K^+)-stimulated ATPase, which is associated with the plasma membrane[20]. (2) Distribution in heart, skeletal muscle and testis followed that of 5′-nucleotidase, which has been shown to be a plasma membrane marker in a variety of tissues[20,21]. (3) The kinase is present in the erythrocyte and on lysis in bicarbonate buffer it behaves like a true constituent of the cell membrane. (4) The enzyme is concentrated in surface membrane fractions prepared from leucocytes. (5) Distribution of phosphatidylinositol kinase resembles that of 5′-nucleotidase and (Na^+-K^+)-stimulated ATPase in brain fractions[3], again indicating the involvement of plasma membrane.

Evidence for two phosphatidylinositol kinases

That there might be more than one kinase in liver tissue was first suggested

* T. A. BRAMLEY, personal communication.

by the effect of Cutscum (Fig. 1) which caused an increase of 400–700% in the activity of the microsomal kinase without affecting the other fractions greatly. The detergent caused no increase in 5'-nucleotidase activity, indicating that the increased kinase activity was not merely due to the rupture of plasma membrane vesicles in the microsomal fraction. Nor is the activation likely to be due to better dispersion of phosphatidylinositol in the aqueous medium, since this would have affected other fractions than the microsomal. It seems therefore, that the Cutscum directly stimulates a kinase of the microsomal fraction which originated in the endoplasmic reticulum rather than the plasma membrane. Table II indicates that this stimulation involves solubilisation of the enzyme from the endoplasmic reticulum. Cutscum liberates more protein from the microsomal fraction than does ultrasonic disintegration, and the kinase liberated has greater activity at pH 8.3. UDP glucuronyltransferase (EC 2.4.1.17) is another microsomal enzyme which is activated by a non-ionic detergent[22].

The two pH optima of Fig. 2 indicate the presence of two enzyme proteins in the microsomal fraction of liver. The enzyme with the pH optimum of 8.3 was not found in the plasma membrane fraction. The brain microsomal phosphatidylinositol kinase of COLODZIN AND KENNEDY[1] had the same pH optimum and was also stimulated by Cutscum. In the absence of the detergent, the kinase activity at pH 8.3 was still seen in liver microsomal fractions.

Cutscum also stimulated the kinase of the kidney microsomal fraction and though further evidence is not available at present, there may be more than one kinase in kidney as well as in liver.

The effects of inhibitors (Table I) also indicate that there is more than one phosphatidylinositol kinase in liver. The plasma membrane kinase was more sensitive to Ca^{2+} and F^- ions than was the microsomal enzyme. K^+ stimulated the latter enzyme, but inhibited the former. The effects of thiol reagents were also different.

Purification of the microsomal kinase

Enzymes acting upon lipid substrates are usually particulate lipoproteins and very few have been purified. The present purification experiments with Sephadex columns were not extended further because the purified soluble enzyme was so unstable. This was presumably due to loss of stabilising lipid. However, the columns produced some separation of the pH 7.4 and pH 8.3 activities, thus confirming the presence of two enzymes.

Subcellular fractionation of rat testis

The authors are not aware of any previous accounts of subcellular fractionation of testis. The present method may provide a starting point. Marker enzymes showed their usual distribution except for glucose-6-phosphatase which was most active in the lysosomal fraction and also appeared in the supernatant. This may be due to differences in the behaviour of the endoplasmic reticulum from the various cell types of testis. More vigorous homogenisation should improve the purity of the fractions. KOUDSTAAL *et al.*[23] describe the histochemical pattern of human testis. Lactate dehydrogenase and 5'-nucleotidase were generally distributed but blood vessels and tunica propria were devoid of acid phosphatase. Subcellular fractionation gives no evidence about the distribution of enzymes between different cell types in a tissue.

It is possible that phosphatidylinositol kinase only occurs in certain cells of the testis. The unusually high recovery of the kinase is probably due to polyphosphoinositide phosphomonoesterase which is very active in the supernatant*. This would reduce the yield of diphosphoinositide from the kinase in the homogenate.

Diphosphoinositide and triphosphoinositide

The formation of diphosphoinositide by the phosphorylation of phosphatidylinositol appears to be widespread among mammalian tissues. The kinase is usually associated with the cell surface membrane (plasma membrane) though in liver and kidney a second kinase occurs, probably in the endoplasmic reticulum. Similar information on triphosphoinositide synthesis is lacking. The assay conditions used in the present work are not designed to measure diphosphoinositide kinase activity, and Cutscum would almost certainly inhibit this enzyme. In brain, diphosphoinositide kinase is found in the supernatant fraction[24]. Synthesis of triphosphoinositide may also take place in the erythrocyte membrane[25].

It has been suggested[26] that the polyphosphoinositides may be important in controlling membrane permeability by their interaction with Ca^{2+}. The association of diphosphoinositide synthesis with the plasma membrane lends support to this idea.

Since this paper was originally submitted the work of HAJRA, SEGUIN AND AGRANOFF[27] has appeared. These authors conclude that in liver, phosphatidylinositol kinase is most active in a mitochondrial fraction. Their microsomal and nuclear fractions had considerable activity, however, and the kinase was assayed under suboptimum conditions, *i.e.* without added phosphatidylinositol. A valid comparison of subcellular fractions requires use of optimum conditions for each fraction. In addition, use of a marker enzyme for plasma membrane might have shown that the mitochondrial fraction of HAJRA, SEGUIN AND AGRANOFF[27] contained such membrane.

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