Rat liver plasma membranes contain a lipid-dependent protein kinase activity

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The treatment of rat liver plasma membranes with EDTA resulted in substantial release of a protein kinase activity. Using histones as external substrates both phosphatidylserine (PS) and diolein activated the enzyme. The activatory effect of PS but not that of diolein depended on Ca^{2+}. In contrast, the enzyme activity was inhibited by both PS and diolein when the substrate was protamine. The inhibition by PS, unlike by diolein, depended on Ca^{2+}. Data suggest that mobilization of plasma membrane-bound Ca^{2+} by physiological stimuli may be accompanied by release of the lipid-dependent protein kinase into the cytoplasm.

Liver plasma membrane *Protein kinase C* *Phosphatidylserine* *Diolein*

1. INTRODUCTION

It is well known that several liver plasma membrane proteins are substrates for the endogenous protein kinase(s) [1]. Characteristics of phosphorylation of liver membrane proteins, such as independence of cyclic nucleotides, have been established [2]; however, the regulatory properties of the membrane bound protein kinase(s) have remained unknown.

It will be shown that liver plasma membranes contain a protein kinase whose activity is regulated specifically by phosphatidylserine and unsaturated diglycerides.

2. MATERIALS AND METHODS

2.1. *Materials*

Histones (types II-S, III-S and VIII-S) protamine (free base, prepared from salmon) and phospholipase C (*Clostridium welchii*) were purchased from Sigma (St Louis MO). Histone 3, purified from calf thymus, was a gift from Dr Anna Faragó (Institute of Biochemistry I., Semmelweis Medical University, Budapest). Phospholipids as well as 1,2- and 1,3-dioleins were bought from Serva. [γ-^{32}P]ATP (> 1000 Ci/mmol) was prepared by the Isotope Institute of Biological Research Center (Szeged).

Abbreviations: PS, PC, PE, PI, serine, choline, ethanolamine and inositol phospholipids, respectively; EGTA, ethylene glycol bis(2-aminoethylether) *N,N,N',N'*-tetraacetic acid

2.2. *Methods*

Female Wistar rats (150–180 g body wt) that had been starved overnight were used. Liver plasma membranes were prepared as in [3].

Solubilization of protein kinase by EDTA as well as its precipitation by $(NH_4)_2SO_4$ was performed as in [4]. The protein content of the solubilized preparation was too low to measure reliably. Therefore, to standardize the amount of enzyme we always used the same volume (0.9 ml), the same amount of membrane protein (1.7 mg) and the same time (1 h) for the solubilization.

The assay mixture (0.1 ml) for the measurement of phosphorylation of histones contained 0.1 mM [γ-^{32}P]ATP (300–500 cpm/pmol), 10 mM $MgCl_2$, 30 mM Tris–HCl (pH 7.8), 20 μg histones and 20 μl solubilized protein kinase. After incubation for 5 min at 33°C, 50 μl aliquots were applied on

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2×2 cm filter (Whatman 31 ET). Further steps were as in [5]. Values will correspond to phosphorylation by 20 μl supernatant.

Separation of phosphorylated membrane proteins by SDS–polyacrylamide slab gel electrophoresis was performed as in [6].

Extraction of lipids and separation of phospholipids were described in [7]. Diacylglycerol was prepared from total liver phospholipid fraction by phospholipase C as in [8] and purified by preparative thin-layer chromatography on silica gel H using petrolether, diethylether, acetic acid, methanol (85:13:1:1, by vol.) as solvent.

Emulsions of phospholipids and diacylglycerols were obtained by ultrasonication in 10 mM Tris–HCl (pH 7.5) with a MSE sonifier.

Protein was determined as in [9] with bovine serum albumin as standard.

All the reported experiments were repeated at least 3 times with similar results.

3. RESULTS

Treatment of liver plasma membranes with 1 mM EDTA for 1 h released ~45% of the total phosphorylating activity. Three successive treatments resulted in 70% solubilization. Similar results were obtained with EGTA. Decay of protein

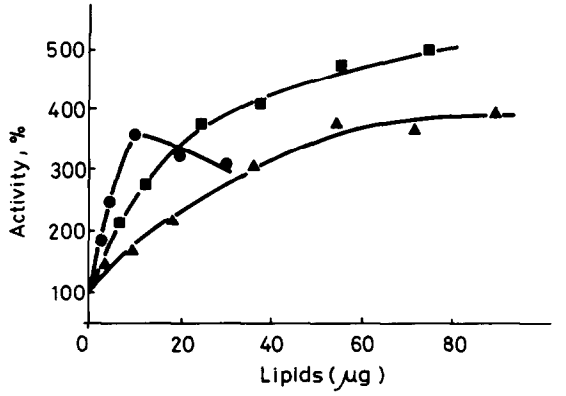

Fig.1. Effect of PS and diacylglycerols on the activity of solubilized protein kinase. Protein kinase activity of EDTA supernatant was determined with H3 (20 μg/assay) as substrate in the absence (100% activity, 345 pmol ^{32}P . mg $H3^{-1}$. 5 min^{-1}) or presence of PS (•—•), diolein (▪—▪) or natural diacylglycerol (▲—▲). Each point represents the mean value of three determinations in one representative experiment.

Table 1

Effect of $CaCl_2$, PS and diolein on the activity of solubilized protein kinase

Additions	No lipid	PS	Diolein	PS + Diolein
None	330±25	1025±31	1820±145	1276±24
$CaCl_2$, 0.01 mM	380±22	1031±80	1917±127	
$CaCl_2$, 0.1 mM	511±17	921±33	1875±119	
CaCl, 1.0 mM	472±47	682±43	1571±228	
EGTA, 4.0 mM	270±11	310±24	1930±129	

20 μg PS and 75 μg diolein/0.1 ml were used. Results are the mean ± SEM of quadruplicate determinations in one representative experiment

Table 2

Effect of PS and diolein on the phosphorylation of exogenous and endogenous substrates (pmol ^{32}P . mg substrate^{-1} . 5 min^{-1})

Addition	H3	Protamine	Total membrane protein
None	35± 2	576± 25	1202±42
PS 20 μg	126± 7	254± 9	1164±30
PS 20 μg + $CaCl_2$ 0.1 mM	186±16	168± 22	1180±23
PS 20 μg + EGTA 4 mM	71± 4	635± 41	1022±35
Diolein 50 μg	315± 8	237± 21	774±54
Diolein 50 μg + $CaCl_2$ 0.1 mM	335± 7	208± 10	724±16
Diolein 50 μg + EGTA 4 mM	420±13	263± 7	757±39
Total lipid 50 μg	–	1325± 98	–
EDTA Supernatant	395±18	2506±116	

Phosphorylation of exogenous substrates (20 μg) was performed with $(NH_4)_2SO_4$ precipitated preparation or equivalent volume of the original EDTA supernatant. For phosphorylation of membrane proteins (30 μg protein) 0.5 mM [γ-^{32}P]ATP was used. $CaCl_2$ or EGTA alone had no significant effect in either case. Results are the mean ± SEM of triplicate determinations in one representative experiment

kinase activity was substantial at 4°C (half-life ~ 50 min); thus, we set the extraction time to 1 h.

Significant protein kinase activity in the supernatant could be measured only with external substrates.

Using H3 as external substrate, PS and diacylglycerols were potent stimulators of the protein kinase activity of the EDTA supernatant (fig.1). Diolein had higher stimulatory effect than PS or the natural diacylglycerol fraction. The latter contains both saturated and unsaturated fatty acids. PC, PE or PI, each tested up to 50 μg/assay, had no significant effect (not shown). Using lysine rich or total histone fractions as substrates the effects of PS and diolein were somewhat less. These lipids did not increase the phosphorylation of protamine (not shown).

In the absence of added lipids phosphorylation of H3 by the EDTA supernatant was increased or decreased by the addition of Ca^{2+} or EGTA, respectively (table 1). The stimulatory effect of PS was markedly reduced by EGTA. In the presence of diolein, however, the protein kinase became insensitive to the externally added Ca^{2+} or EGTA (table 1).

The various plasma membrane preparations contained variable amounts of loosely attached lipids which were partly released by EDTA. We determined that precipitation of protein kinase from the EDTA supernatant by ammonium sulphate resulted in an essentially lipid free preparation. One would expect that the amount of free Ca^{2+} in this precipitate is also greatly reduced. Thus, to examine the dependence of protein kinase activity on lipids and Ca^{2+} more properly we used salt precipitated protein kinase preparation.

With H3 as substrate, the lipid free protein kinase showed low activity (table 2). Addition of PS stimulated the enzyme activity in a Ca^{2+}-dependent manner. Diolein had more marked effect than PS. In contrast to our expectation, 4 mM EGTA did not reduce, instead slightly increased the effect of diolein.

The activity of the protein kinase of the precipitate was relatively high with protamine as substrate and was inhibited by both PS and diolein (table 2). The inhibitory effect of PS was increased by Ca^{2+} and was completely abolished by EGTA. The inhibitory effect of diolein appeared to be independent of Ca^{2+}. Even with protamine as substrate, the enzyme remains partially dependent on lipids judged from the stimulatory effect of total lipid fraction (table 2). Preliminary experiments indicate that an unsaturated fatty acid is the active component of the total lipid fraction.

We attempted but failed to show specific increase of labelling of any membrane proteins in the presence of PS or diolein. In fact, diolein markedly reduced phosphorylation of proteins (table 2). The inhibitory effect of diolein was again Ca^{2+}-independent.

4. DISCUSSION

These data demonstrate that liver plasma membranes contain a protein kinase whose activity is regulated by lipids; therefore, it may belong to the recently discovered C category [10–16].

In deviance from the hitherto characterized lipid-dependent protein kinases [11,17] we could not convincingly show the dependence of diolein effect on either Ca^{2+} or PS. The effect of PS was clearly dependent on Ca^{2+}.

Depending on the substrate, PS or diolein either increased (histones) or decreased (protamine) the activity of protein kinase. Although two-dimensional separation of proteins might reveal exceptions, it appears that phosphorylation of most membrane proteins is regulated negatively by diolein. However, membranes probably contain saturating levels of PS in respect to enzyme activity.

Removal of Ca^{2+} from membranes resulted in the release of lipid-dependent protein kinase in our case and in other cases too [4,18]. Thus, binding of this protein kinase to membranes presumably occurs through Ca^{2+} bridges and, therefore, the involvement of anionic phospholipids might be expected. Ca^{2+}-dependent binding of the soluble protein kinase C to membranes has been demonstrated [14]. All these suggest that mobilization of membrane-bound Ca^{2+}, which occurs by stimulation of turnover of phosphatidylinositol [19–20], may be accompanied by release of protein kinase into the cytoplasma together with the concomitantly formed diacylglycerols. Liver cells are known to contain soluble protein kinase C [21]. The membrane-bound protein kinase C may constitute a reserve of the soluble form which is released when the Ca^{2+}-mobilizing stimulus acts on the membrane.

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