Effect of ionic strength on production of cAMP- and Ca^{2+}-independent protein kinase from rat liver plasma membrane

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Production of cAMP- and Ca^{2+}-independent protein kinase was stimulated when rat liver plasma membrane was incubated with increasing concentrations of NaCl. This protein kinase release was diminished by addition of protease inhibitor. The molecular mass of this enzyme was approx. 50 kDa and a high concentration of Mg^{2+} was required for whole histone phosphorylation. These properties are similar to those of the protease-activated form of protein kinase C. The NaCl effect could be replaced by other salts such as LiCl and $NaHCO_3$. These results suggest that membrane-bound protein kinase C is activated by limited proteolysis corresponding to an increase in ionic strength.

Protein kinase C *Limited proteolysis* *Membrane-bound enzyme* *Ionic strength* *Na^+ influx* *Growth factor*

1. INTRODUCTION

Experimental results have accumulated indicating that ribosomal protein S6 is phosphorylated in response to growth-promoting stimuli such as serum or growth factor treatment and viral transformation [1–5]. Although protein kinase activity corresponding to S6 protein phosphorylation has been detected in various cellular systems, the mode of activation of this enzyme and the relation to the previously isolated protein kinases have remained obscure [6–10]. It has been described repeatedly that the activity of this S6 kinase is not affected by various protein kinase activators such as cyclic nucleotides and Ca^{2+} [7–10]. However, Perisic and Traugh [11,12] showed that the peptide map of S6 protein phosphorylated by protease-activated kinase II was nearly identical with that obtained from 3T3 cells stimulated by insulin or epidermal growth factor. For this S6 protein phosphorylation, we suppose that plasma membrane-bound, protease-activated kinase may be one of the candidates for the catalyst [13,14]. Another line of evidence indicates that an increase in Na^+ influx is one of the earliest responses of quiescent cells to the addition of growth promoting factors [15]. In an attempt to obtain some insight into the role of this Na^+ influx, we examined the effect of salt on the activation of membrane-bound protein kinase. When rat liver plasma membrane was incubated with NaCl, production of cAMP- and Ca^{2+}-independent protein kinase was stimulated according to the increase in this salt concentration. This protein kinase showed similar properties to those of the protease-activated form of protein kinase C. These results suggest that membrane-bound protein kinase C may be involved in the protein phosphorylation induced by various growth-promoting stimuli [16–18].

2. MATERIALS AND METHODS

Rat liver plasma membrane was prepared as described in [13] except that 2 μg/ml of leupeptin, antipain, chymostatin and pepstatin were added to

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the isolation buffers. Finally, the membrane was suspended in buffer containing 50 mM Tris-HCl at pH 8.0 and 250 mM sucrose and stored at −70°C. Calf thymus whole histone was prepared as specified in [19]. Protein inhibitor of cAMP-dependent protein kinase was purified as in [20]. [γ-^{32}P]ATP was prepared by the method described in [21]. Bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor were obtained from Sigma. Other materials were obtained from commercial sources.

Protein kinase (active form) was assayed as in [22] except that 40 mM Tris-HCl at pH 7.5 was employed and incubation was performed for 7.5 min. Other modifications of the assay method were described in [13]. Other reaction conditions were described in each experiment.

Protein was determined by the method of Bradford [23] with bovine serum albumin as a standard. Phosphorylated amino acid was determined by the method in [13]. Radioactivity was determined with an Aloka LSC-950 liquid scintillation counter, by Cerenkov radiation. The molecular mass of protein kinase was estimated as described in [13].

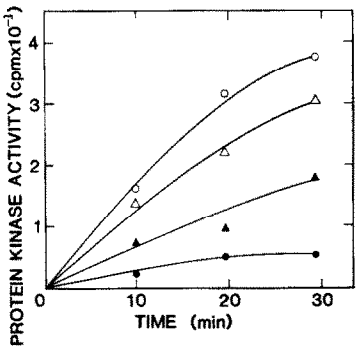

Fig.1. Effect of NaCl on release of protein kinase from rat liver plasma membrane. Rat liver plasma membrane (380 μg protein) was incubated at 30°C with various NaCl concentrations (as indicated below) in a solution (0.2 ml) which contained 25 mM Tris-HCl at pH 8.0, 5 mM 2-mercaptoethanol and 125 mM sucrose. At the time indicated, the reaction was stopped by the addition of 40 μg leupeptin. After centrifugation at 16000 × g for 10 min, an aliquot of the supernatant (70 μl) was assayed for protein kinase under the conditions described in section 2 except that NaCl concentration was adjusted to be the same in all reaction tubes. NaCl concentrations (mM): (●) 0, (▲) 70, (△) 140 and (○) 210.

3. RESULTS

When rat liver plasma membrane was incubated in the presence of NaCl, time-dependent production of whole histone phosphorylating activity was stimulated according to the increase in this salt concentration as shown in fig.1. This protein kinase release was greatly inhibited by the addition of protease inhibitors such as leupeptin and bovine pancreatic trypsin inhibitor (table 1). However, soybean trypsin inhibitor was less effective. These results suggest that some membrane-bound protease may be involved in this protein kinase production. This enzyme showed high Mg^{2+} requirement when activities were compared between 5 and 75 mM Mg^{2+} using whole histone as phosphate acceptor. The histone kinase activity at 75 mM Mg^{2+} was not affected by cAMP (1 μM) or EGTA (1 mM). Inhibitor protein of cAMP-dependent protein kinase was also ineffective and the major phosphorylated amino acid was identified as serine (not shown). The molecular mass of this protein kinase was estimated to be 5×10^4 Da by the gel filtration method (fig.2). The results in table 2 indicate that the NaCl effect could be replaced by other salts such as LiCl and NH_4Cl. This effect was not specific for chloride salt because $NaHCO_3$ and CH_3COONa were also effective in producing the protein kinase from plasma membrane (not shown).

Table 1

Effects of protease inhibitors on release of protein kinase from rat liver plasma membrane

Protease inhibitor	Inhibition (%)
Leupeptin	84
Bovine pancreatic trypsin inhibitor	89
Soybean trypsin inhibitor	24

Rat liver plasma membrane was incubated for 30 min in the presence and absence of each protease inhibitor (182 μg/ml) under the condition described in the legend to fig.1 except that 210 mM NaCl was employed. After centrifugation, protein kinase activity was determined as described in the legend to fig.1. The values are the average of 2 determinations

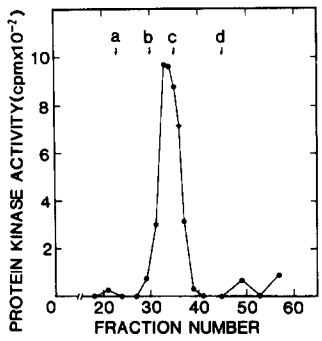

Fig.2. Molecular mass analysis of protein kinase by gel filtration. Rat liver plasma membrane was incubated for 30 min under the condition described in the legend to fig.1 except that 210 mM NaCl was employed and reaction volume was scaled up by 22-fold. After stopping the reaction by the addition of leupeptin (182 μg/ml), the mixture was centrifuged at 16000 × g for 10 min. An aliquot of the supernatant (600 μl containing 360 μg protein) was applied to a Sephadex G-150 column (0.8 × 101 cm) equilibrated with buffer which contained 20 mM Tris-HCl at pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA and 70 mM NaCl. Elution was performed downward with the same buffer at a flow rate of 3 ml/h. Fractions of 0.9 ml each were collected. Protein kinase activity was determined using 205 μl of each fraction under the conditions described in section 2 except that Tris-HCl and 2-mercaptoethanol were omitted. Molecular mass standards: a, human γ-globulin; b, bovine serum albumin; c, ovalbumin; d, horse heart cytochrome *c*.

Table 2

Effects of various salts on release of protein kinase from rat liver plasma membrane

Salt		Protein kinase activity (cpm)
NaCl	+	3720
	−	850
LiCl	+	2660
	−	370
NH_4Cl	+	2500
	−	200

Rat liver plasma membrane was incubated for 30 min in the presence (+) and absence (−) of 210 mM of each salt under the condition described in the legend to fig.1. After centrifugation, protein kinase activity was determined as described in the legend to fig.1 except that salt concentration was adjusted to be the same in each pair of experiments

4. DISCUSSION

The protein kinase activity detected in this study was not affected by cAMP and Ca^{2+}. In addition, the results on the high Mg^{2+} requirement in whole histone phosphorylation and the molecular mass analysis suggest that this enzyme corresponds to the protease-activated form of protein kinase C [22,24,25]. Previous reports from this laboratory showed that membrane-bound protein kinase C could be activated by exogenously added trypsin or endogenous trypsin-like protease [13,14]. Judging from the sensitivity to various types of protease inhibitors, the trypsin-like protease discovered by Tanaka et al. [26] may be involved in the activation of membrane-bound protein kinase observed in this study [14]. However, Melloni et al. [27] showed that protein kinase C is activated by calpain on the neutrophil membrane under physiological concentration of Ca^{2+}.

Recently, experimental results have accumulated indicating that Na^+ plays an important role in the protein phosphorylation including ribosomal protein S6. Pouysségur et al. [28] showed that growth factor-induced Na^+ influx and the extrusion of H^+ via activation of Na^+/H^+ exchanger are coupled to S6 protein phosphorylation. Cuny et al. [29] described that phosphorylation of 40 S ribosomal protein analogous to S6 in growth-arrested *Tetrahymena* was rapidly induced by Na^+ in the starvation buffer. They also pointed out the possibility of activation of preexisting protein kinase by Na^+ [29]. These results suggest that Na^+ influx may be linked to the metabolic regulation through the activation of some protein kinases. The possible role of protein kinase C is currently studied in our laboratory.

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