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DETERMINATION OF Ca^{2+}- AND PHOSPHOLIPID-DEPENDENT PROTEIN KINASE IN RAT LIVER MEMBRANES

BENGT JERGIL and MARIANNE SOMMARIN

Biochemistry, Chemical Centre, University of Lund, P.O.B. 740, S-220 07 Lund (Sweden)

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A method has been developed to measure the Ca^{2+}- and phospholipid-dependent protein kinase in membrane fractions. The method is based on the fact that this enzyme is resistant to comparatively high concentrations of octylglycoside. Rat liver membranes were treated with octylglycoside and the phosphate incorporation from [γ-^{32}P]ATP was measured in the presence of histone H1. The enzyme activity was determined as the difference between the incorporation obtained after addition of Ca^{2+} and phosphatidylserine and the incorporation obtained without these additions but with EGTA. The endogenous incorporation of phosphate to membrane components was constant under these incubation conditions. The conditions for determination of the membrane-bound enzyme were optimized. Two thirds of the total enzymic activity was attached to membranes in rat liver cells. A highly purified plasma membrane preparation had the highest specific activity, while most of the bound enzyme was found in microsomes, and only traces were found in mitochondria.

Introduction

The Ca^{2+}- and phospholipid-dependent protein kinase (Ca protein kinase), originally described by Takai et al. [1] is widely distributed in various organisms and tissues [2,3]. The phospholipid dependency of the enzyme has been taken to indicate that it may bind to cellular membranes [1] under appropriate conditions. Support for this suggestion has come from the recent finding that Ca protein kinase apart from being present in the cytosol also is attached to membrane fractions in various tissues [2,4].

One difficulty which has hampered studies on membrane-bound Ca protein kinase, for instance studies designed to examine possible regulatory mechanisms for the binding of Ca protein kinase to membranes, is the lack of a convenient assay procedure for the bound enzyme. The membrane-bound enzyme has been measured in the high-speed supernatant after extraction with EGTA and Triton X-100 [4]. Apart from the time-consuming centrifugation step, this procedure is limited by the inhibiting effect of Triton X-100 on Ca protein kinase activity. Detergent concentrations above 0.0075% are inhibitory [4], and this is far below what is required to solubilize the enzyme, necessitating extensive dilution of the extracts before enzyme analysis.

In this paper we describe a simple and sensitive procedure for analysis of Ca protein kinase bound to membranes. The procedure is based on the fact that the enzyme tolerates the detergent octylglycoside rather well. The conditions for analysis have been optimized, and the kinase activity in rat liver membrane fractions determined.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*-*N'*-tetraacetic acid; Ca protein kinase, Ca^{2+}- and phospholipid-dependent protein kinase; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid.

Experimental

Materials. *n*-Octyl-β-D-glucopyranoside (octylglycoside), histone H1 and protamine sulfate were obtained from Sigma Chemicals Co. [γ-^{32}P]ATP was synthesized according to Chang et al. [5]. Phosphatidylserine was a generous gift from Dr. R. Sundler, Department of Physiological Chemistry, Lund. All solutions were made up in double distilled water filtered through a Milli-Q™ system (Millipore).

Preparation of liver membrane fractions. These were prepared from male Sprague-Dawley rats which had been starved for 18 h before being killed. Microsomes were prepared either in the absence or presence of 1 mM EDTA and 0.5 mM EGTA. Livers were homogenized in 3 vol. per g tissue of 50 mM Tris-HCl, pH 7.4, and 0.35 M sucrose, with or without the chelators, by six strokes in a glass-teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged at 9000 × *g* for 10 min, and the resulting supernatant at 105 000 × *g* for 90 min. The microsomal pellet was washed once in 10 mM Tris-HCl, pH 7.4, also containing 0.3 M KCl, and once in buffer alone. Smooth microsomes were obtained from the microsomal pellet by suspension in the homogenization medium and discontinuous sucrose gradient centrifugation [6] with or without 1 mM EDTA and 0.5 mM EGTA. The 0.9 M/1.3 M sucrose interface was aspired, diluted with 10 mM Tris-HCl, pH 7.4, and made 0.3 M in KCl before centrifugation for 90 min at 105 000 × *g*. The resulting pellet was washed once in 10 mM Tris-HCl, pH 7.4, and resuspended in the same buffer.

Plasma membrane-enriched fractions were prepared according to Aronson and Touster [7]. Two fractions were obtained; the N_2-fraction primarily contains bile canalicular membranes [8], while the P_2-fraction is a mixture of blood sinusoidal membranes and Golgi [8]. Mitochondria were prepared according to Greenawalt [9]. The membrane fractions were suspended in 10 mM Tris-HCl, pH 7.4. Rat liver cytosol was obtained as the 105 000 × *g* supernatant of homogenates prepared in the presence of 1 mM EDTA and 0.5 mM EGTA. It was chromatographed through Sephadex G-25 before use.

Protein was determined by the procedure of Lowry et al. [10] in the presence of 3% (w/v) sodium dodecyl sulfate with bovine serum albumin as standard.

Protein kinase analyses. The Ca^{2+}- and phospholipid-dependent protein kinase was analyzed in a mixture containing (unless otherwise is indicated) 2.5 μmol Hepes, pH 6.5, 1 μmol $MgCl_2$, 5 nmol [γ-^{32}P]ATP (spec. act. 400–800 cpm/pmol), 10 μg histone H1, 15–30 μg membrane protein, 0.175 mg octylglycoside and either 10 nmol EGTA, or 25 nmol $CaCl_2$ and (optional for membrane fractions) 5 μg phosphatidylserine; the volume was 100 μl. Membranes were preincubated with detergent for at least 5 min before starting the reaction with ATP. Incubations were for 3 min at 25°C, and were stopped by the addition of 10 μl 12 M HCl. The reaction mixtures were quantitatively transferred to filter paper discs, which were washed and analyzed in a scintillation counter as described earlier [11]. Endogenous phosphorylation was measured in the same way but omitting histone H1. The kinase activity was obtained as the Ca^{2+}- and phospholipid-dependent increase in phosphorylation of histone H1 after subtraction of endogenous phosphorylation.

Soluble Ca protein kinase from rat liver cytosol was partially purified by chromatography on DEAE-Sephacel (Sommarin, M. and Jergil, B., unpublished data). The preparation was virtually free from cyclic AMP-dependent protein kinase activity. Protamine kinase activity was measured as described [11] omitting NaCl. All analyses were performed in duplicate.

Results

The effect of detergents on Ca protein kinase activity

Soluble Ca protein kinase is characterized by its dependency on Ca^{2+} and phospholipids for full activity when histone H1 is used as substrate [1]. The enzyme also phosphorylates protamine efficiently in a Ca^{2+}- and phospholipid-independent manner [12]. The effect of detergents on Ca protein kinase and protamine kinase activities was studied using partially purified enzyme from rat liver cytosol (Fig. 1). Triton X-100 at concentrations below 0.01% slightly stimulated Ca protein kinase activity. This stimulation ranged from 25 to

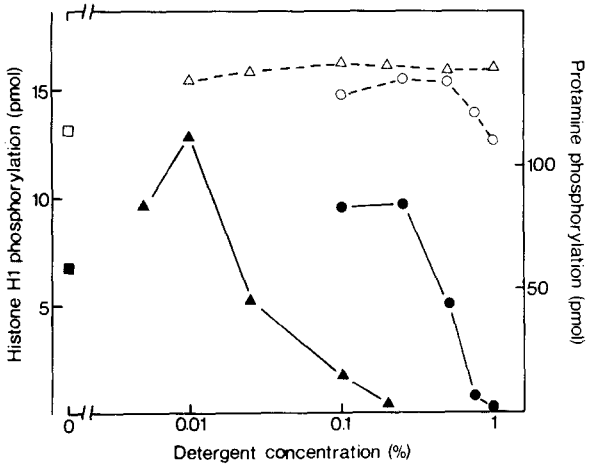

Fig. 1. The effect of detergents on protein kinase activity. Partially purified Ca protein kinase from rat liver cytosol (12 μg per sample) was analyzed for Ca^{2+}- and phospholipid-dependent phosphorylation of histone H1 (closed symbols) or protamine phosphorylation (open symbols) in the presence of various concentrations of Triton X-100 (△) or octylglycoside (○). The phosphate incorporation is expressed as pmol per min.

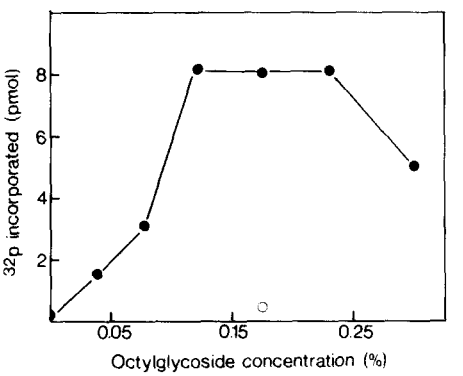

Fig. 2. Ca^{2+}- and phospholipid-dependent phosphorylation of histone H1 by smooth microsomes at various concentrations of octylglycoside. Smooth microsomes (30 μg of membrane protein) were preincubated with octylglycoside for 5 min before a 10-fold dilution in the kinase reaction mixture to the indicated concentrations. The open circle shows the kinase activity when measured directly at 0.175% octylglycoside without preincubation at a high detergent concentration.

90% in different experiments. Above 0.01% Triton was strongly inhibitory, thus corroborating earlier results [4]. In a search for a less inhibitory detergent, we found that octylglycoside was the only one examined which could be used with advantage. Indeed, Ca protein kinase entirely retained its Ca^{2+}- and phospholipid-dependent activity in the presence of 0.25% octylglycoside (Fig. 1), i.e. at a 25-fold higher concentration than with Triton X-100. When the concentration was increased further the activity decreased rapidly. At concentrations below 0.25% the enzyme was slightly stimulated (up to 30%). The enzyme also retained its activity after treatment with high concentrations of octylglycoside, provided that the extract was properly diluted before analysis (see below).

The influence of Triton X-100 and octylglycoside on the Ca^{2+}- and phospholipid-independent phosphorylation of protamine by the enzyme was also examined (Fig. 1). In this case the enzyme was still fully active in the presence of more than 1% (Triton X-100) or close to 1% (octylglycoside) of the detergents.

Octylglycoside concentrations

The effect of octylglycoside on membrane-bound protein kinase was examined next. To this end smooth microsomes were preincubated with various concentrations of octylglycoside and then diluted tenfold in the Ca protein kinase reaction mixture. No enzyme activity could be detected in the absence of detergent. After addition of octylglycoside a Ca^{2+}- and phospholipid-dependent histone H1 kinase activity was observed, however. The activity increased with increasing concentrations of detergent (Fig. 2) reaching a maximum in the concentration range from 0.12 to 0.23%, i.e. the membranes had been pretreated with 1.2–2.3% (w/v) of octylglycoside. The lower activity observed at 0.3% octylglycoside was due to inhibition of the enzyme at this concentration and could be overcome through a further dilution before analysis (not shown). If smooth microsomes were mixed directly with a reaction mixture containing 0.175% octylglycoside virtually no Ca^{2+}- and phospholipid-dependent kinase activity could be detected. This shows that solubilization of the membrane, at least in part, is a prerequisite for measuring bound activity.

Effect of Ca^{2+} and phospholipids

The Ca^{2+} dependency of histone H1 phosphorylation by smooth microsomes treated with octylglycoside was examined. A maximum rate of

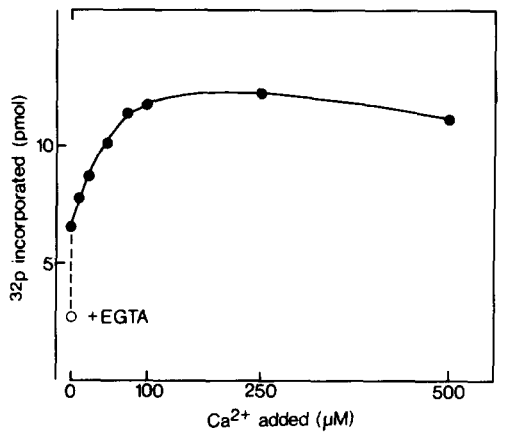

Fig. 3. The effect of Ca^{2+} on histone H1 phosphorylation by smooth microsomes. Kinase activity was measured with 40 μg of membrane protein at different Ca^{2+} concentrations, or after the addition of 50 μM EGTA. The incorporation of phosphate is expressed as pmol per min.

phosphorylation was obtained at approx. 100 μM Ca^{2+} (Fig. 3). Without added Ca^{2+} the rate was still comparatively high and around half of that at the optimum concentration. This could be diminished further, however, by the addition of 50 μM EGTA, yielding a basal rate of histone phosphorylation that was approx. 20% of the rate at optimum conditions. This basal rate corresponds well to the histone H1 phosphorylation expected from the cyclic AMP-dependent protein kinase present in smooth microsomes [13]. The partial activation of the enzyme in the absence of EGTA was probably due to the release of Ca^{2+} from microsomal vesicles on solubilization with octylglycoside; microsomes together with mitochondria contain the major intracellular pool of Ca^{2+}.

Further aspects on the effects of Ca^{2+} and EGTA, together with the effect of phosphatidylserine, on the incorporation of phosphate into endogenous membrane components and histone H1 are shown in Table I. None of these additions had any appreciable effect on the phosphorylation of endogenous components. Furthermore, the addition of phosphatidylserine did not increase the incorporation of phosphate into histone H1. Thus, there is a sufficiently high endogenous concentration of phospholipids in the membrane extract to obtain optimum Ca protein kinase activity provided that the observed enzyme is phospholipid-dependent. The enzyme activity most likely represents Ca protein kinase, since the only protein kinase fraction obtained by chromatography of microsomal extracts on DEAE-cellulose that phosphorylates histone H1 in a Ca^{2+}-dependent manner also is phospholipid-dependent (Jergil, B., unpublished data).

TABLE I

THE EFFECT OF Ca^{2+}, EGTA AND PHOSPHATIDYLSERINE ON THE PHOSPHORYLATION OF HISTONE H1 BY SMOOTH MICROSOMES

Smooth microsomes were prepared in the presence of EDTA and EGTA. The incorporation of ^{32}P-labelled phosphate was measured with (total) or without (endogenous) addition of histone H1. The difference is taken as a measure for the incorporation into histone H1. Incubations were performed with additions as indicated (250 μM $CaCl_2$, 5 μg phosphatidylserine (PS), 100 μM EGTA).

Substrate	^{32}P-phosphate incorporation (nmol/min per mg protein)					
	−	−	−	−	+	+ $CaCl_2$
	−	−	+	+	−	+ PS
	−	+	−	+	−	− EGTA
Total	0.142	0.088	0.164	0.085	0.266	0.271
Endogenous	0.057	0.053	0.052	0.052	0.042	0.045
Histone H1	0.085	0.035	0.112	0.033	0.224	0.226

ATP concentration

Usually 5 μM ATP has been used for the analysis of Ca protein kinase [1,2]. This is far from a saturating concentration for the microsomal en-

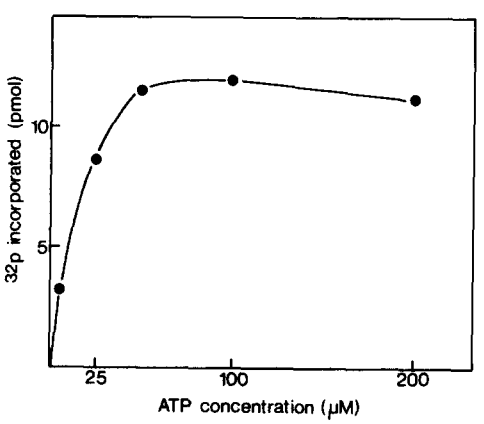

Fig. 4. Histone H1 phosphorylation by smooth microsomes at different ATP concentrations. Kinase activity was measured using 30 μg of membrane protein.

zyme which phosphorylates histone H1 most actively at 50 μM ATP (Fig. 4). At this concentration the activity is 3.5-times higher than with 5 μM ATP.

Histone H1 concentration

The effect of variations in the concentration of histone H1 on phosphate incorporation is presented in Fig. 5. Maximum incorporation was obtained at 100 μg per ml incubation medium when Ca^{2+} and phosphatidylserine were present. Without addition of these compounds the incorporation increased up to 30 μg histone per ml and was then constant.

Other parameters

We have also tested several other parameters using smooth microsomes. Mg^{2+} is necessary for the phosphorylation of histone H1 and maximum activity is obtained at 5 mM concentration. The pH optimum for the reaction is around pH 6.5. These properties together with the saturation curves for ATP and histone H1 concentrations are consistent with those found for partially purified Ca protein kinase from rat liver cytosol (Sommarin, M. and Jergil, B., unpublished data).

The phosphorylation is proportional to the amount of membrane up to 30 μg of membrane protein per incubation for smooth and unfractionated microsomes, and up to 10 μg for plasma membranes. Using these amounts of membranes the reaction is linear with time for 10 and 3 min for smooth microsomes and plasma membranes, respectively. We have also added various amounts of cytosolic Ca protein kinase to smooth microsomes before analysis under standard conditions and found the expected activities within a wide range of enzyme concentrations.

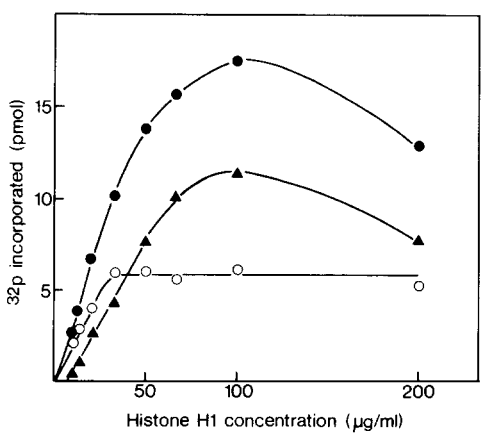

Fig. 5. Histone H1 phosphorylation by smooth microsomes at different histone concentrations. The histone H1-dependent phosphorylation was measured using 40 μg of membrane protein in the presence (●) or absence (○) of Ca^{2+} and phosphatidylserine. In the latter measurements 100 μM EGTA was present. The difference between the two sets of experiments has also been plotted (▲).

TABLE II

Ca^{2+}- AND PHOSPHOLIPID-DEPENDENT ACTIVITIES IN RAT LIVER SUBCELLULAR FRACTIONS

The microsomal fractions were prepared in the absence (−) or presence (+) of EDTA and EGTA. The data represent means ± S.E. for the number of determinations given (*n*).

Fraction	Specific activity (nmol/min per mg protein)	*n*
Smooth microsomes		
− EDTA/EGTA	0.239 ± 0.059	5
+ EDTA/EGTA	0.397 ± 0.077	6
Unfractionated microsomes		
− EDTA/EGTA	0.242 ± 0.113	6
+ EDTA/EGTA	0.290 ± 0.048	6
Plasma membranes		
N_2-fraction	1.25 ± 0.20	4
P_2-fraction	0.542 ± 0.100	4
Mitochondria	< 0.015	2
Cytosol	0.041 ± 0.008	3

Ca protein kinase activities in subcellular fractions

The Ca^{2+}- and phosphatidylserine-dependent phosphorylation of histone H1 catalyzed by various rat liver subcellular fractions is shown in Table II. The highly purified plasma membrane N_2-fraction had the highest specific kinase activity, approximately twice that of the less homogeneous P_2-fraction. Both smooth and unfractionated microsomes showed a slightly lower activity when prepared in the absence rather than in the presence of EDTA or EGTA. The specific activity of these fractions was 6–10-times higher than that found in the cytosol. The activity of mitochondria was close to the detection limit.

Discussion

The results presented show that the Ca^{2+}- and phospholipid-dependent phosphorylation of histone H1 catalyzed by membrane-bound protein kinase can be measured directly after treatment of membranes with octylglycoside. The enzyme is much less sensitive to this detergent than to other detergents, including Triton X-100 which has been used earlier to extract Ca protein kinase from membranes [4]. As a result no extensive dilution of the detergent-treated material had to be done before enzyme analysis. Centrifugation to remove particulate material after the addition of detergent, thereby diminishing the substantial endogenous incorporation of phosphate, and hence the Ca^{2+}-independent phosphorylation, could also be avoided. The endogenous phosphorylation was 2–4-times lower (depending on the membrane material examined) in the high-speed supernatant than in the membrane-detergent mixture before centrifugation. There was no difference in the Ca^{2+}- and phospholipid-dependent phosphorylation of histone H1 catalyzed by the supernatant and the mixture, unless the solubilization of the enzyme was incomplete. In fact, more than 95% of the enzyme was solubilized from microsomes by 1.2% octylglycoside, the detergent concentration yielding the highest kinase activity in membrane-detergent mixtures.

The incorporation of phosphate into histone H1 is catalyzed by at least two enzymes present in membrane fractions. The catalytic subunit of cyclic AMP-dependent protein kinase, presumably present on the luminal surface of microsomes [13] and the external surface of plasma membranes [14], will phosphorylate histone H1 in a Ca^{2+}- and phospholipid-independent manner. The phosphorylation observed without addition of these substances is approximately that expected from the amount of catalytic subunit present in the membrane fractions. The Ca^{2+}-dependent phosphorylation of histone H1 is most likely catalyzed by Ca protein kinase, although the phospholipid-dependency is masked by endogenous phospholipids. It seems less likely that this reaction should be due to other Ca^{2+}-dependent protein kinases, since a Ca^{2+}- and phospholipid-dependent enzyme (corresponding to peak MII in Ref. 13) can be recovered after chromatography of microsomal extracts on DEAE-cellulose. The membrane-bound enzyme also has the same catalytic properties as partially purified Ca protein kinase from rat liver cytosol (Sommarin, M. and Jergil, B., unpublished data).

The endogenous components phosphorylated in membranes include protein [15,16] as well as polyphosphoinositides that are only partly removed in the analytical procedure used. Since the endogenous incorporation of phosphate was constant in incubations with and without Ca^{2+} and phosphatidylserine (this applies to all membrane preparations examined), the endogenous phosphorylation can be omitted from the calculations of Ca protein kinase activity. Thus, the enzyme activity is the difference between the total incorporation of phosphate obtained with added Ca^{2+} and (optional) phosphatidylserine and that obtained without these additions but with EGTA. The presence of 100 μM EGTA together with Ca^{2+} does not affect the Ca^{2+}-dependent phosphorylation under standard conditions. The endogenous phosphorylation has been included in all enzyme determinations presented in this paper, however, in order to check its extensiveness under various incubation conditions.

One problem concerning the accuracy of the determination of membrane-bound Ca protein kinase is the rather high activity obtained without addition of Ca^{2+} and phosphatidylserine. This amounts to approximately 25% in microsomal fractions prepared in the presence of chelators and the N_2-fraction, and to around 50% in microsomes prepared without chelators and the P_2-fraction. We have obtained good correlation, however, between repetitive analyses of various membrane preparations. The Ca^{2+}-dependent increases in membrane kinase activities found here were also substantially larger than those observed earlier [4] in corresponding guinea pig heart membranes after extraction with Triton X-100. Although we obtained similar Ca^{2+}-dependent protein kinase activities in microsomes after treatment with octylglycoside and Triton X-100, the Ca^{2+}-independent incorporation of phosphate was more than two times higher with Triton X-100.

As to the distribution of Ca protein kinase in liver cells approx. two thirds of the total activity is

found in the microsomal fraction and around one third in the cytosol, assuming a recovery of 15 and 65 mg of protein per g tissue in the two fractions, respectively. Only a few percent of the activity resides in plasma membranes, although this fraction shows the highest specific activity of the enzyme. The total incorporation of phosphate catalyzed by the enzyme is approx. 7.5 nmol incorporated/min per g liver tissue. This is nearly 4-times more than was reported previously [2], but corresponds rather well with the very recent results by Kikkawa et al. [17]. These latter authors found, however, that only 20% of the liver Ca protein kinase resided in the particulate fraction.

One question regards the possibility that Ca protein kinase becomes bound to membranes after homogenization of the tissue. We have found in binding experiments of partially purified cytosolic Ca protein kinase and microsomes that the enzyme will only bind to the membranes if Ca^{2+} is also added. This, together with the finding that the activity in smooth and unfractionated microsomes is slightly higher after homogenization in the presence rather than absence of chelators, suggests that the enzyme largely exists in a bound state in the intact liver cell. However, so far it is not known whether any specific factor(s) determines the subcellular distribution of Ca protein kinase. It should be possible to examine this question further using the described procedure for determination of Ca protein kinase bound to membranes.

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