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PROTEIN KINASE IN THE SEA URCHIN EGG CORTICES

ITS PURIFICATION AND CHARACTERIZATION AND SOME PROPERTIES OF AN ENDOGENOUS PROTEIN KINASE IN THE CORTICES

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SUMMARY

A protein kinase was purified from the cortices of sea urchin eggs. It preferred casein as a substrate protein and the activity was independent of adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate. This enzyme was strongly inhibited by *p*-chloromercuriphenyl sulfonate and the activity was recovered with further incubation with dithiothreitol. The estimated molecular weight of the enzyme was about $5 \cdot 10^4$.

The isolated cortices showed a high endogenous protein kinase activity. The endogenous activity was also cyclic nucleotide independent and showed a periodic change along with the cell cycle.

INTRODUCTION

It has been considered that the cortical layer of the egg cell plays a significant role in cytokinesis. Microsurgical studies by Hiramoto [1, 2] have actually confirmed that the motive force of cleavage of the sea urchin egg is derived from the cortical layer at the equatorial plane. Since a large scale preparation technique of sea urchin egg cortices was established by Sakai [3], many investigations have been performed to clarify the mechanism of cleavage of the cell at the molecular level [4–12].

In the course of the investigation of protein kinases (EC 2.7.1.37) in the sea urchin eggs, the author found that more than one-third of protein kinase activity in the total homogenate of eggs was localized in the cortices when casein was used as a substrate protein and that an endogenous protein kinase activity was high in this fraction.

In the present paper, the purification and characterization of a protein kinase in the cortices of sea urchin eggs and some characteristics of the endogenous protein kinase–protein substrate system are described.

MATERIALS AND METHODS

Isolation of egg cortices. Eggs of the sea urchin, *Hemicentrotus pulcherrimus*

were used as the biological material. After the fertilization, membranes were removed with 1 M urea treatment and the fertilized eggs were allowed to develop at 20 °C in artificial sea water. The cortices were isolated from the eggs at metaphase before the first cleavage according to the procedure of Mabuchi and Sakai [9] with the following modification: instead of 0.1 M $MgCl_2$ solution, eggs were washed successively with 50 and 25 mM $MgCl_2$ and were homogenized in the latter solution. The cortex fraction was confirmed to be devoid of nuclear contamination since no DNA was detected in this fraction.

Solubilization of protein kinase. Isolated cortices were treated with 1 mM EDTA containing 10 mM Tris–HCl (pH 7.2) and 1 mM GSH [9] to remove water-soluble materials which contaminated the cortex fraction owing to the high concentration of $MgCl_2$ in the procedure of the cortex preparation. The "EDTA-treated cortex fraction" was further extracted with 0.6 M KCl containing 10 mM Tris–HCl (pH 8.4) [9] to solubilize the protein kinase.

Protein kinase assay. The assay method was essentially the same as that previously reported [13]. A standard assay mixture, unless otherwise indicated, contained 5 μmoles of potassium phosphate buffer (pH 7.5), 1 μmole of $MgCl_2$, 0.1 μmole of dithiothreitol, 0.2 mg of casein, 0.01 μmole of [γ-^{32}P]ATP ($2 \cdot 10^5$–$2 \cdot 10^6$ cpm) and the enzyme fraction in a final volume of 0.1 ml. Incubation was performed at 25 °C for 10 min. The reaction was linear within this period.

Endogenous phosphorylation assay. The endogenous phosphorylation in the cortices was determined with the standard assay mixture free of casein. The reaction was allowed to proceed at 25 °C for 2 min. The rate of ^{32}P incorporation was constant for this period and proportional to the amount of cortices added (0.02–0.5 mg of protein per assay).

Protein phosphatase assay. Protein phosphatase activity in the cortices was measured by the following procedure. After the cortices were labeled for 2 min by the endogenous protein kinase assay system in a volume of 1 ml, the incubation mixture was quickly chilled in an ice-water bath along with the addition of 2 vol. of ice-cold 1 mM unlabeled ATP solution. The cortices were precipitated by centrifugation and washed three times with cold distilled water. During this period no detectable loss of protein-bound radioactivity was observed. The washed cortices were suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 mM unlabeled ATP and incubated at 25 °C. At every 2-min interval, an aliquot was removed to determine the decrease in the acid-precipitable radioactivity.

ATPase assay. ATPase activity in the cortices was determined using the same incubation mixture as that for the endogenous protein kinase assay. After incubation at 25 °C, the reaction was terminated by the addition of 1 ml of ice-cold 5% $HClO_4$, followed by the addition of 0.5 ml of 10% Norit A suspension to adsorb the remaining [^{32}P]ATP. After the Norit was precipitated by centrifugation, the radioactivity of the supernatant was measured.

Other methods. The procedure for sodium dodecylsulfate polyacrylamide gel electrophoresis and the molecular weight estimation with Sephadex G-200 gel filtration were described previously [13].

The amount of protein was determined by the method of Lowry et al. [14] using bovine serum albumin as a standard.

All the chemicals and proteins were the same as those used in the previous paper [13]. Cyclic GMP was purchased from Boehringer. *p*-Chloromercuriphenyl sulfonate and RNAase A were from Sigma and RNAase-free DNAase from Worthington.

RESULTS

Enzyme purification

Enzyme solubilization. From about 280 ml of packed eggs, 210 ml of cortex fraction was obtained. Although about 30% of the total protein in the cortex fraction was extracted with the 1 mM EDTA treatment, almost all of the protein kinase activity remained in the precipitates ("EDTA-treated cortex fraction"). The extraction of the EDTA-treated cortex fraction with 0.6 M KCl caused almost all of the protein kinase to be in a soluble form leaving less than 10% of the total kinase activity in the pellet. The protein kinase activity in the KCl extract was most prominent when casein was used as the substrate protein. It was used as the substrate throughout the purification steps of the enzyme.

$(NH_4)_2SO_4$ fractionation. To the KCl extract solid $(NH_4)_2SO_4$ (0.31 g/ml) was added. The precipitates formed were collected by centrifugation, dissolved and dialyzed against 0.6 M KCl containing 5 mM potassium phosphate (pH 6.7) to yield the $(NH_4)_2SO_4$ fraction.

Hydroxyapatite batchwise fractionation. Hydroxyapatite previously washed with 0.6 M KCl–5 mM potassium phosphate buffer (pH 6.7) was added to the $(NH_4)_2SO_4$ fraction (about 0.1 ml of packed hydroxyapatite per mg of protein). After the gel was stirred for 30 min, hydroxyapatite was precipitated by low speed centrifugation. The pellet was washed twice with an equal volume of 0.6 M KCl–5 mM phosphate buffer (pH 6.7). All the supernatant was combined. All of the protein kinase activity was detected in this fraction and no further activity was eluted from the hydroxyapatite gel with an increasing concentration of phosphate buffer up to 0.5 M (containing 0.6 M KCl). The enzyme fraction was dialyzed against 0.1 M potassium phosphate buffer (pH 7.4).

Hydroxyapatite column chromatography. When the hydroxyapatite batch fraction was chromatographed on a hydroxyapatite column, three peaks of protein kinase activity were detected (Fig. 1). They were called Protein kinase A, B and C. Further purification of the major component, Kinase B, was performed. The peak fractions (Tubes 29–40) were combined, concentrated through ultrafiltration (Diaflo, Amicon, membrane UM-20) and dialyzed against 0.6 M KCl containing 10 mM potassium phosphate buffer (pH 7.1). The fraction was called the "hydroxyapatite column fraction".

Sephadex G-200 gel filtration. For further purification, the hydroxyapatite column fraction was gel filtered through a Sephadex G-200 column. As was shown in Fig. 2, the protein kinase activity was eluted in a single peak. The peak fractions (Tubes 49–60) were combined, concentrated and dialyzed against 50% (v/v) glycerol containing 0.6 M KCl and 10 mM potassium phosphate buffer (pH 7.1). To the concentrated enzyme fraction, dithiothreitol was added to a final concentration of 5 mM and stored at −20 °C. This fraction was called the "Sephadex G-200 fraction". It was completely free from ATPase activity and was used for the characterization of Kinase B. The purification steps were summarized in Table I.

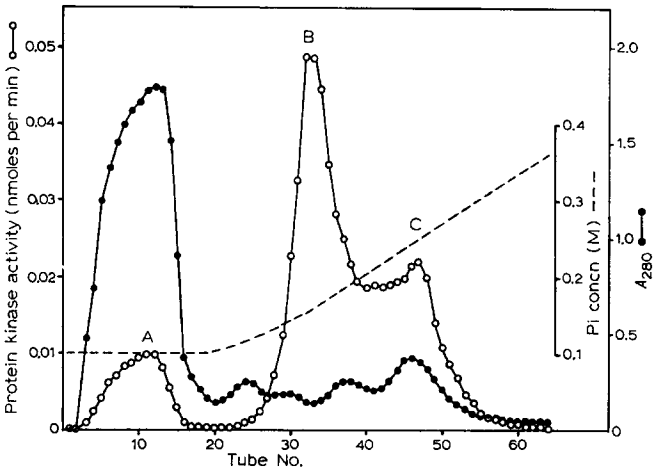

Fig. 1. Hydroxyapatite column chromatography of the hydroxyapatite batch fraction. The hydroxyapatite batch fraction (130 ml) was applied to a hydroxyapatite column (1.6 cm × 10.5 cm) equilibrated with 0.1 M potassium phosphate (pH 7.4). After the column was washed with about 40 ml of the same buffer, the adsorbed materials were eluted with a linear gradient of phosphate buffer (pH 7.4) (from 0.1 to 0.5 M, total 400 ml), collecting fractions of 10 ml. An aliquot of 50 μl from each fraction was assayed using the standard assay mixture.

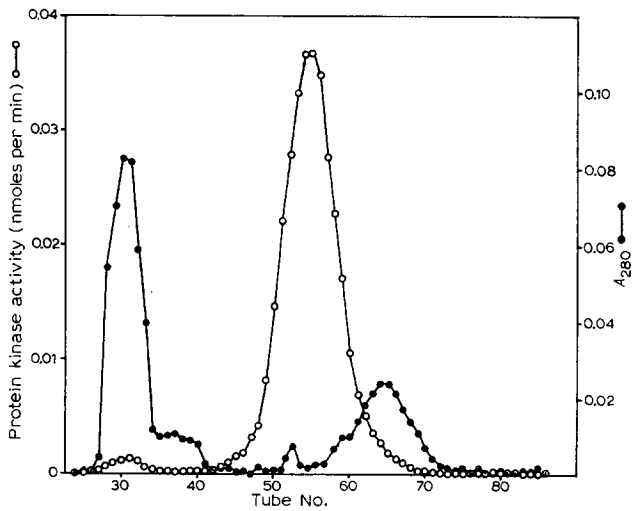

Fig. 2. Gel filtration of the hydroxyapatite column fraction on the Sephadex G-200 column. The hydroxyapatite column fraction (3.7 ml) was loaded onto a Sephadex G-200 column (2.1 cm × 58 cm) equilibrated with 10 mM potassium phosphate (pH 7.1) containing 0.6 M KCl and eluted with the same buffer solution, collecting fractions of 2.5 ml. The enzyme activity was assayed with an aliquot of 50 μl from each fraction using the standard assay mixture.

General properties of Protein kinase B

The pH optimum of the enzyme was 7.5 and a second peak was detected at around pH 10 (Fig. 3).

TABLE I

Purification steps of protein kinase from the cortices of sea urchin eggs. The enzyme activity was determined with the standard assay system. The stars indicate the kinase activity measured in the presence of 0.3 M KCl added to the standard incubation mixture (in the absence of the salts, precipitation of the enzyme occurred in those steps). The values in parentheses show the endogenous protein kinase activity.

	Protein (mg)	Specific activity (nmoles/mg of protein per min)	Total activity (nmoles/min)
Cortex fraction	1043	0.858 (0.520)	895
EDTA-treated cortex fraction	739	1.138 (0.629)	841
KCl extract	469	*1.355	*635
$(NH_4)_2SO_4$ fraction	328	*1.694	*555
Hydroxyapatite batch fraction	39.4	10.53	415
Hydroxyapatite column fraction	2.13	51.5	109.7
Sephadex G-200 fraction	0.43	104	45.1

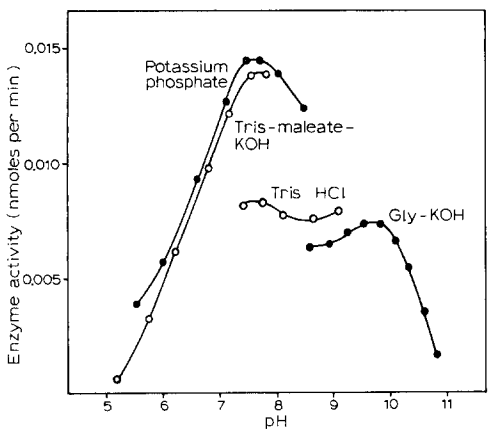

Fig. 3. Effects of pH on the protein kinase activity. The reaction rate was determined with the standard incubation mixture except for the buffers indicated. The concentration of each buffer was 50 mM. The Sephadex G-200 fraction (0.12 μg of protein per assay) was used as an enzyme source.

The apparent K_m for ATP was 14 μM (Fig. 4B). The divalent cation requirement was illustrated in Fig. 5. Among the cations tested, Mg^{2+} was the most effective revealing the optimal concentration at 10 mM. Mn^{2+} and Co^{2+} were less effective with their optima at 0.1 and 0.5 mM, respectively.

Among the substrate proteins tested, only casein could be phosphorylated with an apparent K_m value of 0.5 mg per ml. Calf thymus whole histone and herring protamine (0.5–8 mg/ml) were much poorer substrates, showing activities less than 2% of that of casein. Ovalbumin, bovine serum albumin and γ-globulin were quite ineffective.

10^{-9}–10^{-4} M cyclic AMP or cyclic GMP showed no activation on the phos-

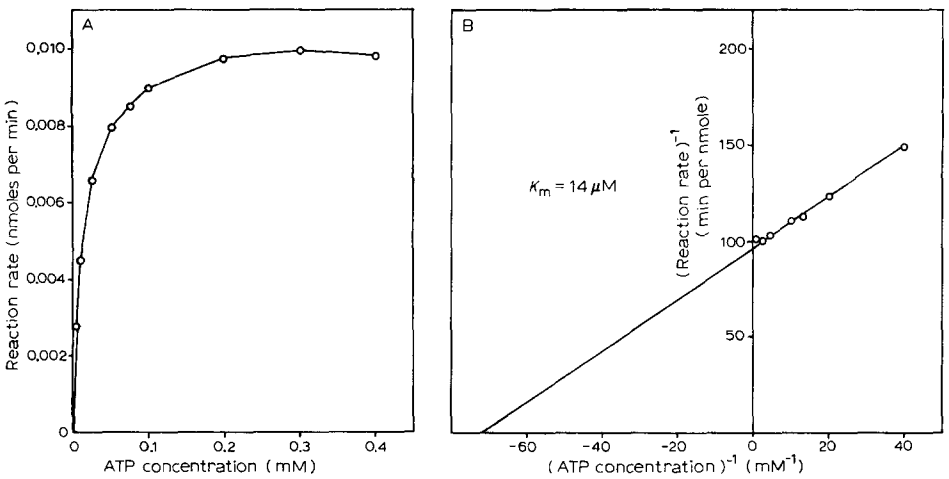

Fig. 4. The effects of ATP concentration on the reaction rate. Protein kinase activity was measured with the standard assay system except for varying concentration of ATP in the reaction mixture. The Lineweaver–Burk plot was shown in B.

phorylation of casein. On the contrary, even a slight inhibition was observed by these nucleotides at higher concentrations (10^{-6}–10^{-4} M).

The enzyme activity was suppressed when an increasing KCl or NaCl concentration was added to the incubation mixture. About half of the enzyme activity was suppressed by 0.25 M KCl or NaCl. The effects of both salts were quite similar.

Ca^{2+} inhibited the protein kinase activity when assayed in the presence of 10 mM Mg^{2+}. 2 mM Ca^{2+} lowered the enzyme activity up to a half of the control level. 1 mM ethyleneglycol-bis-(2-aminoethylether)-*N*,*N*′-tetraacetic acid added to the incubation mixture caused a slight activation of the enzyme activity (Tris–HCl buffer (pH 7.5) was used instead of phosphate buffer in these experiments).

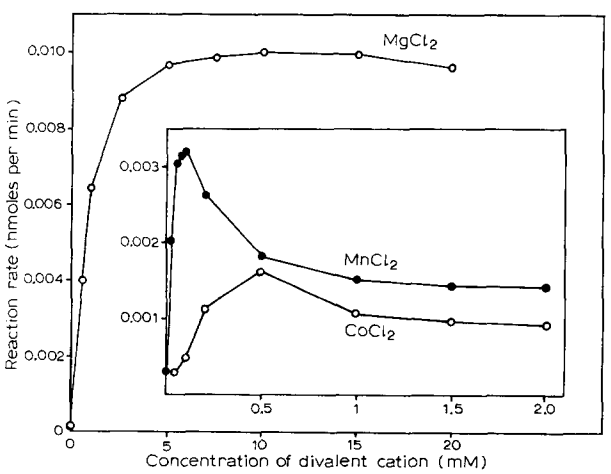

Fig. 5. Divalent cation requirement. The reaction rate was determined with the standard assay system except that 10 mM $MgCl_2$ was substituted by the various concentrations of divalent cations indicated. The Sephadex G-200 fraction (0.12 μg of protein per assay) was used as the enzyme fraction.

The enzyme was strongly inactivated by *p*-chloromercuriphenyl sulfonate. The inhibition with *N*-ethylmaleimide was less prominent. The organic mercurial-treated enzyme restored its activity to a value of 73% of the original level through successive incubation with 3 mM dithiothreitol. The effects of –SH reagents were summarized in Table II.

TABLE II

Effects of –SH reagents on the protein kinase activity. The reaction rate was measured using the standard incubation mixture except for the omission of dithiothreitol and the addition of the –SH reagents indicated. The hydroxyapatite column fraction (final 8.3 μg/ml) was preincubated at 0 °C for 20 min with –SH reagents at the concentrations indicated. Then an aliquot of 60 μl was used to measure the enzyme activity. The last column in the table indicates the results of the following experiment; the enzyme fraction was treated with 0.2 mM *p*-chloromercuriphenyl sulfonate at 0 °C for 20 min followed by the addition of dithiothreitol to a final concentration of 3 mM. After incubation at 0 °C for 30 min, an aliquot of 60 μl was used for the assay.

	Concentration (mM)	Enzyme activity (nmole/mg of protein per min)	Percent activity (%)
Control		0.0172	100
Dithiothreitol	2	0.0196	114
p-Chloromercuriphenyl sulfonate	0.1	0.0020	12
	0.2	0.0016	9
	1	0.0010	6
	2	0.0005	3
N-Ethylmaleimide	1	0.0090	58
	2	0.0076	44
	4	0.0053	31
p-Chloromercuriphenyl sulfonate + Dithiothreitol	0.2 + 3	0.0126	73

The molecular weight of Kinase B was estimated with the aid of the Sephadex gel filtration technique. The apparent molecular weight was about $5 \cdot 10^4$ (Fig. 6). When Peak C fractions (Fig. 1, Tubes 43–50) were combined, condensed and gel filtered through a Sephadex G-200 column, two peaks of enzyme activity were observed at the position of the apparent molecular weights of $5 \cdot 10^4$ and $5.6 \cdot 10^4$. The former value was consistent with that of the major protein kinase (Kinase B) and this peak was considered to be due to the contamination of the Protein kinase B in Peak C fractions. It was reasonable to consider that the latter value was attributed to Kinase C. Owing to the difficulty in eliminating the contamination of Protein kinase B from Kinase C fraction, the nature of the latter has not yet been investigated.

Endogenous phosphorylation in the egg cortices

Isolated cortices incorporated ^{32}P from [γ-^{32}P]ATP when incubated without exogenous substrate protein. Using 0.2–0.5 mg of cortical protein per assay, the reaction was linear for 2 min and reached a plateau at 10 min. Labeled cortices were prepared and the nature of the radioactive materials was examined. As was shown in

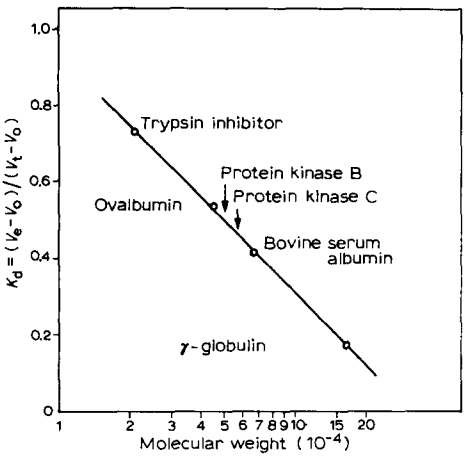

Fig. 6. Molecular weight estimation of the enzyme with Sephadex G-200 gel filtration. Kinase B (hydroxyapatite column fraction, 2 ml) was applied onto a column (2.1 cm × 58 cm) equilibrated with 10 mM potassium phosphate (pH 7.1) containing 0.6 M KCl. The elution was performed with the same buffer collecting 2.5-ml fractions. The details were described previously [13].

Table III, incorporated ^{32}P was not released with hydroxylamine treatment [15–17], RNAase and DNAase treatments and lipid extraction. About 80% of the radioactivity remained in the acid precipitates after hot trichloroacetic acid treatment. In contrast, all of the ^{32}P was released with hot alkali treatment [18]. From these results, the incorporated radioactivity was considered to be of phosphoester nature.

TABLE III

The nature of the bond between the incorporated ^{32}P and the materials in the cortices. Labeled cortices were prepared using the endogenous assay system on a large scale (2 ml). Cortices (5.6 mg of protein) were labeled for 10 min at 25 °C followed by precipitation with 10% trichloroacetic acid containing 1% sodium pyrophosphate. They were successively washed four times with 5% trichloroacetic acid–1% sodium pyrophosphate and twice with distilled water. The precipitates were then homogeneously suspended in distilled water and aliquots of 0.2 ml underwent the indicated treatments, followed by the measurement of the radioactivity remaining in the acid-insoluble materials. Hydroxylamine: 0.8 M, pH 5.3, 10 min, 30 °C. RNAase: 0.1 mg per ml, pH 6.9, 15 min, 37 °C. DNAase: 0.1 mg per ml, pH 6.9, 15 min, 37 °C. Lipid extraction: extraction twice with 95% ethanol, twice with ethanol–chloroform (3:1 v/v), twice with ethanol–ether (3:1, v/v) and once with ether. Hot trichloroacetic acid: final 5%, 15 min, 95 °C. Hot alkali: 1 M NaOH, 5 min, 95 °C.

Treatment	Specific activity in acid precipitates (nmoles per mg of protein)	Percent specific activity (%)
Control	2.39	100
Hydroxylamine	2.34	98
RNAase A	2.37	99
DNAase	2.42	101
Lipid extraction	2.32	97
Hot trichloroacetic acid	1.84	77
Hot alkali	0.0055	0.23

The pH dependence of the ^{32}P incorporation into the cortices was similar to the pH curve of the purified Protein kinase B with the exceptions that the pH optimum in the endogenous phosphorylation was slightly higher than that of the purified enzyme and that the second peak at around pH 10 was more prominent in the former case (Fig. 7). These results suggested that Kinase B was responsible for the endogenous phosphorylation. In addition, the ^{32}P incorporation was inhibited by Ca^{2+} and was slightly activated by ethyleneglycol-bis-(2-aminoethylether)-*N*,*N*'-tetraacetic acid as was already shown for the purified enzyme.

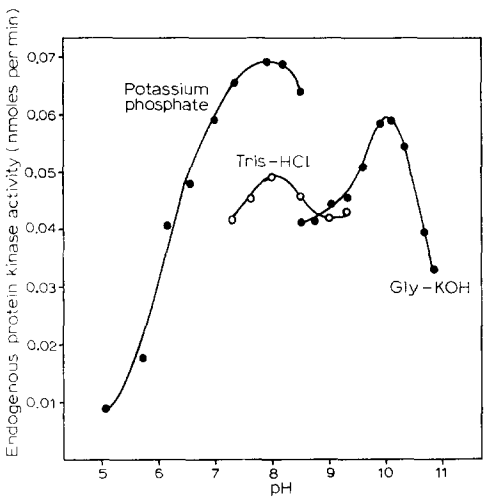

Fig. 7. Effect of pH on the endogenous protein kinase activity in the cortices. The reaction rate was measured with the endogenous protein kinase assay system except for the buffers indicated. The cortex fraction (0.15 mg of protein per assay) was used as the enzyme–substrate system.

The endogenous phosphorylating activity in the cortices was not affected by the addition of 10^{-9}–10^{-4} M cyclic AMP or cyclic GMP, even in the presence of 1 mM theophylline. Therefore, the purified protein kinase was considered to be cyclic nucleotide independent in situ in the cortices.

Labeled proteins in cortices were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (Fig. 8). Many peaks of radioactivity emerged. It appeared that many species of polypeptides could be phosphorylated, but the nature of these labeled materials is quite unclear at present. An experiment of ortho[^{32}P]phosphate incorporation into eggs (10-min pulse at metaphase) revealed that more than one-fourth of the hot trichloroacetic acid-insoluble radioactivity in the whole homogenate was incorporated into the cortex fraction. Therefore, it was confirmed that the phosphorylation of the cortical proteins did occur in the living cells. The analysis of the proteins phosphorylated in vivo has not yet been performed.

Along with the cell cycle, a periodic change in the endogenous phosphorylation was observed in the cortex fraction (Fig. 9). It reached a maximum at around metaphase and a minimal level after cleavage. This periodicity was not due to the difference in the yield of cortices during the cell cycle. After the cortex fractions were extracted

with 0.6 M KCl, the protein kinase activities in the KCl extract were measured using casein as the substrate protein. This experiment revealed very little variation of the protein kinase activity in the KCl extract during the cell cycle. As the KCl extraction solubilized almost all of the protein kinase which had existed in the cortices, it could be considered that the kinase activity in the KCl extract reflected the amount of enzyme which had been bound in the cortices. Therefore, it was reasonable to consider that the periodic change in the endogenous phosphorylating activity was not due to the change in the "amount" of the enzyme in the cortices.

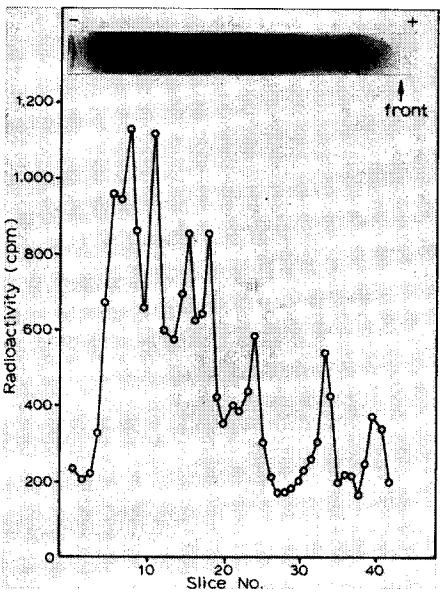

Fig. 8. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of labeled proteins in the cortices. Isolated cortices (2.5 mg of protein) were labeled by endogenous protein kinase using the endogenous assay system on a large scale (1 ml). The incubation was performed at 25 °C for 10 min. About 100 μg of protein was applied per gel. Details were presented in the previous paper [13].

The ATPase activity in the cortices is very high [10, 19]. The activity of the cortical ATPase was maximal at around metaphase and minimal after cleavage of the egg in accordance with the results of Miki [19]. When cortices containing 0.3 mg of protein per assay was used, about one-fourth of [^{32}P]ATP in the reaction mixture was hydrolyzed during the assay of the endogenous phosphorylation (2 min). Judging from the K_m value for ATP of the purified Kinase B (14 μM), such a decrease in ATP concentration was considered to be insignificant for the endogenous phosphorylation assay. The protein phosphatase activity in the cortices was measured to be quite low throughout the cell cycle. The genuine activity could be lower than the apparent values obtained because of the possible presence of proteolytic activity. Therefore, the periodic change in the endogenous activity in the cortices was not due to the fluctuation of ATPase and protein phosphatase activities but was due to the change in the rate of phosphorylation itself.

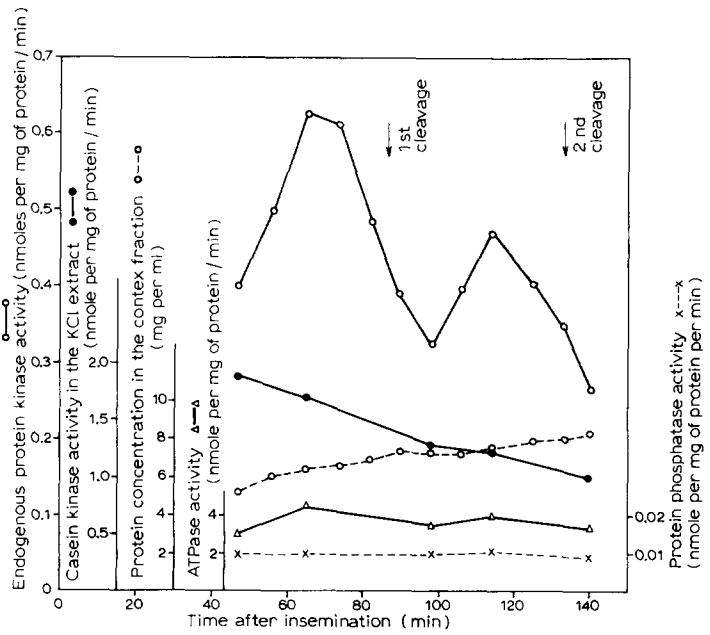

Fig. 9. Periodic change in the endogenous protein kinase activity in the cortices along with the cell cycle. From the suspension of eggs developing at 20 °C, aliquots were taken out every 9 min, from which the cortex fractions were prepared. The endogenous protein kinase activity was measured using the endogenous assay system (0.25–0.4 mg of protein per assay). The time indicated shows the time of the homogenization of the cells. Protein kinase activity in the KCl extracts was measured in the following manner. The cortices (2.0 mg of protein) obtained from eggs at various stages of the cell cycle were extracted with 0.6 M KCl containing 10 mM Tris-HCl (pH 7.2) (final 1.25 ml) overnight at 0 °C. After centrifugation, an aliquot of 12.5 μl from the supernatant was used per assay. The enzyme activity was measured using the standard assay system with casein as the substrate protein.

DISCUSSION

Several characteristics of the endogenous protein kinase–substrate system in the egg cortices were presented in this paper. As was mentioned in Introduction, a relatively high activity of protein kinase was detected in the cortex fraction and the specific activity of the endogenous phosphorylating activity in the cortices was several times higher than in other structural protein kinase–substrate systems such as brain membranes [17, 20, 21], muscle membranes [22] and the ghosts of erythrocytes [18, 23, 24].

As was shown in Fig. 8, many species of cortical protein were phosphorylated in vitro. However, the functions of these proteins and the significance of their phosphorylation are not clear. According to Mabuchi and Sakai [9], the cell membranes were included in the isolated cortices and the KCl extraction caused solubilization of the materials in the cortical layer leaving the cell membranes in the precipitates. Preliminary experiments revealed that about half of the protein-bound radioactivity was extractable from labeled cortices with 0.6 M KCl extraction, with the other half remaining in the pellet. Therefore, it was considered that not only the proteins in the cortical layer but also those of the cell membranes were phosphorylated. The periodic

change of the endogenous protein kinase activity in the cortices might be in relation to the cell division and or the permeability of the cell membrane. It is of interest that the -SH content of the cortex protein [5, 8], the cortical ATPase activity [19] and the endogenous protein kinase activity in the cortices fluctuate periodically in the same manner during the cell cycle. Further investigations are required on the nature of the phosphoproteins in the cortices and the significance of the phosphorylation of these proteins.

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