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PP2A Type Phosphatases in Sea Urchin Eggs

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ABSTRACT—Four peaks of the p-nitrophenyl phosphate (pNPP) splitting activity were obtained by QAE-Toyopearl chromatography in the extract of sea urchin eggs from homogenate in 0.2M KCl solution containing 0.1% Triton X100 and so on. In 2 among these 4 peaks, pNPP splitting reactions were strongly inhibited by 10nM okadaic acid (OA) and calyculin A (CLA), potent and specific inhibitors of protein phosphatase 2A (PP2A). High sensitivities of pNPP splitting reaction to OA and CLA in these 2 peaks suggest that pNPP splitting results from the reaction catalyzed by PP2A, which reaction is expected to play roles in gene expression, signal transduction and cell movement such as cell division. OA sensitive pNPP splitting activities in these 2 peaks were eluted by FPLC gel permeation chromatography (Superdex 200HR) with approximate molecular mass of 160 kDa, corresponding to that of PP2A trimeric holoenzyme in mammalian cells. By immunoblot analyses with anti-human PP2A catalytic subunit antibody, an immunoreactive 36 kDa protein was found by SDS-PAGE in a peak of OA-sensitive pNPP splitting activity, obtained by QAE-Toyopearl chromatography. Sea urchin eggs have at least 2 PP2A like enzymes with similar molecular masses to that of mammalian PP2A and one of them contains human type catalytic subunit.

INTRODUCTION

Serine/threonine protein phosphatases, expected to participate in the process of signal transduction, cell movement such as cell division and gene expression (Kinoshita *et al*., 1990, 1993; Healy *et al*., 1991; Mayer-Jaekel *et al*., 1993; Mumby and Walter,1993), are classified into type 1 (PP1), type 2A (PP2A), type 2B (PP2B) and type 2C (PP2C) in mammalian cells. Among these protein phosphatase species, PP1 and PP2A are known to be strongly inhibited by okadaic acid (OA) (Bialojan and Takai, 1988; Haystead *et al*., 1989), polyether fatty acid isolated from the marine sponges of genous *Halicondria okadai* (Tachibana *et al*., 1981). OA is also known to inhibit PP2A at lower concentrations than those to block PP1 in mammalian cells (Ishihara *et al*., 1989). It has also been found that OA exhibits high affinities to catalytic subunits of PP1 and PP2A, which are structurally alike to each other with 50% identity in their amino acid sequences (Cohen, 1989; Cohen and Cohen, 1989). PP1 and PP2A are also inhibited by calyculin A (CLA), isolated from the marine sponges of genous *Discodermia calyx* (Kato *et al*., 1986), at the same concentrations to each other enzyme. Deltamethrin hardly inhibits PP1 and PP2A but strongly blocked PP2B, calcineurin (Enan and Matsumura ,1992).

In sea urchin eggs, PP2B has been identified as a protein composed of 55 and 17 kDa subunits (Iwasa and Ishiguro, 1986). PP1 type enzyme activity, strongly reduced by OA, is found in the extract from sea urchin spermatozoa (Hosino and Suzuki, 1996). It has been reported that formation of irregular mitotic apparatus (Johnston *et al*., 1994) and abnormal cleavage (Tosuji *et al*., 1992) are induced by OA in unfertilized eggs and developmental events in early development are blocked by OA (Troll *et al*., 1995). These findings suggest that the reactions catalyzed by these protein phosphatases are indispensable for some events in early development. However, protein phosphatases in sea urchin eggs and embryos seem to be not sufficiently characterized at present. In the present study, several trials were made on sea urchin eggs to characterize these protein phosphatases.

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MATERIALS AND METHODS

Preparation of sea urchin eggs and embryos

Eggs and sperm of the sea urchin (*Hemicentrotus pulcherrimus*) were obtained by filling the body caving with 0.5 M KCl. Eggs were collected in artificial sea water (ASW; Jamarin Laboratory, Osaka, Japan) and washed twice in ASW. The washed eggs were collected by centrifuge at 1,000g for 2 min and the pellet was quickly frozen in liquid nitrogen and stored at –80°C. Eggs in an aliquot obtained before freezing were fertilized and incubated in ASW at 18°C. At the late gastrula stage, embryos were collected by centrifuge at 1,000g for 2 min, and the pellet was quickly frozen in liquid nitrogen and stored at –80°C.

Enzyme sources for p-nitrophenyl phosphate (pNPP) phosphatase

All procedures were carried out in the cold. Frozen egg pellet in about 1.5g was homogenated by sonication (40W, 30 sec×6) in 7.5

ml of homogenizing solution containing 200 mM KCl, 50 mM Tris/HCl pH 7.4, 5 mM $MgCl_2$, 1 mg/ml BSA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 0.1% (v/v) Triton-X100. The supernatant obtained by centrifugation of the egg homogenate at 25,000g for 15 min, as well as the homogenate, was used as the enzyme source to estimate the pNPP splitting activity (the activity of pNPP phosphatase). The protein amount and the pNPP splitting activity in the supernatant were more than 90% of those in egg homogenate. The precipitate obtained by above centrifuge was suspended in extraction mixture containing 1.5 M NaCl, 50 mM Tris/HCl pH 7.4, 5 mM, $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A, again homogenized by sonication and centrifuged as above. The precipitate thus obtained hardly exhibited any pNPP splitting activity. Hence, the activity in this extract in extraction mixture is regarded as the activity in the precipitate obtained by centrifugation of egg homogenate in homogenizing solution and shown as the activity in the precipitate. Enzyme source was also obtained from isolated nuclei, homogenized by sonication in the extraction mixture. Nuclei fraction was isolated from 20g of late gastrulae pellet by the method of Albanese *et al.* (1980).

pNPP splitting reaction

The pNPP splitting activity was measured in the reaction mixture (250 μl) containing 40 mM Tris/HCl pH 8.2, 30 mM $MgCl_2$, 1 mg/ml BSA, 1 mM $MnCl_2$, 1 mM DTT, 0.5 mM PMSF, 5 mM pNPP (Sigma Chemical Co., St. Louis, MO, USA), and appropriate amount of the enzyme source. In some experiments, the reaction mixture also contained 1mM Ca^{2+} and 40 μg/ml calmodulin (Calbiochem). The reaction for 30 min at 18°C was initiated by addition of pNPP, and terminated by 250 μl of 0.5 M NaOH addition. The release of p-nitrophenol (pNP) is determined spectrophotometrically by the absorbance at 410 nm. The pNPP splitting activity is expressed as nmol pNP produced/mg protein/30 min. Protein concentration was determined by the method of Bradford (1976) using BSA as a standard. The activity was also estimated in the presence of protein phosphatase inhibitors, OA (okadaic acid; Calbiochem, San Diego, CA, USA), CLA (calyculin A; Sigma), Microcystin-LR (Calbiochem) and deltamethrin (ICN, Costa Mesa, CA, USA). Solutions of these inhibitors in DMSO (dimethyl sulfoxide) were diluted in ethanol and used as the stock solutions. Even when the estimation of pNPP splitting activity was carried out in the presence of these compounds at the highest examined concentrations, the concentration of ethanol was less than 4%(v/v), at which pNPP splitting reaction was not affected by ethanol. All assays were carried out in duplicate or triplicate.

Chromatographies

All procedures were performed in the cold except for Superdex 200HR column chromatography, which was carried out at the room temperature. The supernatant fraction was diluted to make KCl 0.05 M by addition of buffer A (10 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.5 mM PMSF) and applied to a 1.5×13 cm column of QAE-Toyopearl (Tosoh Corp., Tokyo, Japan) equilibrated in buffer A. The column was washed with 5-column volume of buffer A and developed with a 8-column volume linear concentration gradient of NaCl from zero to 0.5 M in buffer A. Fractions of 3.5 ml each were collected and assayed for pNPP splitting activity. The fractions which had OA-sensitive pNPP splitting activity were assembled in their respective peaks and solid ammonium sulfate was added to make the final concentration 1.2 M. The solutions were applied to a Prepacked Macroprep HIC methyl column (Bio-Rad, Hercules, CA, USA) equilibrated in buffer A containing 1.2M ammonium sulfate, respectively. The column was washed with 5-column volume of buffer A containing 1.2 M ammonium sulfate and eluted with buffer A. Fractions of 2.0 ml each were collected and analyzed for pNPP splitting activity. The fractions which had OA-sensitive pNPP splitting activity were pooled and 378g of solid ammonium sulfate was added to each liter of solution to make the degree of saturation 60%. The solution was then adjusted to pH 7.5 with 1.0 M Tris. After standing for 60min at 0°C, the suspension was centrifuged for 15 min at 25,000g at 4°C. The pellets were resuspended in buffer B (150 mM NaCl, 10 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF) in a volume as small as possible and centrifuged for 15 min at 25,000g at 4°C. The supernatant was applied to a Superdex 200HR column (Amersham Pharmacia Biotech.,Uppsala, Sweden) equilibrated in buffer B. The column was eluted with Buffer B at room temperature and the eluent was fractionated by 0.5 ml and assayed for pNPP splitting activity.

SDS-PAGE and immunoblot analysis

Aliquots of the fractions, which exhibited the OA-sensitive pNPP splitting activity, were concentrated by Macroprep HIC methyl column, were analyzed by SDS-PAGE using 12.5% polyacrylamide gel (Laemmli, 1970). Electrophoresed proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane by semi-dry electrophoreictransfer (30 min, 2 mA/cm^2) and blocked in blocking solution (Kirkegaard & Perry Labo.,Gaithersburg,MD). The blots were incubated with primary anti-human PP2A catalytic subunit antibodies (goat IgG, polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1/200 dilution in blocking solution for 1hr. Antibody binding was detected by further incubation with biotinylated secondary antibodies (mouse anti-goat IgG, ICN). The blot was incubated with Streptoavidine-Gold conjugated (British Biocell,UK) to detect the secondary antibody, and visualized by Silver enhancing Kit (British Biocell, UK).

Immunoprecipitation

The all procedures were performed at 4°C. Frozen eggs pellet in about 1g were homogenated by sonication (40W, 30 sec×6) in 5vol of buffer C (200 mM KCl, 50 mM Tris/HCl pH 7.4, 10% glycerol, 5 mM $MgCl_2$, 1 mg/ml BSA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin and 5 μg/ml pepstatin A) containing 0.1% (v/v) Triton-X100, 10 mM β-glycerophosphate and 5 mM sodium pyrophosphate, and centrifuged at 12,000g for 30 min. Anti human PP2A catalytic subunit antibodies (5 μg) were added to the egg extract (1 ml) and incubated for 24 hr. Protein G plus agarose (Calbiochem) suspension in 60 μl was added to this egg extract-antibody mixture and incubated for another 24hr. Immunoprecipitates were collected by centrifugation and were washed three times with buffer C containing 0.5% (v/v) Triton-X100 and once with buffer C containing 0.1% (v/v) Triton-X100. In the suspension (150 μl) in buffer C, the immunoprecipitates were analyzed for pNPP splitting activity.

RESULTS

As shown in Table 1, the supernatant obtained by centrifugation at 25,000g for 15 min from the homogenate of unfertilized sea urchin eggs in 0.2 M KCl containing Triton X-100 and so on, contained markedly larger amount of protein than the precipitate. The specific activity of pNPP splitting in the precipitate was alike to the specific activity in the supernatant. OA evidently inhibited pNPP splitting in the supernatant and the precipitate, as well as egg homogenate (Table 1). It is demonstrated that OA inhibits pNPP splitting catalyzed by OA sensitive protein phosphatases but does not block the splitting by other phosphatase species, such as alkaline phosphatase (Ishihara *et al.*, 1989). Certainly, the supernatant and the precipitate, as well as egg homogenate, contain OA-sensitive protein phosphatases.

CLA and microcystin-LR, potent inhibitors of protein phosphatases such as PP1 and PP2A in mammalian cells (Ishihara

Table 1. pNPP splitting activity in unfertilized eggs of sea urchin.

	Protein (%)	pNPP splitting activity (nmol pNP produced/mg protein/30min) None	+10 nM Okadaic acid (% Inhibition)
Homogenate	100.0	96.7± 4.9	51.0± 2.9 (47.3±0.9)
Supernatant	91.4±21.8	100.5± 3.7	49.7± 2.6 (50.5±0.8)
Precipitate (re-extract)	1.3± 0.3	105.2±11.1	83.1±11.1 (23.6±3.2)

Eggs of sea urchin were homogenated in 0.2 M KCl containing Triton X-100 and regents shown in MATERIALS AND METHODS. Supernatant was obtained by centrifuge at 25,000g for 15 min. Precipitate obtained by above centrifuge was extracted in extraction medium (shown in Materials and Methods) and protein amount and pNPP splitting activity were estimated. Protein amount in homogenate were 14.5±0.5 mg/ml in 4 experiments. The activity of pNPP splitting is expressed as nmol pNP (p-nitrophenol) produced/mg protein/30 min, estimated in the presence and absence of 10 nM OA. In parenthesis, values for % inhibition of pNPP splitting by OA are shown and are close to the values for the maximum inhibition in homogenate and supernatant. Each value is mean±SE (Standard error) for 3 experiments.

et al., 1989; Honkanen *et al.*, 1990), inhibited pNPP splitting in the supernatant and the precipitate obtained from homogenate of unfertilized eggs (Table 2). OA-caused inhibition of pNPP splitting at 10 nM in the supernatant and egg homogenate was evidently stronger than in the precipitate as shown in Table 2, as well as Table 1, whereas the inhibition by CLA and microcystin-LR at 10nM in the precipitate was as strong as in the supernatant (Table 2). Inhibition of pNPP splitting by OA at 10 nM was only slightly lower, if any, than at 100 nM and was markedly stronger than at 1 nM in the supernatant and the precipitate in almost the same manner as CLA and microcystin-LR (data not shown). Probably, maximum inhibition of pNPP splitting by OA as well as CLA occurs at 10 nM in the supernatant.

In the precipitate, pNPP splitting is less sensitive to OA than CLA even at the concentrations for these compounds to cause the maximum inhibition in the supernatant (Table 2). It has been demonstrated that OA-sensitivity of PP1 is markedly lower than that of PP2A (Ishihara *et al.*, 1989). It seems probable that pNPP splitting catalyzed by PP2A is predominant in the supernatant obtained from the homogenate of sea urchin eggs. In the precipitate, pNPP splitting catalyzed by PP1 is probably made apparent by elimination of the supernatant exhibiting high PP2A activity. In the present study, therefore, characterization of pNPP splitting reaction with high OA sensitivity was made mainly on the supernatant obtained from egg homogenate. On the other hand, deltamethrin, inhibiting PP2B, calcineurin, hardly exerted any effect on pNPP splitting in the precipitate and the supernatant, as well as in egg homogenate (Table 2). Even in the presence of 1 mM Ca^{2+} and 40 μg/ml calmodulin, the pNPP splitting activity was not enhanced in these enzyme sources and was not inhibited by deltamethrin, though OA, CLA and microcystin-LR inhibited pNPP splitting in the same manner as in their absence. The values obtained in the presence of Ca^{2+}/calmodulin were completely the same to those in their absence (Table 2) and hence, those values were not shown in the present paper. The activity of PP2B, to be enhanced by Ca^{2+}/calmodulin, was not detectable by pNPP splitting in the present study.

Further studies were made on the supernatant obtained from egg homogenate. The supernatant from egg homogenate is referred to, hereafter, as the extract of eggs. Fig. 1 shows representative elution profile of pNPP splitting activity in the extract of sea urchin eggs obtained by QAE-Toyopearl column chromatography. Considerable pNPP splitting activity, which was not affected by OA, CLA and deltamethrin, was found to pass through the column on application of the extract onto the column. As shown Fig. 1, 4 peaks of pNPP splitting activity, specified to as peak I, II, III and IV, were found on the chromatogram. In the peaks I and IV, pNPP splitting was hardly blocked by OA, CLA and microcystin LR as well as deltamethrin (data not shown). As shown in Fig. 2, OA concentration-dependently inhibited the pNPP splitting in peaks II and III with IC_{50} value of 0.1–1 nM. The effect of OA on pNPP splitting in the fractions containing peak II and III was as strong as the effect on PP2A in mammalian cells (Ishihara *et al.*, 1989). Provided that pNPP splitting with high OA sensitivity is due to the reaction catalyzed by PP2A, sea urchin eggs are assumed to have 2 species of PP2A isozymes. On the other hand, it was found that pNPP splitting activity in the extract obtained from nuclei isolated from embryos at the gastrula stage was not evidently inhibited by OA, unless OA concentration was made higher than 10 nM, at which pNPP

Table 2. Effects of protein phosphatase inhibitors on pNPP splitting in sea urchin homogenate and 25,000g centrifuged fractions.

	% Inhibition of pNPP splitting activity 10 nM OA	10 nM CLA	10 nM Microcystin-LR	40 nM Deltamethrin
Homogenate	47.3±0.9	44.1± 9.9	44.7±7.1	–2.5± 4.1
Supernatant	50.5±0.8	48.6± 4.5	51.9±1.1	0.3± 2.2
Precipitate (re-extract)	23.6±3.2	39.6±12.4	39.9±8.3	–0.9±24.9

Enzyme sources are those shown in Table 1. Values for percent inhibition of pNPP splitting by the inhibitors of protein phosphatases are shown in Table 2. Activities of pNPP splitting in homogenate, supernatant and precipitate in the absence of inhibitors are those shown in Table 1. Values shown are means±SE for 3 experiments.

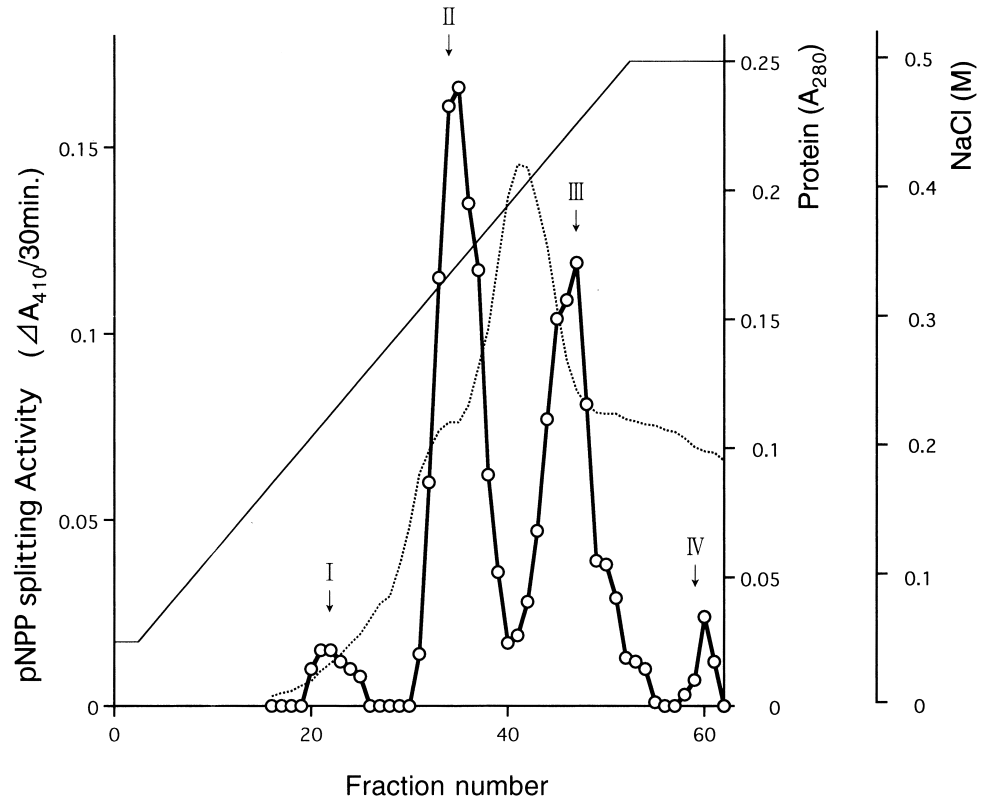

Fig. 1. Elution profile of pNPP splitting activity by QAE-Toyopearl column chromatography in extract of sea urchin eggs. All procedures are shown in Materials and Methods. Activity of pNPP splitting, expressed as ΔA_{410}/30 min are shown with open circle (○) connected with solid lines. Peaks of pNPP splitting activity are specified with symbols I, II, III and IV. Changes in absorbance at 280nm, corresponding to amount of protein, are shown with dotted line. Thin line shows change in NaCl concentration (M). Elution pattern shown in figure is typical among 8 experiments.

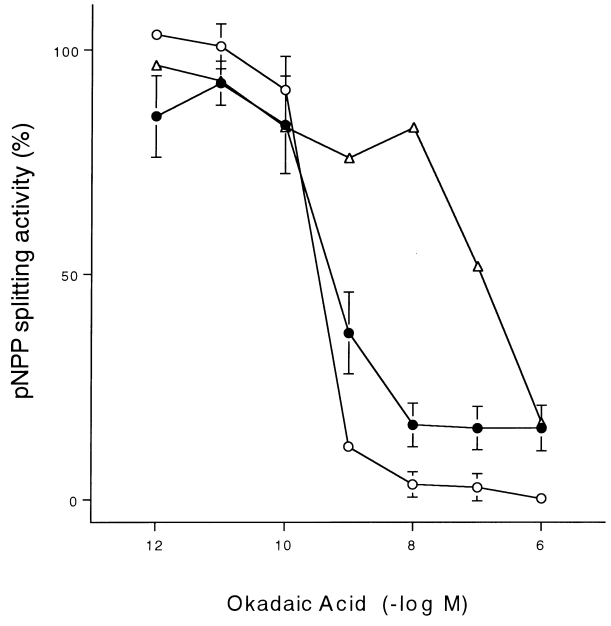

Fig. 2. Effect of okadaic acid on pNPP splitting in peak II and III obtained by QAE-Toyopearl chromatography. Values shown are % of pNPP splitting activity estimated in the presence of okadaic acid in peak II (○) and peak III (●), as well as in extract from nuclei (△) isolated from late gastrulae (24 hr at 18°C). All values with vertical bars are means±SE (vertical bar) for 3 experiments. Values for nucleus extract are means for 2 experiments.

splitting in the extract from eggs became close to the minimum. Inhibitory effect of OA on this activity in gastrula nuclei was lower than OA effect on the activity in peaks II and III (Fig. 2). OA-sensitivity of pNPP splitting in gastrula nuclei was similar to PP1 type activity in sea urchin sperm (Hosino and Suzuki, 1996). Markedly high sensitivity to OA in pNPP splitting in peaks II and III suggests that these pNPP splitting is mediated by PP2A type enzymes. In these two peaks exhibiting OA-sensitive pNPP splitting, and in other peaks, pNPP splitting was not inhibited by deltamethrin an inhibitor of PP2B (data not shown).

Superdex 200HR column chromatography of the peak II, obtained by QAE-Toyopearl chromatography showed a major peak of OA-sensitive pNPP splitting activity at about 160 kDa in the molecular mass and a small peak at about 60 kDa (Fig. 3). Major peak of OA-sensitive pNPP splitting was also found at about 160 kDa and quite small peaks at lower than 35 kDa were found on Superdex 200HR chromatography of peak III obtained by QAE-Toyopearl chromatography, as shown in Fig. 3. On the basis of the molecular mass in the major peaks on Superdex 200HR column chromatography, the molecular mass of proteins catalyzing pNPP splitting in peak II obtained by QAE-Toyopearl chromatography is about 160 kDa and is practically the same as in peak III. The molecular masses of proteins catalyzing pNPP splitting in peak

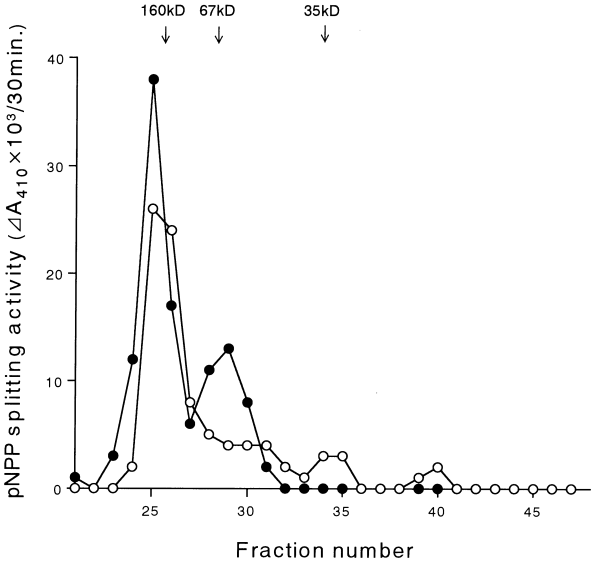

Fig. 3. Elution profiles of pNPP splitting activity on Superdex 200HR column chromatography. Figures show elution profiles obtained by Superdex 200HR chromatography of peak II (●) and peak III (○), obtained by QAE-Toyopearl chromatography (Fig.1). Numbers with arrows show molecular masses of standard proteins, IgG (160 kDa) , BSA (67 kDa) and β-lactogloblin (35 kDa).

II and III are alike to the molecular mass of trimeric PP2A holoenzyme, reported in mammalian cells (Kamibayashi *et al.*, 1992), and is somewhat different from the masses of many PP1 holoenzymes as well as PP2B, reported in mammalian cells (Cohen, 1989).

The activity of pNPP splitting was found in immunoprecipitate obtained from egg homogenate by anti-human PP2A catalytic subunit antibody (Fig. 4). Probably, pNPP splitting in egg extract is mediated by PP2A. As shown in Fig. 5, immunoblot analysis by anti-subunit antibody, made on SDS-PAGE of proteins in the peaks II (lane 1) and III (lane 2), indicates that peak III contains a protein reacting with this antibody. The molecular mass of catalytic subunit in human PP2A is known to be 36 kDa (Van Eynde *et al.*, 1995). The protein to react with this antibody in peak III was about 36 kD in its molecular mass (Fig. 5). These observations indicate that pNPP splitting in peak III is due to the reaction catalyzed by PP2A composed of catalytic subunit, reacting with this antibody. On the other hand, proteins in peak II exhibiting pNPP splitting activity with the same OA-sensitivity as in peak III did not show reactivity with anti-human PP2A catalytic subunit antibody. At present, we assumed that the enzymes in peak II is also PP2A having other type of PP2A catalytic subunit. The enzyme in peak II is now under investigation.

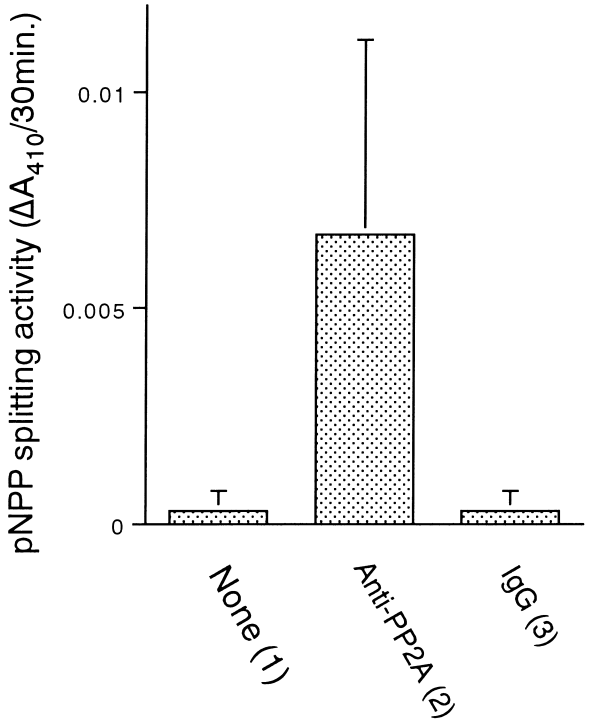

Fig. 4. Activity of pNPP splitting in immunoprecipitate obtained by anti-human PP2A catalytic subunit antibody. Extract of unfertilized egg was incubated with **(1)** none; **(2)** anti-human PP2A catalytic subunit antibody; and **(3)** goat IgG in the presence of Protein G plus-agarose beads. Immunoprecipitate obtained by centrifugation was analyzed for pNPP splitting. Values of pNPP splitting activity (ΔA_{410}) are presented as difference between pNPP splitting activity in the presence and absence of 10 nM OA. All values are expressed as mean±SE (vertical bar) for 3 experiments.

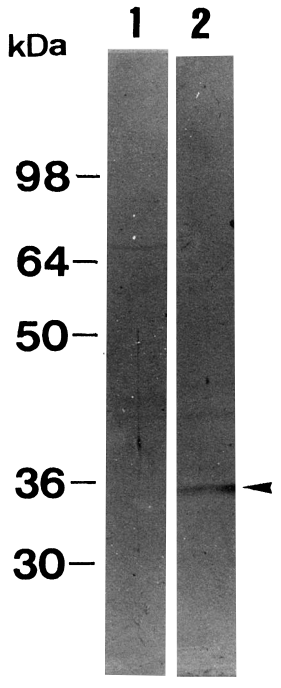

Fig. 5. Immunoblotting analysis with anti-human PP2A catalytic subunit antibody. Immunoblotting analysis was made on SDS-PAGE of peak II (lane 1) and peak III (lane 2) obtained by QAE-Toyopearl chromatography (Fig. 1). All procedures are described in Materials and Methods. Values shown are molecular masses obtained using prestained protein standards (NOVEX, San Diego, CA, USA) BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), and myoglobin (30 kDa).

DISCUSSION

In the present study, trials were made on sea urchin eggs to characterize protein phosphatases using pNPP as the substrate. Serine/threonine protein phosphatases are known to catalyze pNPP splitting (Takai and Mieskes, 1991), in the same manner to other phosphatases, such as alkaline phosphatase, and inhibitors of protein phosphatase, such as OA, inhibit solely pNPP splitting catalyzed by protein phosphatases (Takai and Mieskes, 1991). It is also expected that the activities of protein phosphatases are to be estimated by pNPP splitting without any consideration of apparent changes in the activity due to difference in substrate protein species and the amount of phosphate moiety in the substrate protein, provided that protein phosphatases catalyze not only dephosphorylation of phosphoproteins but also pNPP splitting. In the extracts of sea urchin eggs and in egg homogenate, pNPP splitting was strongly inhibited by nano-moler level of OA, CLA and microcystin-LR, known to inhibit PP1 and PP2A in mammalian cells (Ishihara *et al.*, 1989; Honkanen *et al.*, 1990). These observations suggest that sea urchin eggs contain at least PP1 and/or PP2A, which also catalyze pNPP splitting in the same manner as in mammalian cells.

On the other hand, pNPP splitting in the homogenate and extract of eggs was not affected by deltamethrin, well known to be a specific inhibitor of PP2B, calcineurin (Enan and Matsumura, 1992). This observation, however, does not deny the presence of PP2B in sea urchin eggs, unless PP2B in sea urchin eggs is confirmed to catalyze pNPP splitting. Furthermore, it has recently been reported that deltamethrin fails to inhibit purified PP2B (Fakata *et al.*, 1998). Thus, it is difficult to identify PP2B on the basis of deltamethrin caused inhibition of pNPP splitting. Thus the failure of deltamethrin to inhibit pNPP splitting does not indicate the absence of PP2B in the egg extract. Indeed, the activity of PP2B-like enzyme is demonstrated in sea urchin eggs (Iwasa and Ishiguro, 1986).

The concentrations of OA to inhibit PP2A are known to be quite low as compared with those to inhibit PP1 in mammalian cells, whereas CLA inhibits PP1 at almost the same concentration as PP2A inhibition (Ishihara *et al.*, 1989; Honkanen *et al.*, 1990). In the egg extract, OA-caused inhibition of pNPP splitting became close to be maximum at the similar concentrations for maximum inhibition of mammalian PP2A. The inhibition of pNPP splitting in egg extract was hardly fortified by OA, even when OA concentration was made as high as those for the maximum inhibition of mammalian PP1. Provided that the OA-sensitivity of PP2A is markedly higher than the sensitivity of PP1 in egg extract as found in mammalian cells, the reaction catalyzed by PP2A certainly takes major part in pNPP splitting in the extract from sea urchin eggs.

In the egg extract, 4 peaks of pNPP splitting activity were obtained by QAE-Toyopearl chromatography and pNPP splitting in 2 among 4 peaks (peak II and III shown in Fig. 1) were strongly inhibited by OA, as well as CLA and microcystin-LR. In these 2 peaks exhibiting high activity of pNPP splitting, OA and CLA caused maximum inhibition of pNPP splitting at the similar concentrations to make the inhibition of mammalian PP2A maximum. The molecular masses of proteins exhibiting pNPP splitting activity in these 2 peaks were found to be about 160 kDa by Superdex 200HR column chromatography. The molecular mass of enzymes catalyzing OA-sensitive pNPP splitting is different from that of mammalian PP1 and PP2B (Cohen, 1989), as well as dimeric PP2A holoenzyme, and similar to that of trimeric holoenzymes in mammalian cells (Tung *et al.*, 1985). OA-sensitive pNPP splitting in egg extract is catalyzed by enzymes with the same molecular mass as trimeric PP2A holoenzyme in mammalian cells.

Immunoprecipitate obtained by anti-human PP2A catalytic subunit antibody from the egg extract showed considerable OA-sensitive pNPP splitting activity. A protein in one of 2 peaks obtained by QAE-Toyopearl chromatography (peak III) reacted with this antibody and the molecular mass of this antibody-reacting protein was found to be about 36 kDa by SDS-PAGE. The molecular mass of the protein to react with this antibody in sea urchin eggs is almost the same as that of human PP2A catalytic subunit (Van Eynde *et al.*, 1995). These observations indicates that 1 among 4 peaks of pNPP splitting activity obtained by QAE-Toyopearl chromatography contains PP2A, composed of similar catalytic subunit as in human enzyme. Although immunoreactive protein was not obtained in another peak exhibiting OA-sensitive pNPP splitting (peak II), the pNPP splitting activity in this peak is to be ascribed to PP2A reaction, because of its OA sensitivity, as well as its similarity in the molecular mass to that of mammalian PP2A. Sea urchin eggs probably have at least 2 isoenzymes of PP2A, composed of different catalytic subunits from each other. In other 2 peaks of pNPP splitting obtained by QAE-Toyopearl chromatography (peak I and IV), pNPP splitting was hardly affected by these examined inhibitors of protein phosphatases. The presence of other types of protein phosphatases than PP2A were not confirmed in the extract of unfertilized eggs by the procedures in the present study.

The extract obtained from nuclei isolated from late gastrulae underwent pNPP splitting, which was blocked by OA at the concentration evidently higher than the concentration for maximum inhibition of PP2A in the egg extract. This pNPP splitting in gastrula nuclei was strongly blocked by CLA at the same concentrations as those to inhibit pNPP splitting in the reaction catalyzed by PP2A of sea urchin eggs. The responses of pNPP splitting reaction to these inhibitors in the nucleus extract are very similar to those of PP1 in mammalian cells. PP1 type enzyme is found in sea urchin sperm (Hoshino and Suzuki, 1996) as in nuclei isolated from late gastrulae. In sperm, the volume of nucleus is predominant and hence, PP1 type enzyme probably distributes in their nuclei. It seems that PP1 distributes in nuclei in sea urchin gametes, in the same manner as in gastrulae. In mammalian cells, PP1 is reported to localize in nuclei (Paulson *et al.*, 1996).

In preliminary experiments, nuclei isolated from sea urchin eggs exhibited the activity of PP1 like enzyme (unpublished). The nucleus number is, of course, only 1 in an egg and more than 800 in a gastrula and hence, nuclei isolated

from gastrulae are used to obtain enough amount of enzyme source in the present study. The precipitate obtained after egg extraction also underwent pNPP splitting which sensitivities to the inhibitors of protein phosphatases were somewhat alike to mammalian PP1. The amount of this precipitate was quite small as the amount of nucleus fraction obtained from eggs. Hence, trials to find out evidences for the presence of nuclei in the precipitate were not successful and further studies were not carried out to characterize PP1 like enzyme in the precipitate. These observations, however, at least indicate that sea urchin eggs and embryos contain PP1 like enzyme with somewhat low OA sensitivity, though the activity of PP1 like enzyme is quite lower than the PP2A activity in whole egg homogenate.

In the extracts and whole homogenate of sea urchin eggs, other types of protein phosphatases, such as PP2B and PP2C, were not confirmed by the procedures using pNPP as the substrate. Further studies will be made to find out these protein phosphatases in eggs and embryos of sea urchin.

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