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Involvement of a calcium-phospholipid-dependent protein kinase in the maturation of *Xenopus laevis* oocytes

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It has been described that phosphorylation, and dephosphorylation, of specific proteins is associated with key events of the cell cycle and is likely to be due to activation of kinase(s). From our results, the presence of calcium-phospholipid-dependent protein kinase (PKC) was clearly demonstrated in both the cytosolic and particulate fractions of immature *Xenopus laevis* oocytes and in the cytosolic fraction of mature oocytes. However, it was less active in metaphase II- than in prophase I-arrested oocytes. The enzyme was partially purified by DEAE-cellulose and phenyl-Sepharose chromatography. It was activated in vitro by the tumor-promoting phorbol ester, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) as already described for PKC from other tissues. On the other hand, a calcium-phospholipid-independent histone kinase activity 4-fold higher in metaphase II- than in prophase I-arrested oocytes was detected. The possible role of PKC and phospholipid-independent histone kinase in the maturation process is discussed.

Protein kinase C; Tetradecanoyl phorbol acetate; Meiose; Oocyte; (*Xenopus laevis*)

1. INTRODUCTION

The control of cellular functions such as proliferation and differentiation is believed to be strongly associated with the phosphorylation state of regulatory proteins [1,2]. Several hormones and growth factors induce biological responses through the modification of kinase-phosphatase activities. Recent attention has focused on the role of protein phosphorylations catalyzed by the calcium-phospholipid-dependent protein kinase (PKC). PKC has been shown to be present in a wide variety of tissues and phyla [3–5]. It may mediate the effects of a variety of extracellular messengers which induce phosphatidylinositol (PI) turnover with transient production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Several observations led to the suggestion that PI turnover is implicated in the cell division process [6,7]. However, it has been shown that, if microinjection of IP_3 triggers activation of amphibian oocytes [8], it does not induce meiotic maturation, the release from the G_2/prophase I block [8,9]. The second product of phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown, DAG, activates PKC. 12-*O*-Tetradecanoyl phorbol 13-acetate (TPA), which contains a DAG-like moiety in its structure, can substitute for DAG in PKC activation. It has been described as a potent activator of PKC [10]. Moreover, it has been reported that TPA induces maturation of oocytes from the rat [11], surf clam [12] and *Xenopus laevis* [9]. These results enhanced the possibility of PKC being found to be involved in the maturation process. Here, we show directly that PKC is present in *Xenopus* oocytes, exhibiting calcium and phospholipid dependence as well as sensitivity to TPA. Furthermore, it was partially purified from prophase I-arrested

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oocytes. PKC was demonstrated to be present at the two key stages of the meiotic cycle: prophase I and metaphase II, with lower activity in metaphase II. On the other hand, as suggested by phosphorylation/dephosphorylation cycles along meioses and mitoses, after DEAE-cellulose chromatography, a histone kinase activity was found to be higher in metaphase II extracts.

2. MATERIALS AND METHODS

2.1. *Preparation of oocytes*

Stage VI, prophase I-arrested oocytes were obtained surgically from ovarian fragments of the frog *X. laevis* primed by injection with pregnant mare's serum gonadotropin. Metaphase II-arrested oocytes were obtained from frog induced to ovulate by injection of human chorionic gonadotropin. Eggs were dejellified by treatment with 2% cysteine (pH 7.8) as in [13].

2.2. *Purification of PKC*

All purification steps were performed at 0–4°C. Approx. 5 ml of prophase I- or metaphase II-arrested oocytes were homogenized by 15 strokes of a glass teflon pestle homogenizer in 50 ml of 20 mM Hepes-NaOH buffer (pH 7.5) containing 2 mM EDTA, 10 mM EGTA, 0.3 M sucrose, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 2 μg/ml pepstatin, 25 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml soybean trypsin inhibitor (buffer A). The homogenate was centrifuged at 100000 × *g* for 1 h. The 100000 × *g* supernatant fluid (0.5–1 g protein, 10 mg/ml), adjusted to an ionic strength of 0.5 mS with 20 mM Hepes-NaOH (pH 7.5) containing 2 mM EDTA, 2 mM EGTA and 1 mM DTT (buffer B), was stirred for 20 min with 1 vol. DEAE-cellulose (1 ml cellulose per 50 mg protein). The resin was poured into a column (2.5 × 4 cm) and washed with 15 vols buffer B. Proteins were eluted by buffer B containing 0.12 M NaCl. Major fractions containing PKC activity were pooled, adjusted to 1.5 M NaCl, and passed through a phenyl-Sepharose CL4B column (1.5 × 4 cm) equilibrated with buffer C (20 mM Hepes-NaOH, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT) containing 1.5 M NaCl. The column was washed with 10 vols buffer C containing 1.5 M NaCl, and proteins were eluted with buffer C.

2.3. *Subcellular distribution of PKC*

When the presence of PKC was investigated in both soluble and particulate fractions of oocytes, Triton X-100 was omitted from buffer A. The 100000 × *g* supernatant will be referred to as the cytosolic fraction. The pellet was homogenized in the original volume of buffer A containing 0.5% Triton X-100, sonicated for 1 min, incubated for 10 min at 4°C, and centrifuged at 100000 × *g* for 1 h at 4°C. This supernatant will be referred to as the membrane fraction. The cytosolic and membrane fractions were subjected to a single-step purification on DEAE-cellulose chromatography as described above. The enzyme from both sources was eluted under identical conditions.

2.4. *Protein kinase assay*

Fractions eluted from the chromatographic steps were tested for their phosphorylating activity as follows: assays (60 μl) contained 20 mM Hepes-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM $CaCl_2$, freshly sonicated phospholipids (80 μg/ml phosphatidylserine, 8 μg/ml diolein), 1 mM DTT, 1 mg/ml histones (IIIS from Sigma) and 10–20 μl of each fraction tested. Assays performed in the absence of $CaCl_2$ and/or phospholipids (PL), or in the presence of 2 μM cyclic AMP were used as controls. The reaction was initiated by the addition of 0.1 mM [γ-^{32}P]ATP (spec. act. 100–200 cpm/pmol) and phosphorylation measured after 5 min incubation at 20°C. The reaction was terminated by pipetting 40 μl of the reaction mixture onto Whatman P-81 phosphocellulose papers [14]. The papers were washed three times in ice-cold water, 5 min in 95% ethanol, dried and assayed for radioactivity in 5 ml RIA Luma scintillant.

Partially purified PKC was assayed for its activation using 10^{-6} and 10^{-7} M TPA as described above except that diolein was omitted. Activities in the presence of either dimethyl sulfoxide (DMSO) alone (TPA solvent) at 0.01% final concentration in the reaction mixture or, 4α-phorbol 12,13-didecanoate (4α-PDD), an analog of TPA known to have no effect on PKC activity and no tumor-promoting activity, at the same concentration employed for TPA, were used as controls.

Protein was determined according to Bradford [15].

3. RESULTS

As in numerous tissues or cells, PKC was not detectable in the crude extract of *Xenopus* oocytes by classical histone kinase assay. Therefore, the 100000 × *g* supernatant of prophase I-arrested oocyte homogenate was chromatographed on a DE52 ion-exchange column under the conditions specified in the legend to fig.1. Homogenization and chromatography buffers contained EDTA and EGTA to prevent proteolysis of native PKC by Ca^{2+}-activated proteases. The DE52 elution profile obtained is given in fig.1: using an elution buffer containing 0.12 M NaCl, PKC was resolved from other contaminating protein kinases and from its proteic inhibitor [16]. A single asymmetric peak of protein kinase was eluted at 0.05 M NaCl. The exact nature of the microheterogeneity of the enzyme illustrated by the shape of the peak of elution remains to be explained. Histone kinase assayed in the presence of cyclic AMP did not reveal the presence of kinase A (protein kinase activated in the presence of cyclic AMP) at such concentrations of NaCl. The majority of protein kinases which were not regulated by PL and even inhibited by PL, or activated by cyclic AMP, were eluted at ionic strength higher than 0.15 M NaCl (not shown). At this purification step, PKC was detected only in the presence of PL and $CaCl_2$.

As already shown for PKC, *Xenopus* oocyte PKC exhibits hydrophobic region(s). As shown in fig.2, it has been found to interact with hydrophobic chromatography resin as described for rat brain PKC. Hydrophobic interaction of the enzyme with the solid phase was favored by high ionic strengths, PKC being eluted by linearly decreasing the concentration of NaCl from 1.5 to 0 M. A single symmetric peak of PKC was obtained which still absolutely required $CaCl_2$ and PL for enzymatic activity, as indicated in fig.2. The sequential use of two chromatographic steps, anion exchange and hydrophobic interaction, resulted in the efficient resolution and enrichment of PKC.

Phorbol esters have a wide range of effects on a large number of tissues and cells. Moreover, they have been shown to activate directly PKC in vitro by enhancing PKC-specific phosphorylations [10]. The next set of experiments was conducted to determine whether TPA activates oocyte PKC directly. Fig.3 shows that *Xenopus* oocyte PKC partially purified by phenyl-Sepharose elution after DE52-cellulose chromatography was effectively activated by TPA. This activation was demonstrated using a reaction mixture containing no diolein. We checked that the concentration of TPA able to trigger the activation of PKC in vitro was the effective concentration inducing oocyte maturation (not shown), in agreement with the observation of Stith and Maller [9]. Neither 4α-

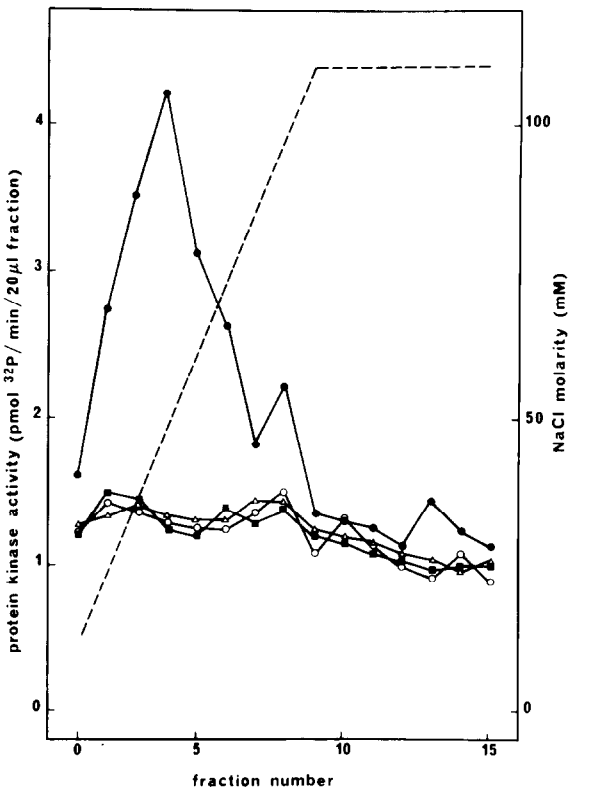

Fig.1. DEAE-cellulose (DE52) column chromatography of cytosolic PKC. The soluble fraction prepared from *X. laevis* oocytes as described in section 2 was applied to a DE52 column (2.5 × 4 cm) equilibrated with buffer B. The column was washed with 10 vols of the same buffer and PKC eluted by 100 ml buffer B, 0.12 M NaCl (NaCl concentration, – – –). Fractions of 6 ml were collected. Each fraction was assayed for histone kinase activity under the conditions described in section 2 with PL and $CaCl_2$ (●—●), without PL and with $CaCl_2$ (■—■), with cyclic AMP in the absence of $CaCl_2$ (△—△), without PL and $CaCl_2$ (○—○).

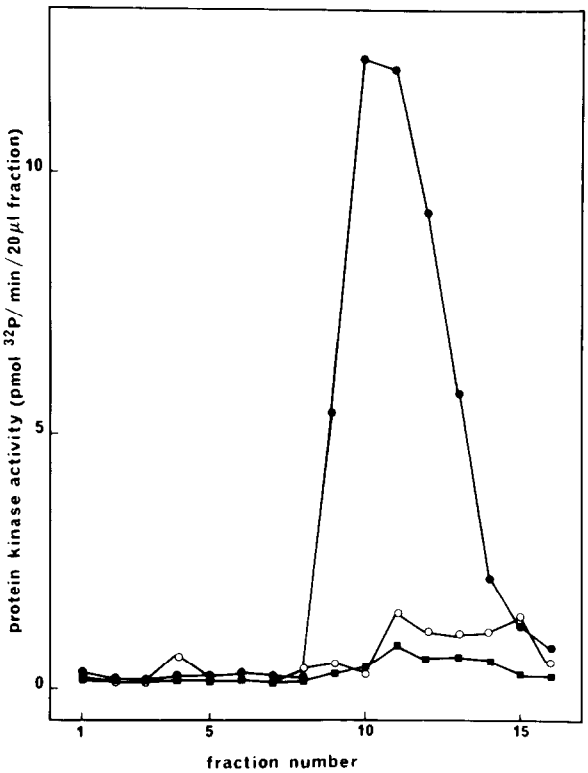

Fig.2. Phenyl-Sepharose CL4B chromatography. Fractions eluted from DE52 containing PKC activity were pooled, adjusted to 1.5 M NaCl and passed through a phenyl-Sepharose column (1.5 × 4 cm) equilibrated with buffer C containing 1.5 M NaCl. The column was washed with 10 vols of the same buffer, PKC being eluted with 30 ml buffer C. Fractions of about 0.6 ml were collected and assayed for histone kinase activity as described in section 2, with PL and $CaCl_2$ (●—●), without PL and with $CaCl_2$ (■—■) and without PL and $CaCl_2$ (○—○).

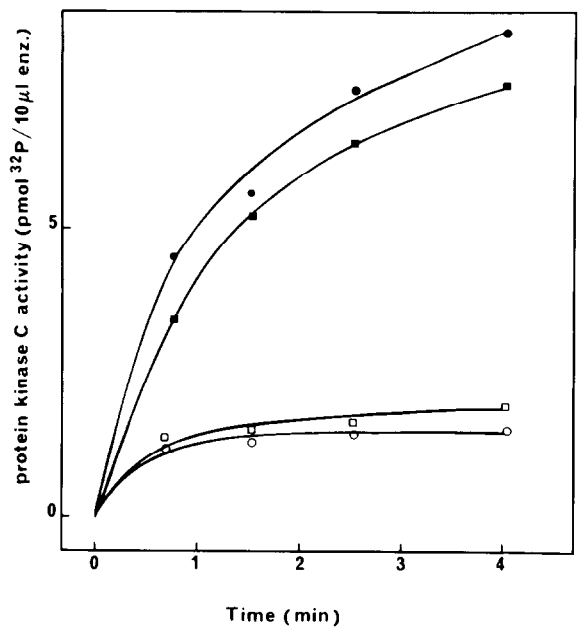

Fig.3. Time course of activation of PKC by TPA. PKC eluted from the phenyl-Sepharose column was assayed in the presence of 10^{-6} M TPA (●—●), 10^{-7} M TPA (■—■), 4α-PDD (□—□) or without activator (○—○), in the absence of diolein.

PDD nor DMSO used as controls was able to activate PKC.

In normal cellular reactions, as a result of receptor-mediated stimulation of inositol lipid turnover, DAG binds to and activates PKC via a kinetic mechanism involving translocation of PKC from the cytosol to membranes. Phorbol esters are believed to activate PKC directly, bypassing formation of DAG [17,18]. In the present study, we investigated the distribution of PKC between two compartments of the oocyte: cytosol and membranes. We looked at PKC activity at the two stages of the meiotic cycle: prophase I and metaphase II, in order to determine whether modification of subcellular repartition of PKC might occur along the cellular cycle. Experiments were performed under the conditions described in section 2. In order to prevent the association of cytosolic PKC with membranes as well as to protect the enzyme from proteolysis by Ca^{2+}-dependent neutral proteases, metal chelators were used during the experiment and a cocktail of antiproteases (at the same final concentrations used in buffer A) was distributed in each DE52 elution tube. The 100000 × g pellet was homogenized and sonicated in buffer A containing Triton X-100 in order to recover PKC activity in membrane fractions.

PKC was demonstrated in the cytosolic fraction of oocytes at both stages investigated and in prophase I membranes. Table 1 lists the relative activity of PKC that was recovered in both soluble and particulate fractions of *Xenopus* oocytes. The

Table 1

Protein kinase activity in cytosolic and membrane fractions of prophase I- and metaphase II-arrested oocytes

	Prophase I			Metaphase II		
	Total histone kinase	PKC	Basal histone kinase	Total histone kinase	PKC	Basal histone kinase
Cytosol	38	28	11	65	24	44
Membrane	9	2.5	5	27	0	20
Total		30.5			24	
% membrane		8			0	

Total histone kinase activity (in the presence of PL and $CaCl_2$), protein kinase C (activity in the presence of PL and $CaCl_2$ minus that in the absence of PL but in the presence of $CaCl_2$) and basal histone kinase activity (in the absence of PL and $CaCl_2$) were measured separately after DE52 chromatography and calculated in pmol [γ-^{32}P]phosphate incorporated/min per mg protein

membrane-associated enzyme was activated in the presence of PL and $CaCl_2$ in a manner similar to that described for the cytosolic enzyme. About 8% of the total PKC activity was associated with membranes in prophase I oocytes. PKC was not detected in metaphase II oocyte membranes unless the detergent was added for the homogenization step. In prophase I, the subcellular distribution pattern of PKC was similar to that described for rat platelets and lymphocytes in which the major portion of PKC was found in the cytosol, and distinct from that in rat brain and liver [19] where a significant quantity of the enzyme was associated with the membranes. As shown in table 1, the histone kinase activity in the presence of PL and $CaCl_2$ was 2-fold (cytosol) and 3-fold (membranes) greater in metaphase II than in prophase I. Although in prophase I, only one protein kinase was present after DEAE-cellulose chromatography, another histone kinase, detected in the absence of PL and $CaCl_2$, appeared in metaphase II, resulting in elevated basal activity of the enzyme preparation. At any rate, cytosolic PKC (activity in the presence of PL and $CaCl_2$ minus that in the absence of PL but in the presence of $CaCl_2$) remained unchanged between prophase I and metaphase II whereas in the membrane fraction, PKC activity was 2.5-fold lower in metaphase II than in prophase I.

4. DISCUSSION

The widespread occurrence of PKC has been demonstrated in various tissues and phyla. Although the presence of PKC was expected in *Xenopus* oocytes as suggested by phorbol ester-induced alteration of a variety of cellular processes such as ion(s) fluxes, protein phosphorylation and maturation, direct demonstration of its presence, in animal oocytes and embryos, has not yet been made. We have established that *X. laevis* oocytes (as well as starfish and sea urchin oocytes, not shown) contain a calcium-phospholipid-dependent protein kinase similar but not identical to that described in other tissues.

Due to the presence of PKC inhibitor(s) and/or phosphatase(s), PKC was undetectable in oocyte homogenates. Therefore, detection and measurement of PKC activity were performed following DEAE-cellulose chromatographic fractionation of the oocyte 100000 $\times$ g supernatant. *Xenopus* oocyte PKC was eluted from DEAE-cellulose at an NaCl concentration below 0.05 M compared with 0.08 M for PKC from other cells and/or tissues. PKC apparently shows no tissue or species specificity in physical, kinetic and catalytic properties but, thus far, four types of PKC have been isolated that can be distinguished by immunological and physico-chemical properties. The

specific behaviour of *Xenopus* oocyte PKC on DEAE-cellulose might be explained either by the existence of different proportions of the isoforms of the enzyme or by a different amino acid composition in even a small domain of the molecule participating in the interaction with the quaternary ammonium groups of the chromatographic gel. The DEAE-cellulose step allows concentration of the enzyme and probably removal of an endogenous inhibitory activity. This activity may be due to the presence of an inhibitor, similar to the low-M_r Ca^{2+}-binding proteic inhibitor previously characterized and purified from rat brain [16,20], present in the crude extract. The presence of competing kinases which can interfere with PKC activity was also prevented after DEAE-cellulose chromatography.

As for the enzyme isolated from other tissues, *X. laevis* PKC exhibits hydrophobic domains. This property was used in the second purification step which allows one to obtain more active and concentrated fractions of PKC. Partially purified *Xenopus* oocyte PKC exhibits properties similar to those of rat brain PKC. Until now, we have failed to characterize the enzyme with respect to its M_r. The enzyme was extremely unstable and was lost on filtration chromatography. On the other hand, the amount of purified enzyme was too small to allow detection after polyacrylamide gel electrophoresis.

It has been shown that the maturation of *Xenopus* oocytes, i.e. the release from prophase I block to metaphase II is concomitant with an increase in protein(s) phosphorylation(s) [1]. This increase is likely to be due to the appearance of new kinase activities [2].

It has been described that TPA rapidly induces (<1 h) maturation of oocytes. Moreover, we found that, in vitro, TPA activated partially purified *Xenopus* oocytes PKC. These results suggest that PKC is implicated in maturation. We showed that membrane-bound PKC was 2.5-fold less active in metaphase II, the stage at which the natural arrest occurs, than in prophase I oocytes. Since there was no decrease in cytosolic PKC, the overall PKC activity was lower in metaphase II than prophase I.

Several hypotheses can explain this result. One may be related to the activation/inactivation mechanism of the enzyme: PKC is reversibly activated by DAG-induced association with membranes. This activation process frequently results in the subsequent degradation of the kinase by endogenous proteases [21], which generate PKM, the PL- and $CaCl_2$-independent form of PKC [22]. TPA was supposed to trigger cellular responses by inducing translocation of the enzyme in an analogous manner to DAG receptor-linked natural extracellular messengers such as growth factors. Thus, in metaphase II-arrested oocytes, PKC might be proteolyzed irreversibly after transient translocation to the membrane. If this is the case, a decrease in PKC should be concomitant with an increase in PKM, its proteolytic form, which in other systems is eluted at 0.2 M NaCl from DEAE-cellulose, a concentration higher than that required to elute PKC.

Alternatively, another possible explanation would be that, in metaphase II PKC is proteolyzed more rapidly than in prophase I by increased protease activity or, after translocation, a subpopulation of PKC may remain associated with the particulate fraction even after treatment with calcium chelators and Triton X-100.

The maturation process is long enough to explain the steady-state level of PKC activity in the cytosol through the de novo synthesis of PKC.

Thus, PKC may interfere during the early stage of the maturation process in the triggering of other phosphorylation activities.

If one could correlate the cellular levels of PKC activity, investigation being facilitated by the ability to detect PKC in oocytes, with the extent of phosphorylation of PKC substrates, it would give a clue to the role of PKC in oocyte maturation.

However, if PKC activity showed sensitive modification at the metaphase II, we point out that a PL- and $CaCl_2$-independent histone kinase activity eluted at 0.12 M NaCl is 4-fold higher in metaphase II-arrested oocytes. This activity might be correlated with the cycling kinase previously described in *Xenopus* oocytes and might participate in the increase in protein phosphorylation observed in metaphase II.

Finally, how PKC activity relates to the cascade of events previously described, including maturation-promoting factor activation, and the identity of the PL- and $CaCl_2$-independent protein kinase of which the activity is enhanced at the metaphase II stage are under investigation.

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