Characterization of protein kinase C in early Xenopus embryogenesis

ARIE P. OTTE, IJSBRAND M. KRAMER, MAURICE MANNESSE, CARO LAMBRECHTS and ANTONY J. DURSTON

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT UTRECHT, The Netherlands

Summary

Recently, we presented evidence that protein kinase C (PKC) is involved in mediating the endogenous signals that induce competent Xenopus ectoderm to differentiate to neural tissue. We report here that PKC is already strongly activated in neural-induced ectoderm from midgastrula embryos and that this activation runs parallel with an increase in the level of inositol phosphates. We further identify several proteins that are phosphorylated, both in natural neural-induced ectoderm and in TPA-treated ectoderm, suggesting that they are phosphorylated through the PKC route. We found no major changes in PKC activity among different pregastrula stages, including the unfertilized egg. However, PKC isolated from animal, ectodermal cells is highly sensitive to Ca\(^{2+}\) and can be activated by low concentrations, (6-25 \(\mu M\)) of arachidonic acid, while PKC isolated from vegetal, endodermal cells is more insensitive to Ca\(^{2+}\) and cannot be activated by arachidonic acid. These results suggest that different PKC isozymes are present in animal and vegetal cells.

Key words: protein kinase C, signal transduction, neural induction, Xenopus.

Introduction

Protein kinase C (PKC) plays an important role in the mechanisms by which external signals are transmitted to the interior of cells. Several growth factors and hormones that bind to, and activate, receptors on the cell surface, stimulate inositol phospholipid turnover via phospholipase C activation, thereby producing the second messengers diacylglycerol and inositol phosphates, of which diacylglycerol activates PKC (Nishizuka, 1984, 1986; Berridge, 1987; Berridge and Irvine, 1989). Several growth-factors and hormones that stimulate inositol phospholipid turnover, induce translocation of PKC from the cytosol to the membrane (Niedel and Blackshear, 1986). PKC also serves as the receptor for phorbol esters, a class of tumour promoters (Castagna et al. 1982). One of them, 12-O-tetradecanoyl phorbol-13-acetate (TPA), activates PKC efficiently and causes PKC to translocate from the cytosol to the membrane.

There are clues suggesting that the PKC/inositol phosphate pathway plays an essential role in early embryogenesis. Fertilization increases the polyphosphoinositide content of sea urchin eggs (Turner et al. 1984) and induces changes in phosphatidylinositol turnover (Dworkin and Dworkin-Rastl, 1989). Iontophoresis of inositol trisphosphate activates Xenopus (Busa et al. 1985) and sea urchin eggs (Whitaker and Irvine, 1984). Also, TPA activates mouse oocytes (Cuthbertson and Cobbold, 1985), sea urchin eggs (Swann and Whitaker, 1985) and Xenopus eggs (Bement and Capco, 1989). In addition, TPA caused a cortical contraction when applied to unfertilized Rana pipiens eggs, suggesting that it causes Ca\(^{2+}\) release (Zimmerman, 1985). In Xenopus oocytes, meiotic maturation is induced by TPA (Stith and Maller, 1987; Smith, 1989) and facilitated by injection of protein kinase C (Kamata and Kung, 1990). These studies indicate a role for PKC and phosphoinositol phosphate turnover in the maturation of oocytes and in fertilization.

Another clue about the roles of PKC and the inositol phosphates in early embryogenesis comes from experiments with Li\(^+\). This ion is thought to block the processing of inositol phosphates, resulting in depletion of the phosphatidylinositol phosphate pools (reviewed in Berridge et al. 1989). Short treatments of 32- to 64-cell Xenopus embryos with Li\(^+\) dorsalizes these embryos (Kao et al. 1986). Injection of Li\(^+\) into a ventral blastomere also leads to dorsalization. Importantly, these teratogenic effects can be annihilated when myo-inositol is coinjected with Li\(^+\), indicating a role for PKC in establishing the dorsoventral polarity of the embryo.

Another clue about the role of PKC in early embryogenesis comes from our own work. We found that PKC is translocated from the cytosol to the
membrane in neural-induced ectoderm, but not in non-induced ectoderm. We also found that TPA causes translocation of Xenopus PKC and induces competent ectoderm to differentiate to neural tissue (Otte et al. 1988).

In this paper, we show that there are several proteins that become strongly phosphorylated in neural-induced ectoderm, either after artificial PKC activation or during natural neural induction. An increased level of inositol phosphates was also detected in neural-induced ectoderm. We show further that PKC, which is present in animal, ectodermal cells, is highly sensitive to Ca\(^{2+}\) and can be activated by low concentrations of arachidonic acid. PKC, which is present in the vegetal, presumptive endodermal cells, is more independent of Ca\(^{2+}\) for its activation and cannot be activated by arachidonic acid. These observations suggest that there are different PKC isozymes present in early embryogenesis.

**Materials and methods**

**Culture conditions**

Embryos were obtained by natural fertilization *via* standard procedures. Embryonic stages were determined according to Nieuwkoop and Faber (1967). Embryos were dejellied in 2% cysteine-hydrochloride (Sigma) (pH 8.0). Explants were cultured in 100% Flickinger solution (NaCl, 58 mM; KCl, 1 mM; NaHCO\(_3\), 0.24 mM; Na\(_2\)HPO\(_4\), 1 mM; KH\(_2\)PO\(_4\), 0.2 mM; CaCl\(_2\), 0.5 mM; MgSO\(_4\), 1 mM) (pH 7.5).

**Protein kinase C assay**

Embryos or explants were washed and homogenized in ice-cold lysis buffer (20 mM Tris pH 7.5, 2.5 mM EDTA, 0.5 mM EGTA, 5 mM b-mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride and 0.01 mg ml\(^{-1}\) leupeptin). The homogenate was centrifuged at 10,000g for 15 s, the pellet was homogenized again, centrifuged and the supernatants were combined.

The pellet, containing yolk platelets and organelles, was discarded, as it contained no PKC activity. The supernatant was spun at 100,000g for 1 h. The 100,000g supernatant, considered to be the cytosolic fraction, was removed. The pellet was used for the *in vitro* phosphorylation experiments described in Fig. 3. To isolate membrane-bound PKC, the pellet was resuspended and shaken regularly for 45 min in lysis buffer containing 1% Nonidet-40 (Sigma). The mixture was centrifuged for 15 min at 10,000g and the pellet discarded. The supernatant was considered to be the particulate or membrane fraction. When total (soluble and particulate) PKC was isolated from tissue, the tissue was homogenized in 1 ml ice-cold lysis buffer containing 1% Nonidet-40 and shaken regularly for 45 min. After that, the homogenate was spun at 10,000g for 10 min. The supernatant was loaded onto a DEAE column. Partial purification of PKC was achieved by washing the DEAE-cellulose with 50 mM Tris pH 7.5, 7.5 mM MgAc, 1 mg ml\(^{-1}\) leupeptin, 10 nM EGF receptor peptide, 0.25 mg ml\(^{-1}\) BSA, 0.2 mM ATP, 1.5 x 10\(^8\) cts min\(^{-1}\) [\(^{32}\)P]-ATP (New England Nuclear) and either 1 mM EGTA (background) or 1 mM Ca\(^{2+}\), 8 \muM phosphatidylserine (PS) (Spinal cord, Lipid Products), 0.8 \muM diacylglycerol (Diolein, Sigma) (assay). Lipids were stored under nitrogen at -20°C in chloroform/methanol 3:1. They were dried using a flow of nitrogen, suspended in 20 mM Tris pH 7.5 and sonicated for 3 min to obtain liposomes. Different Ca\(^{2+}\)-concentrations were obtained by using Ca\(^{2+}\)-EGTA buffers (Bartfai, 1979). The reactions were started by adding 25 \muL PKC eluate to 60 \muL reaction mix. The incorporation of \(^{32}\)PO\(_4\) at 30°C was linear with time up to 60 min. After 15 min, the reactions were stopped by adding 25 \muL 25% TCA. After 20 min on ice, the samples were spun (4°C) at 10,000g for 10 min and 90 \muL of the supernatant was then spotted onto a Whatman P-81 phosphocellulose filter and this was immediately transferred to 400 ml 75 mM phosphoric acid solution in which a magnetic stirrer rotated slowly. After changing the phosphoric acid solution 4 times (10-15 min rotation each), the filters were dried and counted. We found identical results using either histone III S or the EGF-receptor nonapeptide as substrate. Only the absolute value of the specific activities differed (compare with Otte et al. 1988), due to different numbers of phosphorylation sites in histone III S and the nonapeptide. Using the nonapeptide as a substrate has the advantage that lower backgrounds can be obtained, leading to a better signal to noise ratio.

**Substrate phosphorylation and gel electrophoresis**

Explants were incubated for 3 h in 5 ml Holtfreter solution (59 mM NaCl, 0.7 mM KCl, 0.9 mM CaCl\(_2\)) containing 80 \muCi \[^{32}\]Pophosphophosphate. After incubation, the explants were washed and homogenized in sample buffer consisting of 8 mM urea, 2% Nonidet P40 and 0.1% ampholytes pH 3-10. Two-dimensional electrophoresis was carried out according to O'Farrell (1975), using isoelectric focusing (2% ampholytes pH 3-10, Sigma) in the first dimension and SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) in the second dimension.

**Measurement of inositol phosphates and phosphatidylinositol phosphates**

Determination of total inositol phosphates was performed essentially according to methods described by Tilly et al. (1987) and by Dworkin and Dworkin-Rastl (1989). Determination of the phosphatidylinositol phosphates was performed essentially according to methods described by Lacal et al. (1987) and by Dworkin and Dworkin-Rastl (1989). Fertilized eggs were microinjected with 40 nl of 20 mM Tris (pH 7.2) containing 3 \muCi of \[^{3}H\]myoinositol (Amersham, 3.5 Ci mmol\(^{-1}\)). The eggs were cultured to stage 10 and entire ectoderm explants were dissected, either with or without a small piece (<20%) of dorsal mesoderm left attached. The explants were cultured for as long as it took control stage 10 embryos to reach stage 114. The explants were then homogenized in 2 ml chloroform:methanol:12 n HCl (200:100:0.75). After a 10 min extraction (RT) 8 \muL Folch II (Sigma) was added and the mixture was vortexed. 500 \muL 0.6 n HCl was added and the mixture was centrifuged at 3000g for 5 min. The upper water phase was removed and the lower phase was washed twice with 1 ml chloroform:methanol:0.6 n HCl (3:5:48:47). The water phases from these sequential washes were combined. The remaining chloroform and methanol was removed by evaporation under a flow of nitrogen. An equal volume of 10% TCA was added to the water phase to remove proteins. The mixture was centrifuged at 4000g for 10 min.
We also cultured stage 10 ectoderm explants against neural tissue (Jones and Woodland, 1989). As a classical histology and using a monoclonal antibody contained invariably neural tissue as determined via these explants till stage 38-40. These explants, for as long as it took control stage 10 embryos to reach these, for as long as it took control stage 10 embryos to reach stages 11, 13 and 16. The translocation of PKC was determined at these stages. As a control, stage 10 ectoderm (ectoderm, stage 10) was cultured as long as it took control stage 10 embryos to reach stage 16. The time scale indicates the time after fertilization (20°C). Values are means±s.E.M. of seven independent experiments, using 40 explants per experiment. In these experiments, the nonapeptide derived from the EGF-receptor was used as substrate for PKC.

without dorsal mesoderm till stages 11, 13 and 16. These cultured explants were not neural induced. We then measured the translocation of PKC from the soluble (cytosolic) to the particulate (membrane) fraction, in both types of explants, as described previously (Otte et al. 1988). As shown in Fig. 1, we found that PKC was translocated from the cytosolic to the membrane fraction in neural-induced ectomesoderm explants from stage 11 onwards. The translocation was maximal at stage 13, the membrane-bound PKC activity being a factor 3.27 higher at stage 13 than at stage 10. At stage 11, however, the PKC translocation was already strong, the membrane-bound PKC activity being a factor 2.45 higher at stage 11 than at stage 10, i.e. 75% of the maximum found at stage 13. The translocation persisted after stage 13, since we found an almost identical translocation in stage 16 ectomesoderm explants as at stage 13. In cultured but non-induced ectoderm explants, we observed no PKC translocation (only stage 10 and stage 16 ectoderm explants are shown).

When whole embryos were used to investigate the PKC translocation, we observed a similar initial strong PKC translocation in stage 11 embryos and only a small

**Fig. 1. Translocation of protein kinase C during neural induction.** The translocation of PKC from the cytosolic fraction (soluble, open bars) to the membrane fraction (particulate, stippled bars) was determined in neural-induced ectoderm. Stage 10 ectoderm was excised together with a small piece of dorsal mesoderm (ectomesoderm, stage 10), and cultured for as long as it took control stage 10 embryos to reach stages 11, 13 and 16. The translocation of PKC was determined at these stages. As a control, stage 10 ectoderm (ectoderm, stage 10) was cultured as long as it took control stage 10 embryos to reach stage 16. The time scale indicates the time after fertilization (20°C). Values are means±s.E.M. of seven independent experiments, using 40 explants per experiment. In these experiments, the nonapeptide derived from the EGF-receptor was used as substrate for PKC.
further increase in PKC translocation at stage 13 (results not shown). We conclude that PKC is translocated from the cytoplasm to the plasma membrane during an early phase in neural induction.

**Protein phosphorylation in neural induced ectoderm.**

We next investigated the phosphorylation of proteins in neural-induced ectoderm. Entire stage 10 ectoderm explants were incubated in \[^{32}P\]orthophosphate-containing medium for 3 h; this is as long as it took control stage 10 embryos to reach stage 11, the period during which PKC is almost maximally translocated (see Fig. 1). We also incubated ectoderm under the same conditions, for the same period, but in the presence of 350 nm TPA or with a small piece (<20%) of the dorsal mesoderm left attached.

After 3 h, the explants were washed, homogenized and analyzed by two-dimensional gel electrophoresis and autoradiography. As is shown in Fig. 2A and B, the pattern of the resolved proteins was highly reproducible and the amounts of the individual proteins were similar in cultured ectoderm explants (Fig. 2A) and in ectomesoderm explants (Fig. 2B), as judged by staining the gels with Coomassie Brilliant Blue.

Protein phosphorylation increased up to 3 h after the incubation was started, and did not increase thereafter (Fig. 2C). Apparently, a maximal phosphorylation level had already been reached at the time (3 h) when we analyzed the phosphorylation pattern in the cultured explants.

As is shown in Fig. 2D–E, we found that several proteins were phosphorylated more strongly in TPA-treated (Fig. 2E) and ectomesoderm explants (Fig. 2F) than in cultured but non-induced ectoderm explants (Fig. 2D). This increase in the degree of phosphorylation of the same proteins in ectomesoderm and TPA-treated ectoderm explants suggests that these proteins are either endogenous PKC substrates or substrates of kinases which are activated by PKC. The proteins numbered 1 to 9 are notably better phosphorylated, both in TPA-treated and in ectomesoderm explants, than in non-induced ectoderm. The characteristics of protein number 1, i.e. its weight of 80×10^3 M_r and its strong acidic character (pI 4.3) resemble those of the well-known 80×10^3 M_r PKC substrate which is found in many cellular systems (Niedel and Blackshear, 1986; Rodríguez-Bena and Rozengurt, 1986; Aderem et al. 1988; Wang et al. 1989; Stumpo et al. 1989; Graff et al. 1989).

There are also, mainly quantitative, differences in

![Fig. 2. Protein phosphorylation in neural-induced ectoderm. Stage 10 ectoderm was incubated in \[^{32}P\]orthophosphate-containing medium, either in the absence (A,D), or the presence of 350 nm TPA (E) or as a ectomesoderm explant (B,F). Phosphorylated proteins were analyzed by two-dimensional gel electrophoresis as described in the Materials and Methods. The gels were stained with Coomassie Brilliant Blue (A,B) and exposed for autoradiography (C,D,E,F). The pI range (pI 4.0–8.5) and relative molecular mass range (15–120×10^3) are indicated in A. Proteins that are more strongly phosphorylated after TPA treatment are indicated by numbers. In another experiment (C), the time course with which the proteins were phosphorylated was established. The proteins from these explants, which were incubated in the \[^{32}P\]orthophosphate-containing medium for the time indicated, were analyzed on a 5–15% SDS–polyacrylamide gradient gel.](image-url)
protein phosphorylation between TPA-treated (Fig. 2E) and ectomesoderm explants (Fig. 2F). Notably proteins 2, 3, 4, 5, 9 and 14 are more strongly phosphorylated in ectomesoderm than in TPA-treated explants.

Some phosphorylated proteins are localized in the membranes of neural-induced ectoderm

It is known of several PKC substrates that they are present in, or become translocated to, the plasma membrane (Aderem et al. 1988; Wang et al. 1989). This localization would facilitate phosphorylation by the translocated and activated membrane-bound PKC. We investigated whether the same is true for the phosphorylated proteins that we identified (above), in neural-induced ectoderm. We therefore isolated membranes from ectoderm and ectomesoderm explants which were cultured as long as it took control stage 10 embryos to reach stage 11. We also isolated membranes from stage 10 ectoderm explants, which had been treated with 500 nM TPA for 30 min, a treatment that causes a maximal PKC translocation (Otte et al. 1988). These membranes were prepared exactly as when the translocation of PKC was measured, i.e. homogenized tissue was centrifuged at 100,000 g for 1 h. The membranes were then homogenized and 1 mM Ca\(^{2+}\), diacylglycerol/phosphatidylserine liposomes, and \([\text{\text{H}}]ATP\) were then added. After 20 min incubation at 30°C, phosphorylation of the membrane proteins was analyzed by two-dimensional gel electrophoresis and autoradiography.

As is shown in Fig. 3, we found that some of the phosphorylated proteins that were identified in intact cells (Fig. 2) are present and strongly phosphorylated in membranes from TPA-treated ectoderm and ectomesoderm explants (Fig. 3B and C) but are much less phosphorylated in membranes from cultured, but non-treated ectoderm explants (Fig. 3A). The more basic proteins (numbers 10 to 14), that are not the most strongly phosphorylated \textit{in vivo}, (compare with Fig. 2), are notably well phosphorylated in membranes that contain translocated and activated PKC (Fig. 3B and C). The most strongly phosphorylated protein in these membranes is the 80x10^5 M\(_r\) protein (protein number 1), which is only poorly phosphorylated in membranes from non-induced ectoderm explants (Fig. 3A).

These results show that some of the major proteins that are phosphorylated \textit{in vivo} are present in membranes from neural-induced ectoderm. This ectoderm contains increased PKC activity in its membranes, due to PKC translocation during neural induction, and it is possible that these proteins are translocated to the membrane, concomitantly with PKC. We cannot, however, exclude from these experiments that these proteins were already present in the membrane.

Neural-induced ectoderm contains a higher level of inositol phosphates

Cell-surface receptor mediated hydrolysis of phosphatidylinositol bisphosphate via phospholipase C raises the levels of diacylglycerol and of inositol phosphates. We tested whether phospholipase C is activated concomitantly with PKC activation, during neural induction, leading to an increase in the level of inositol phosphates. Fertilized eggs were injected with 3 μCi [\text{\text{H}}]myoinositol per egg and cultured to stage 10. This period has been shown to be long enough to label the endogenous phosphatidylinositol lipid pools in \textit{Xenopus} embryos to equilibrium (Lacal et al. 1987; see Materials and methods). At stage 10, ectoderm and ectomesoderm explants were excised and cultured as long as it took control stage 10 embryos to reach stage 11. We then measured the levels of inositol phosphates and phosphatidylinositol phosphates in neural-induced and non-induced ectoderm at stage 11, since PKC is still in the process of being activated during neural induction at this stage (Fig. 1).

The stage 11 explants were homogenized and total

Fig. 3. Some of the \textit{in vivo} phosphorylated proteins are present in the membranes of neural-induced ectoderm. Stage 10 ectoderm was excised without dorsal mesoderm (A and B), and either treated with 500 nM TPA for 30 min (B) or not (A) or else was excised with the dorsal mesoderm left attached (C). The non-treated ectoderm explants and ectomesoderm explants were then cultured as long as it took control stage 10 embryos to reach stage 16. Membrane preparations were isolated from the explants and incubated in the presence of \([\text{\text{P}}]ATP\), diacylglycerol/phosphatidylserine liposomes and 1 mM Ca\(^{2+}\) at 30°C for 20 min. After incubation, the proteins were analyzed by two-dimensional gel electrophoresis and autoradiography. The pi and molecular weight range is as indicated as in Fig. 2A. The phosphorylated proteins, which are numbered, correspond to the phosphorylated proteins in Fig. 2.
levels of inositol phosphates (including IP, IP₂, IP₃ and IP₄), and of phosphatidylinositol monophosphate (PI), phosphatidylinositol monophosphate (PIP) and phosphatidylinositol bisphosphate (PIP₂) were determined. The usual procedure that is followed when phosphatidylinositol turnover is investigated, is to add Li⁺ to the culture medium. This causes an accumulation of inositol phosphates, which can then be assayed more easily (Tilly et al. 1987). Since Li⁺ has strong teratogenic effects, including effects on neural induction (Masui, 1960; Nieuwkoop et al. 1985), which we wished to avoid, we measured the levels of phosphatidylinositol phosphates and inositol phosphates during neural induction in the absence of Li⁺. As is shown in Table 1, we found a consistent increase (145±5 %) in the level of inositol phosphates in neural-induced ectoderm during neural induction above that in non-induced ectoderm. We found no changes in the levels of PI, PIP and PIP₂ between neural-induced and non-induced ectoderm. Increases in the level of inositol phosphates without changes in the levels of the phosphoinositides have been found in other systems (Brammer et al. 1988; Augert et al. 1989; Van Haastert et al. 1989).

We conclude from these results, that phospholipase C is activated in neural-induced ectoderm during neural induction, leading to higher concentrations of the inositol phosphates.

**Protein kinase C activity is constant throughout early Xenopus embryogenesis**

Since indirect evidence suggests a role for PKC during fertilization, and in establishing the dorsoventral polarity of the embryo, as well as in neural induction (see above), we wondered how the level of total PKC activity is regulated through early development. We isolated total (cytosolic and membrane) PKC from unfertilized and fertilized eggs, and stage 2, 5, 8, 10, 11, 13 and 16 embryos. As is shown in Fig. 4, we found no differences in total PKC activities among unfertilized and fertilized eggs and stage 2 to stage 10 embryos.

**Table 1. An increased inositol phosphate level in neural induced ectoderm**

<table>
<thead>
<tr>
<th></th>
<th>InsPn</th>
<th>PI</th>
<th>PIP</th>
<th>PIP₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectoderm (st 114)</td>
<td>100</td>
<td>97.3±5.5</td>
<td>1.8±0.5</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>Ectomesoderm (st 114)</td>
<td>145±5</td>
<td>98.5±6.6</td>
<td>2.1±0.4</td>
<td>0.9±0.2</td>
</tr>
</tbody>
</table>

100 % InsPn=2709±318 disnts min⁻¹ (n=5)/500 μg protein

PI=169 170±9348 (n=5)/500 μg protein

PIP=3148±872 (n=5)/500 μg protein

PIP₂=1535±374 (n=5)/500 μg protein

Fertilized eggs were injected with 3 μCi [3H]myoinositol each. At stage 10 entire ectoderm was excised with or without a small piece of dorsal mesoderm. The explants were cultured till control stage 10 embryos reached stage 114. At this stage, total inositol phosphates were isolated and polyphosphoinositides were separated by using thin-layer chromatography. The levels of the inositol phosphates are presented as percentages in which 100 % represents the level in 20 uninduced ectoderm explants. The levels of the polyphosphoinositides are presented as percentages in which 100 % is the sum of PI, PIP and PIP₂ in uninduced ectoderm.

Only during neural induction (between stages 10 and 16), did we find a small increase in total PKC activity, a result reported previously (Otte et al. 1988). Note that, although there is only a small increase in total (cytosolic and membrane) PKC activity, there is a strong translocation of PKC from the cytosol to the membrane (see above) between these stages. Since transcription increases strongly only after the midblastula transition (Newport and Kirschner, 1982), it is likely that, up to stage 8, the PKC is of maternal origin. Whether the PKC that is involved in neural induction is also of maternal origin or zygotic, we do not know.

**PKC variants derived from animal and vegetal tissues have different biochemical properties**

PKC is known to consist of a large family of isoforms (Nishizuka, 1988; Kikkawa et al. 1989). The different isoforms have subtly different individual biochemical properties. It has previously been shown that different PKC isoforms have different sensitivities to Ca²⁺, and that they are activated differentially by arachidonic acid (Naor et al. 1988; Nishizuka, 1988; Kikkawa et al. 1989). We investigated whether PKC derived from the distinct, prospective tissues in the early embryo have different biochemical properties. We dissected stage 10 ectoderm and isolated PKC from this and the remainder (vegetal, prospective endodermal cells and mesoderm). We investigated the sensitivity of PKC from each of these parts of the embryo to Ca²⁺. As is shown in Fig. 5A, ectodermal PKC was highly sensitive to Ca²⁺. At Ca²⁺ concentrations lower than 100 μM, no PKC activity was measured. When only phosphatidylserine (PS) was added, the dependence on Ca²⁺ was even higher. When PKC was isolated from the rest of the embryo (prospective endoderm and mesoderm),
Protein kinase C in early Xenopus embryogenesis

Fig. 5. PKC derived from animal and vegetal cells from the early gastrula have different sensitivities to Ca\(^{2+}\). Stage 10 entire ectoderm explants were dissected and total PKC was isolated from the animal, ectodermal (A) cells and from the rest of the embryo (B). The PKC was activated in the presence of diolein/phosphatidylserine or phosphatidylserine only. A range of free Ca\(^{2+}\) concentrations was added as indicated. Histone III S was used as substrate. Values are the average from three independent experiments (S.E.M. less than 10%).

and tested for its Ca\(^{2+}\) dependence, we found a different picture. This PKC activity was more independent of Ca\(^{2+}\) (Fig. 5B). At 1 μM Ca\(^{2+}\), PKC still exhibited 80% of the activity measured with 1 mM Ca\(^{2+}\). But in the absence of Ca\(^{2+}\), the activity declined sharply, indicating that this PKC activity is not entirely Ca\(^{2+}\)-independent. In addition, when diacylglycerol was omitted and only PS was present, this PKC was strongly sensitive to Ca\(^{2+}\).

It is important that under the PS/diolein and PS stimulation conditions, both the ectodermal and vegetal PKC activities fall to background levels when no Ca\(^{2+}\) is present. This rules out the possibility that the observed difference in Ca\(^{2+}\) sensitivity between ectodermal and vegetal PKC results from different Ca\(^{2+}\) concentrations due to Ca\(^{2+}\) which is released during homogenization and remains in the PKC preparations. We do not think this latter possibility very likely in any case, since homogenization, PKC purification and storage occur in a lysis buffer that is devoid of Ca\(^{2+}\) and contains 2 mM EDTA and 0.5 mM EGTA.

It is also important to note that 80% of the total PKC activity in the early gastrula embryo is localized in the ectoderm, thereby confirming our earlier results (Otte et al. 1988).

It has been shown (Naor et al. 1988; Kikkawa et al. 1989) that the type I mammalian PKC isozyme can be activated by low (6–25 μM) concentrations of arachidonic acid (AA) and that the type III mammalian isozyme can be activated by a higher AA concentration (100 μM). As is shown in Fig. 6, the PKC isolated from ectoderm could be stimulated via 25 μM AA to ±50% of the activity found when PS and diolein were used as PKC activators (compare with Fig. 4). The PKC activity increased from 6 to 25 μM AA and declined at 50 μM AA. PKC isolated from the rest of the embryo, however, could not be stimulated by AA at any of the concentrations tested and the activity never exceeded the background activity, measured in the presence of 1 mM EGTA and arachidonic acid (background) with histone III S as substrate. Values are the average of three independent experiments (S.E.M. less than 10%).

Fig. 6. Arachidonic acid activates PKC from ectodermal cells only. PKC was isolated from different tissues of the stage 10 gastrula embryo as in Fig. 5. Equal amounts of PKC as in Fig. 5 were activated in the presence of 1 mM Ca\(^{2+}\) and a range of arachidonic acid concentrations (animal and vegetal), as indicated, or in the presence of 1 mM EGTA and arachidonic acid (background) with histone III S as substrate. Values are the average of three independent experiments (S.E.M. less than 10%).

These results show that the different tissues in the early Xenopus embryo contain PKC with different biochemical properties, suggesting that these are distinct PKC isozymes.

Discussion

The two major results from this paper are that we substantiate the notion that protein kinase C is involved in mediating neural induction and show that this possibly occurs via phospholipase C activation, and that we also show that the different tissues of the embryo contain PKC with different biochemical properties.

We showed previously that PKC is translocated from the cytosol to the membrane, specifically in the neural-induced ectoderm, in response to the neural-inducing signals (Otte et al. 1988). In this paper, we report that PKC is already strongly translocated at stage 11. We
found earlier that the levels of cAMP and adenylate cyclase (AC) increase from stage 12 onward (Otte et al. 1989). We also found that the increase in AC activity is dependent on PKC activation, indicating cross-talk between these pathways (Otte et al. 1989). The result shown here, that PKC is activated at an earlier stage than the cAMP pathway, fits our model that PKC needs to be activated first, before the cAMP pathway can be activated (Otte et al. 1989).

It is important to note that PKC translocation lasted, even after the involution of the neural inducing mesoderm was complete and the area which becomes neural tissue had been established (stage 13) (Nieuwkoop and Faber, 1967). This might mean that this translocated PKC is still active, but it might also be present in an inactive form. This last possibility is proposed in a recent model, which postulates a role for sustained PKC translocation in cellular memory (Alkon and Rasmussen, 1988). This quasi-permanently translocated PKC might become reactivated whenever the intracellular Ca²⁺ concentration is raised.

Two of our present observations strengthen our previous suggestion that PKC is involved in mediating neural induction:

(i) The neural-induced ectoderm contains a higher level of inositol phosphates, indicating phospholipase C activation. We have not tested though, whether phospholipase C activation also leads to the production of diacylglycerol and PKC activation in Xenopus ectoderm. Since we find PKC activation at a similar time, this appears, however, to be a very likely possibility.

(ii) The existence of a set of proteins which become strongly phosphorylated in induced neur ectoderm, and which also become phosphorylated after PKC activation by TPA, makes it likely that PKC activation is the causal event in both of these situations. Whether these phosphorylated proteins are actual direct PKC substrates is difficult to determine. It is quite possible that PKC activation leads to the activation of other kinases and that the phosphorylated proteins are substrates of these kinases. We can also not exclude the possibility that natural neural induction is mediated by kinases other than PKC that phosphorylate the same set of proteins as those phosphorylated via TPA activated PKC. We feel, however, that, considering the close similarity between the phosphorylation patterns in TPA-treated ectoderm and in ectomesoderm explants and the observation that both TPA (Otte et al. 1988) and dorsal mesoderm induce PKC translocation (see above), these results at least indicate that PKC is involved in mediating natural neural induction.

One of these phosphorylated proteins resembles the well-known 800 X 10³ M₀ PKC substrate (Rodriguez-Pena and Rozengurt, 1986; Niedel and Blackshear, 1988). We base this suggestion on the molecular weight of the protein and on its highly acidic character (pI 4.3). We found further that some of these phosphorylated proteins are present in the membranes of neural-induced ectoderm. The 800 X 10³ M₀ substrate was especially abundant. It was long thought that this substrate needs to be present in the membrane in order to become phosphorylated (Aderam et al. 1988; Stumpo et al. 1989; Wang et al. 1989), although recent experiments now challenge this idea (Graff et al. 1989). In our case, however, some, but certainly not all of the proteins that are phosphorylated in vivo appear to be present in the membrane when they are phosphorylated. Since we find these proteins in membranes after a short (30 min) TPA treatment, they are likely to be actual, direct PKC substrates. This leaves open the possibility that the other, in vivo phosphorylated proteins are substrates of kinases that are activated by PKC, or that they are PKC substrates that have not been translocated to the membrane.

The observation that the PKC types derived from prospective ectoderm and from vegetal tissue, respectively, have different sensitivities to Ca²⁺ and arachidonic acid suggest that these are different PKC isozymes. The different PKC isozymes exhibit distinct patterns of tissue-specific expression (Kikkawa et al. 1989; Hidaka et al. 1988; Huang et al. 1987; Brandt et al. 1987), suggesting important, different, but as yet unknown, physiological functions. In a recent study Noar and coworkers (1988) showed that PKC type I or γ-PKC is highly dependent on Ca²⁺ for its activation and that it can be activated by 12-25 μM arachidonic acid. It is therefore tempting to speculate that the PKC isolated from ectodermal cells represents the mammalian type I PKC isozyme. This is all the more interesting because this isozyme has been found exclusively in neural tissue (Kikkawa et al. 1989), which is derived from ectoderm. As long as there are no nucleotide sequence data available from Xenopus PKC genes, this point remains speculative, however.

The presence of different PKC isozymes with different biochemical properties might throw an interesting light on the various putative roles of PKC in early embryonic events like fertilization (Turner et al. 1984; Whitaker and Irvine, 1984; Busa et al. 1985; Dworkin and Dworkin-Rastl, 1989), the establishment of the dorsoventral polarity of the embryo (Kao et al. 1987; Busa and Gimlich, 1989; Berndge et al. 1989) and neural induction (Otto et al. 1988 and this present work). Since the establishment of the dorsoventral polarity of the embryo occurs in the vegetal cells (Boterenbrood and Nieuwkoop, 1973; Gimlich and Gerhart, 1984) and neural induction in the ectodermal cells, it is tempting to speculate that different PKC isozymes function in these two distinct, important embryological events. More detailed studies will, however, be needed to substantiate this idea.

We thank Drs R. Van Driel and S. de Laat for critically reading this manuscript.

References

Protein kinase C in early Xenopus embryogenesis


A. P. Otte and others


(Accepted 26 June 1990)