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Evidence for a Role of Protein Kinase C ζ Subspecies in Maturation of *Xenopus laevis* Oocytes

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A number of studies have demonstrated the activation of phospholipase C-mediated hydrolysis of phosphatidylcholine (PC-PLC) both by growth factors and by the product of the *ras* oncogene, p21^{ras}. Evidence has been presented indicating that the stimulation of this phospholipid degradative pathway is sufficient to activate mitogenesis in fibroblasts as well as that it is sufficient and necessary for induction of maturation in *Xenopus laevis* oocytes. However, the mechanism whereby PC-PLC transduces mitogenic signals triggered by growth factors or oncogenes remains to be elucidated. In this study, data are presented that show the involvement of protein kinase C ζ subspecies in the channelling of the mitogenic signal activated by insulin-p21^{ras}-PC-PLC in *Xenopus* oocytes as well as the lack of a critical role of protein kinase C isotypes α, β, γ, δ, and ε in these pathways.

Considerable effort has been expended to identify critical steps in mitogenic signal transduction pathways. Particularly, phospholipid degradation, which is potently activated after stimulation with growth factors (2, 8), is the core of recent intense research. Although most of the work has been focused on phosphoinositide turnover, a number of studies demonstrate the existence of phosphoinositide-independent signal transduction cascades involving the phosphodiesterase-mediated hydrolysis of phosphatidylcholine (PC) (3, 8, 12, 13, 23, 24). Recently, evidence has accumulated showing that activation of phospholipase C (PLC)-catalyzed hydrolysis of PC (PC-PLC) is sufficient to mimic a significant portion of the platelet-derived growth factor (PDGF) mitogenic signal (14). PC-PLC has also been shown to be stimulated by the product of the *ras* oncogene, p21^{ras} (5, 12, 16, 24), whose role in mitogenic cascades has been documented (26). Therefore, all these results permit one to suggest that PC-PLC activation could be critically involved in pathways controlling cell growth and tumor transformation. Oocytes from *Xenopus laevis* are a suitable system for investigating the involvement of different enzymatic activities in relevant signal transduction mechanisms controlled by oncogenes (11, 13). *Xenopus* oocytes undergo a maturation program after stimulation with either insulin or progesterone, and several lines of evidence indicate the specific involvement of p21^{ras} in the maturation-signalling cascades activated by insulin-insulin growth factor-1 (4, 11). More definitive proof of the involvement and importance of PC-PLC in the oocyte maturation pathway activated by insulin-p21^{ras} has recently been obtained (9). Thus, we have shown that PC-PLC is both necessary and sufficient for activation of maturation in *X. laevis* oocytes by insulin-p21^{ras}, as measured by the induction of germinal vesicle (nuclear) breakdown (GVBD) and activation of maturation-promoting factor histone 1 (1) kinase (9). The mechanism whereby PC-PLC transduces growth factor mitogenic signals remains to be elucidated. In this regard, since PC-PLC generates diacylglycerol, which is an important activator of protein kinase C (PKC) (19), the involvement of this kinase in the mitogenic signalling cascades activated by PC-PLC is an intriguing possibility. Previous data demonstrate that down-regulation of PKC isotypes sensitive to chronic exposure to phorbol esters does not affect the ability of PC-PLC to promote mitogenesis in fibroblasts (14). Furthermore, expression of the stromelysin gene in response to PDGF-PC-PLC-p21^{ras} is not affected by chronic treatment of cells with phorbol myristate acetate (PMA) (6). Interestingly, transient expression experiments with plasmids harboring different deletions and mutations in the stromelysin promoter region linked to a reporter gene demonstrate that the tetradecanoyl phorbol acetate-responsive element located in that promoter is not required to transmit signals generated by PDGF-PC-PLC (6). Taken together, all these results strongly suggest at the least that classical PKC isotypes sensitive to down-regulation by chronic treatment with PMA do not appear to be involved in PC-PLC signalling.

In the present report, we contribute data that demonstrate the involvement of the PKC ζ subspecies (ζ-PKC) in channelling of the mitogenic signal activated by insulin-p21^{ras}-PC-PLC in *Xenopus* oocytes as well as the lack of a critical role of PKC isotypes α, β, γ, δ, and ε in these pathways.

MATERIALS AND METHODS

Oocyte culture. Oocytes were prepared by following standard procedures (9). Briefly, ovaries from *X. laevis* frogs (Blades Biologicals, Kent, United Kingdom) were incubated with 2 mg of collagenase (Boehringer GmbH, Mannheim, Germany) per ml for 45 min in modified Barth solution without Ca^{2+} (110 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5]). After extensive washing, stage VI oocytes were selected and incubated overnight at 20°C.

cDNA cloning of *Xenopus* oocyte ζ-PKC. For cDNA cloning of the ζ-PKC homolog in *Xenopus* oocytes, a probe was generated by amplification of a DNA fragment directly on a lambda-ZAP library from rat brain tissue by the polymerase chain reaction with the following oligonucleotides: 5′-ATGAATTCTGAAGGCGCACTAC-3′ and 5′-ATGAATTCTCGATGACAGGCTTA-3′. This resulted in a fragment of

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656 bp, encompassing nucleotides 52 to 708 of rat brain ζ-PKC (21). This fragment was labelled with ^{32}P by random priming (Multiprime DNA labelling system; Amersham International) and used to screen an oligo(dT)-primed *Xenopus* oocyte cDNA library (generously provided by D. Melton). Hybridizations were carried out in 50% formamide at 42°C, and filters were washed at 65°C with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS). Positive signals were picked, and phages were purified. Inserts were subcloned into pBluescript plasmid. Clones were analyzed by DNA sequencing with the fmol DNA sequencing system (Promega, Madison, Wis.).

In vitro transcription. The bacteriophage T3 or T7 RNA polymerase was used to synthesize sense and antisense RNAs in the presence of the cap analog GpppG by using 10 μg of linearized DNA as template (mCAP mRNA capping kit; Stratagene, La Jolla, Calif.).

Oligonucleotides. Fifteen-mer oligonucleotides identical or complementary to the beginning (starting at the initiation codon) of the coding region of *Xenopus* ζ-PKC were synthesized and modified on the backbone to phosphorothioates (Operon Technologies, Alameda, Calif.). As a further control, a nonsense oligonucleotide with a random sequence of nucleotides identical to those present in the antisense oligonucleotide was synthesized.

Isolation of PC-PLC from *Bacillus cereus*. PC-PLC was isolated from cultures of *B. cereus* SE-1 essentially as described previously (9, 14). Following this protocol, the enzyme preparation was purified to complete homogeneity as confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) and then by silver staining. The specific activity of the purified enzyme was 1.5 U/μg.

Preparation of $p21^{ras}$ proteins. Transforming and normal $p21^{ras}$ proteins were expressed in bacteria as previously described (7). A final step of purification consisted of gel filtration chromatography through a Sephadex G-100 column (2.5 by 90 cm); fractions containing the purified protein were pooled and dialyzed extensively against 20 mM Tris-HCl (pH 7.5) to remove urea and kept at −70°C until utilized.

Immunoblot analyses of different PKC isotypes. Extracts from *Xenopus* oocytes containing 100 μg of total cell protein were resolved in SDS–10% polyacrylamide gels after being denatured in SDS sample buffer. Afterwards, they were transferred electrophoretically onto polyvinylidene difluoride membrane (Immobilon; Millipore Continental Water Systems, Bedford, Mass.) and incubated with antibodies specific for PKC isotypes α, β, and γ or ζ either in the absence or in the presence of the corresponding competition peptides. PKC isotypes α, β, and γ, on the one hand, and ζ, on the other, were visualized with the AuroProbe BL plus system (Amersham International) after incubation of blots with the corresponding antibodies. For detection of isotypes α + β + γ, an antipeptide antibody generated against the peptide ILKKDVVIQDDDVE corresponding to amino acids 381 to 394 of γ-PKC was used. For detection of isotype ζ, an antipeptide antibody generated by using the peptide corresponding to amino acids 577 to 592 of ζ-PKC (sequence GFEYINPLLLSAEESV) was used. These antibodies were purchased from GIBCO Bethesda Research Laboratories, Gaithersburg, Md.

Analysis of oocyte maturation. Groups of 20 oocytes were cultured at 20°C in modified Barth solution, and GVBD was assessed by the appearance of a white spot in the animal pole. In some cases, nuclear breakdown was confirmed by dissection of trichloroacetic acid (10%)-fixed oocytes (9).

Maturation-promoting factor H1 kinase assay. Twenty oocytes were homogenized in a buffer containing 20 mM HEPES (pH 7.0), 10 mM β-glycerophosphate, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 5 mM $MgCl_2$, 50 mM NaF, 2 mM dithiothreitol, 100 μg of leupeptin per ml, and 100 μM phenylmethylsulfonyl fluoride. After centrifugation at 13,000 × *g* for 15 min, extracts (1 to 2 mg per assay) were incubated with $p13^{suc1}$ linked to agarose beads, and histone 1 kinase activity was determined in the precipitates which were then separated by SDS-PAGE (7).

Effect of different inhibitors on PMA-sensitive PKC activities in extracts from *X. laevis* oocytes. Stage VI oocytes were homogenized in 20 mM Tris-HCl (pH 7.4)–5 mM β-mercaptoethanol–0.5 mM EGTA–2 mM EDTA–10 μM phenylmethylsulfonyl fluoride–10 μM leupeptin–1% Triton X-100. After 45 min on ice, extracts were centrifuged at 100,000 × *g* for 30 min, after which PKC was purified by sequential chromatography as described previously (32), except that separation on hydroxyapatite was omitted. Of note is the finding that after anion-exchange chromatography on DEAE-Sephacryl, the sample was devoid of calcium-independent PKC isozymes (ζ in the oocyte), as demonstrated earlier (32) and confirmed by Western immunoblot (not shown). Incubations were performed in the presence of Ca^{2+} (100 μM) either with or without phosphatidylserine (PS) plus PMA (5 μg/ml). The kinase activity was determined as described previously (32) in the absence or the presence of peptide A, Z, or the PKC inhibitor GF 109203X.

Immunoprecipitation and autophosphorylation assay. Oocyte extracts were incubated with 10 μg of anti-ζ-PKC antibody, and immunocomplexes were recovered with protein G-agarose. Immunoprecipitates were assayed for the autophosphorylation kinase activity of ζ-PKC in a mixture of 300 μM [γ-^{32}P]ATP with or without 80 nM Ca^{2+} either in the absence or in the presence of 50 μg of PS per ml. Incubations were carried out with different concentrations of peptide A or Z (described in the legend to Fig. 3). Reactions were stopped after 45 min, and proteins were resolved by SDS-PAGE. No kinase activity was detected when oocyte extracts were incubated with protein G-agarose in the absence of antibody.

RESULTS

Identification of PKC isotypes present in extracts of *X. laevis* oocytes. We initially determined which PKC isotypes were present in stage VI oocytes from *X. laevis*. To this aim, antibodies specific for each PKC isotype were used in immunoblots of oocyte extracts. Results from Fig. 1A demonstrate that immunoblotting with an antibody specific for isotypes α, β, and γ detected an 80-kDa band in oocyte extracts, which is inhibited by the isozyme-specific peptide. Similarly, immunoblotting with an antibody specific for ζ-PKC clearly detected a band with an approximate molecular mass of 65 kDa (Fig. 1B); this size is consistent with the sequence data of its cDNA from rat brain tissue (21) and from *Xenopus* oocytes (see below). These bands were specifically eliminated by blockade of the antibody with the corresponding isoenzyme-specific peptide (Fig. 1B). When immunoblotting of oocyte extracts was carried out with antibodies monospecific for isotypes δ and ε, respectively, no bands were observed, although these antibodies readily detected the corresponding PKC isotypes in rat brain extracts (not shown).

Role of PMA-sensitive PKC isotypes in stimulation of mat-

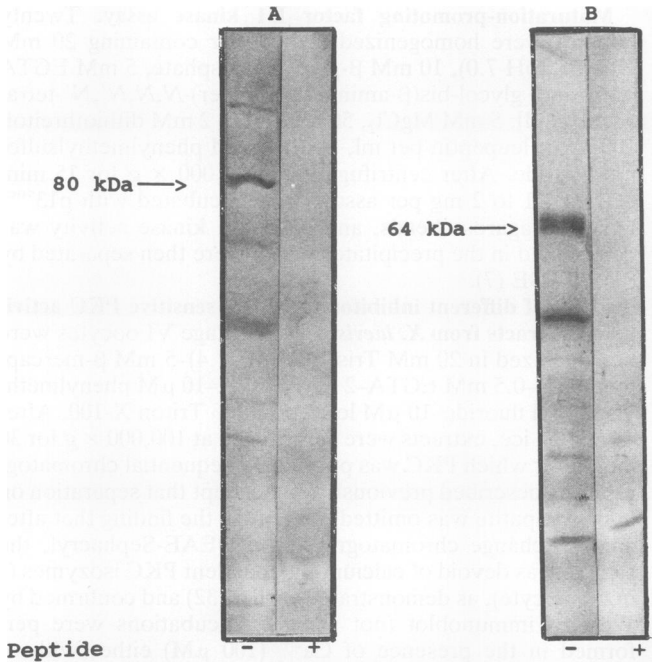

FIG. 1. Immunoblot analyses of different PKC isotypes in extracts from *Xenopus* oocytes. Oocyte extracts were resolved by SDS-PAGE, electroblotted, and incubated with antibodies specific for PKC isotypes α, β, and γ (A) or ζ (B), either in the absence (lanes −) or in the presence (lanes +) of the corresponding competition peptides. Essentially identical results were obtained in three independent experiments.

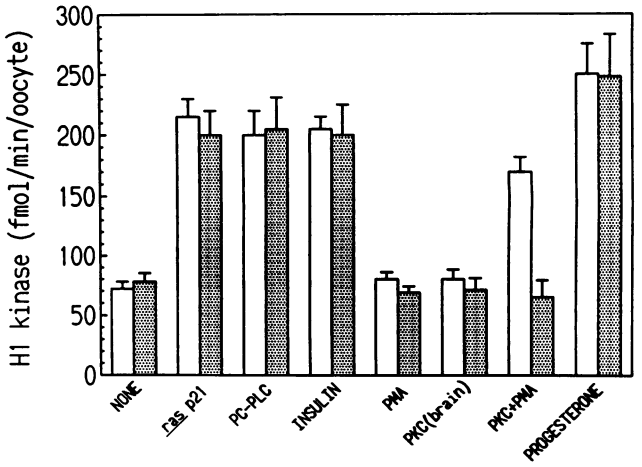

FIG. 2. Lack of involvement of PMA-sensitive PKC isotypes in activation of H1 kinase in *Xenopus* oocytes. Stage VI oocytes were either untreated (open bars) or incubated with 5 μM GF 109203X (shaded bars) for 15 min before microinjection or before the addition of different stimuli. Some oocytes were microinjected with 1 ng of purified bovine brain PKC prepared as described in Materials and Methods for the oocyte. The specific activity of this preparation was 2.3 μmol/min/mg of protein. Subsequently, oocytes were microinjected with 20 ng of transforming $p21^{v\text{-}H\text{-}ras}$ or with 25 μU of *B. cereus* PC-PLC or incubated in the presence of PMA (100 ng/ml), insulin (1 μM), or progesterone (1 μM). Afterwards, H1 kinase activity was determined in the extracts as described previously when oocytes displayed a 50% induction of GVBD. Results are mean ± standard deviation of three independent experiments, with incubations in duplicate.

uration-promoting factor H1 kinase activity. To assess the functional importance of PKC in maturation of *Xenopus* oocytes, we initially determined whether PMA, a very well-known activator of classical PKC subspecies (19), stimulated maturation-promoting factor H1 kinase activity in oocytes. This enzymatic activity has been extensively characterized as a complex which includes the catalytic subunit $p34^{CDC28/cdc2+}$ (18) and can be specifically measured after its precipitation with $p13^{suc1}$-agarose beads. Results from Fig. 2 clearly show that the addition of PMA up to 100 ng/ml produces only very weak or no stimulation of H1 kinase compared with that elicited by either microinjection of transforming $p21^{v\text{-}H\text{-}ras}$ or microinjection of a permanently activated PC-PLC from *B. cereus* (described in references 9 and 14) (Fig. 2). These results can be interpreted as confirmation that no PMA-sensitive PKC appears to be involved in the activation of H1 kinase. We next attempted to reconstitute a PKC-dependent pathway for the activation of H1 kinase by PMA in the oocyte. Thus, PKC was partially purified from bovine brain tissue by following previously standardized protocols and was microinjected into *X. laevis* oocytes. This did not produce any effect on H1 kinase activity (Fig. 2); however, incubation of PKC-microinjected oocytes with 100 ng of PMA per ml significantly stimulated H1 kinase. Therefore, it is possible to reconstitute a PMA-activatable, PKC-dependent pathway for activation of oocyte maturation by microinjecting a purified preparation of PKCs. Accordingly, a PMA-PKC-dependent pathway does not appear to play a decisive role in oocyte maturation, unless bovine brain PKCs are provided.

Most of the pharmacological inhibitors of PKC so far described in the literature are poorly selective against other protein kinases. Recently, a novel PKC inhibitor, the bisindolylmaleimide GF 109203X, has been reported to be a potent and selective inhibitor of PKC subspecies α, β, and γ in vitro, displaying little or no activity against the other five protein kinases (30). Furthermore, GF 109203X potently and selectively inhibits PMA-PKC-dependent functions in vivo, including the inhibition of PMA-induced proliferation in fibroblasts (30). In order to test whether GF 109203X affects maturation pathways in *X. laevis* oocytes, experiments were performed by incubation of oocytes with up to 10 μM GF 109203X, after which they were microinjected with 25 μU of *B. cereus* PC-PLC or with 20 ng of transforming $p21^{v\text{-}H\text{-}ras}$ or they were incubated with insulin (1 μM) or progesterone (1 μM). Another group of oocytes were microinjected with bovine brain PKC and incubated afterwards with 100 ng of PMA per ml. From the results shown in Fig. 2, it can be concluded that GF 109203X did not affect the ability of either insulin, $p21^{ras}$, or *B. cereus* PC-PLC to induce H1 kinase, although it completely abolished the activation of this parameter by PMA in PKC-microinjected oocytes. Progesterone, which activates maturation through a pathway completely independent of that of $p21^{ras}$ or PC-PLC (4, 9, 11), is not affected by the presence of this novel PKC inhibitor (Fig. 2). Taken together, all these results strongly suggest that no PMA-sensitive PKC is involved in maturation in *Xenopus* oocytes.

Use of pseudosubstrate peptide inhibitors to study the involvement of ζ-PKC subspecies in stimulation of H1 kinase activity and GVBD in *Xenopus* oocytes. The results presented so far are consistent with previous observations demonstrating that down-regulation of PMA-sensitive PKC isotypes by chronic exposure of fibroblasts to phorbol esters did not

affect signalling responses to p21^{ras}-PC-PLC (6, 14). One important property of the other PKC isotype present in *Xenopus* oocytes, namely ζ-PKC, is its lack of sensitivity to PMA (21). Therefore, if a PKC isotype is to play any role in maturation of *Xenopus* oocytes, the isotype ζ appears to be a good candidate. In order to test this hypothesis, we initially cloned the CDNA encoding the *X. laevis* ζ-PKC homolog. We used a cDNA library from *Xenopus* oocytes (generously provided by D. Melton), a 656-bp probe generated by polymerase chain reaction from a rat brain cDNA library, and the appropriate primers (6a). This probe encompasses the regulatory portion of the enzyme, including the cysteine-rich domain. From the sequence of this clone it is clear that *Xenopus* ζ-PKC displays a 72% identity at the amino acid level to its rat brain homolog. All the important signatures of the enzyme, including the cysteine-rich and ATP-binding domains, are perfectly well conserved. Interestingly, the sequence corresponding to the pseudosubstrate region is 100% identical at the amino acid level to that from its rat brain homolog.

Several kinases have an autoinhibitory region called the pseudosubstrate domain (27). We reasoned that experiments with oocytes from *X. laevis* permit one to investigate the role of different PKC isotypes in mitogenic signalling by microinjection of specific pseudosubstrate peptide inhibitors of the different PKC isozymes. Taking into account the PKC isotypes present in oocytes, peptides A and Z were synthesized. Peptide A has a sequence conserved in the pseudosubstrate region of PKC isotypes α, β, and γ and, therefore, should be a good candidate inhibitor of these isotypes (22). Peptide Z has a sequence identical to that of the ζ-PKC pseudosubstrate region, which significantly differs from that of isotype α, β, or γ (22). Accordingly, oocytes were microinjected with peptide A after which they were microinjected with 25 μU of *B. cereus* PC-PLC or with 20 ng of transforming p21$^{v\text{-}H\text{-}ras}$ or they were incubated with insulin (1 μM) or progesterone (1 μM). Control oocytes were microinjected with bovine brain PKC and incubated afterwards with 100 ng of PMA per ml. Results from Fig. 3 clearly demonstrate that the presence of peptide A did not affect the ability of either progesterone, insulin, transforming p21$^{v\text{-}H\text{-}ras}$, or *B. cereus* PC-PLC to activate H1 kinase, although it completely abolished the stimulation of this parameter by PMA in PKC-microinjected oocytes. This is consistent with the results from experiments with the PKC inhibitor GF 109203X. Therefore, these data strongly indicate that neither α, β, nor γ PKC isotype appears to be implicated in the transmission of the maturation signal by insulin-p21^{ras}-PC-PLC.

In the next series of experiments, we used peptide Z to investigate the possible involvement of ζ-PKC in mitogenic signalling in oocytes. Therefore, oocytes were microinjected either with peptide Z or with buffer control, after which they were microinjected with either 25 μU of *B. cereus* PC-PLC or with 20 ng of transforming p21$^{v\text{-}H\text{-}ras}$ or they were incubated with insulin (1 μM) or progesterone (1 μM). Again, as a control, oocytes were microinjected with bovine brain PKC and incubated afterwards with 100 ng of PMA per ml. Results from Fig. 3 demonstrate that oocytes microinjected with peptide Z did not respond to activation with insulin, p21^{ras}, or PC-PLC although they did respond to the addition of progesterone. The ability of PMA to stimulate H1 kinase in PKC-microinjected oocytes was not affected by the microinjection of peptide Z. This finding strongly suggests that ζ-PKC is a specific critical step in maturation signal transduction in response to insulin-p21^{ras}-PC-PLC. The fact that the progesterone-activated maturation program is not affected by peptide Z is a good control of the specificity of its effect on the insulin pathway.

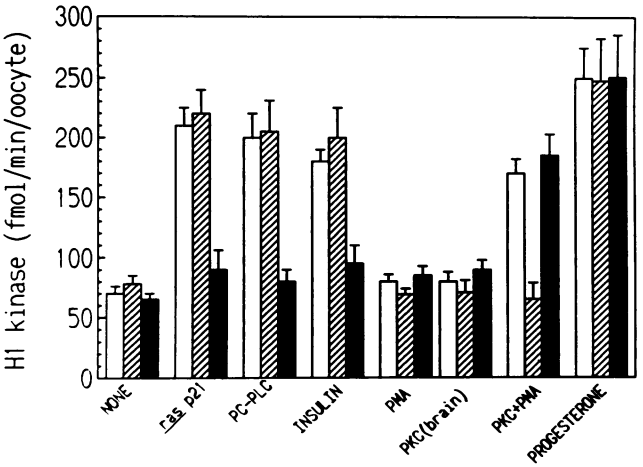

FIG. 3. Involvement of ζ-PKC in activation of H1 kinase in *Xenopus* oocytes. Stage VI oocytes either untreated or microinjected with 1 ng of purified bovine brain PKC were microinjected with distilled water (open bars) or with 5 μM (final concentration into the oocyte) peptide A (hatched bars) or Z (solid bars). Subsequently, oocytes were microinjected with 20 ng of transforming p21$^{v\text{-}H\text{-}ras}$ or with 25 μU of *B. cereus* PC-PLC or were incubated in the presence of PMA (100 ng/ml), insulin (1 μM), or progesterone (1 μM). Afterwards, H1 kinase activity was determined in the extracts as described in Materials and Methods, when oocytes displayed a 50% induction of GVBD. The amino acid sequences of peptides A and Z were (single-letter code) RKGALRQKN and RRGARRWRK, respectively. Results are mean ± standard deviation of three independent experiments, with incubations in duplicate.

In order to confirm that peptide A does actually inhibit the PMA-sensitive PKC activity present in *X. laevis* oocytes, they were extracted and PKC activity was purified through DEAE-Sephacryl, which removes nonclassical Ca^{2+}-dependent isotypes (ζ in the oocyte) and the effect of different concentrations of inhibitor peptides on the ability of PMA to activate this PKC preparation was determined. Results from Table 1 indicate that peptide A and GF 109203X potently

TABLE 1. Effect of different inhibitors on PMA-sensitive PKC activities in extracts from *X. laevis* oocytes[a]

Inhibitor (concn, μM)	PKC activity (dpm/10 min/μg of protein)	
	None	PS-PMA
Control	250 ± 15	750 ± 45
Peptide A		
0.1	240 ± 22	500 ± 40
0.5	220 ± 21	270 ± 35
2.0	222 ± 18	246 ± 25
Peptide Z		
0.1	250 ± 12	720 ± 55
0.5	245 ± 25	700 ± 50
2.0	240 ± 20	550 ± 38
GF 109203X (2.0)	220 ± 30	235 ± 25

[a] Results are mean ± standard deviation of three independent experiments, with incubations in duplicate.

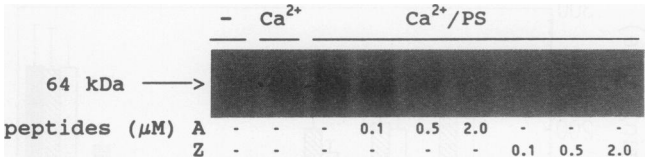

FIG. 4. Effect of peptides A and Z on autophosphorylation activity of ζ-PKC. Oocyte extracts were incubated with 10 μg of anti-ζ-PKC antibody (described in the legend to Fig. 1), and immunocomplexes were recovered with protein G-agarose. Immunoprecipitates were assayed for the autophosphorylation kinase activity of ζ-PKC in a mixture of 300 μM [γ-^{32}P]ATP with or without 50 μg of PS per ml. Incubations were carried out with different concentrations of peptides A or Z (described in the legend to Fig. 3). Reactions were stopped after 45 min, and proteins were resolved by SDS-PAGE. No kinase activity was detected when oocyte extracts were incubated with protein G-agarose in the absence of antibody. Essentially identical results were obtained in three independent experiments.

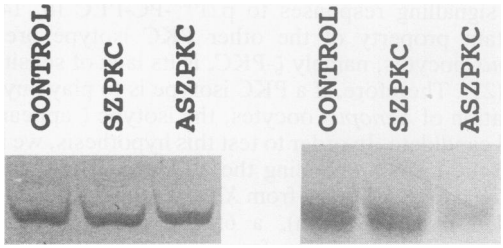

FIG. 5. Immunoblot analyses of ζ-PKC levels in extracts from RNA-microinjected oocytes. Sense and antisense RNAs synthesized in vitro from a plasmid harboring a fragment from the regulatory domain of ζ-PKC were microinjected into stage VI oocytes. Forty-eight hours after microinjection of RNAs or distillated water, oocytes were homogenized and extracts were resolved by SDS-PAGE, electroblotted, and incubated with antibodies specific for PKC isotypes α, β, and γ (left) or ζ (right). Essentially identical results were obtained in three independent experiments.

inhibited this activity, whereas peptide Z was dramatically less efficient.

In order to determine whether peptide Z actually blocks the enzymatic activity of ζ-PKC, the following experiment was carried out. Oocyte extracts were immunoprecipitated with the specific anti-ζ-PKC antibody described above, and autophosphorylation of ζ-PKC was measured in the protein G-agarose-recovered immunocomplexes. Results from Fig. 4 demonstrate that a dramatic autophosphorylation of ζ-PKC was observed in the immunoprecipitates incubated in the presence of PS. Interestingly, as little as 0.1 μM peptide Z completely abolished ζ-PKC autophosphorylation (Fig. 4). A comparable level of inhibition of this activity was detected only with a concentration of peptide A which was 20-fold higher than that of peptide Z. These results demonstrate that the inhibition of ζ-PKC by peptide Z was actually specific.

Since activation of H1 kinase is critical for the control of maturation in *Xenopus* oocytes, conceivably, microinjection of peptide Z will inhibit GVBD in response to the stimulation of the insulin pathway but not to progesterone. Results from Table 2 indicate that this is actually the case. Thus, microinjection of transforming p21$^{v\text{-}H\text{-}ras}$ or *B. cereus* PC-PLC promotes a potent maturation response comparable to that produced by the addition of insulin or progesterone. Addition of PMA does not induce GVBD unless oocytes were previously microinjected with purified bovine brain PKC, which is consistent with the data on H1 kinase. Of note is the finding that microinjection of peptide A inhibited PMA-induced maturation in PKC-microinjected oocytes but produced little or no effect on the induction of GVBD in response to insulin-p21^{ras}-PC-PLC or progesterone. Interestingly, microinjection of peptide Z completely abolished GVBD in response to microinjection of transforming p21$^{v\text{-}H\text{-}ras}$ or *B. cereus* PC-PLC or to the addition of insulin. It is noteworthy that GVBD induction in response to progesterone or PMA, in PKC-microinjected oocytes, was not affected by microinjection of peptide Z.

TABLE 2. Effect of pseudosubstrate peptides A and Z on maturation of *X. laevis* oocytes

Treatment	GVBD (%)[a]		
	Control	Peptide A	Peptide Z
None	0	0	0
Insulin	65 ± 8	68 ± 6	20 ± 2
Progesterone	89 ± 6	90 ± 8	88 ± 9
PKC (brain)	8 ± 2	2 ± 1	9 ± 2
PKC + PMA	45 ± 8	4 ± 2	40 ± 4
p21^{ras}	80 ± 7	78 ± 6	12 ± 5
PC-PLC	74 ± 9	70 ± 8	14 ± 9

[a] Oocytes were either microinjected with buffer control or with 1 ng of purified bovine PKC prepared as described in the legend to Fig. 2. Afterwards, corresponding oocytes were microinjected with either water or peptide A or Z (5 μM final concentration into the oocyte). Subsequently, they were incubated in the presence of PMA (100 ng/ml), insulin (1 μM), or progesterone (1 μM) or microinjected with transforming p21$^{v\text{-}H\text{-}ras}$ (20 ng) or with *B. cereus* PC-PLC (25 μU), and GVBD was determined 6 h thereafter. Results are mean ± standard deviation of three independent experiments, with incubations in duplicate.

Effect of antisense RNA from ζ-PKC on activation of maturation-promoting factor H1 kinase and GVBD in *Xenopus* oocytes. In order to further demonstrate the importance of ζ-PKC in oocyte maturation, a fragment of the regulatory domain of this PKC isotype, encompassing bases −10 to +600, was subcloned into pBluescript. Sense and antisense RNAs were synthesized in vitro from that plasmid, and both RNAs were microinjected into stage VI oocytes. At different times after microinjection, the oocytes were extracted and the levels of different PKC isotypes were determined by immunoblotting. Results from Fig. 5 demonstrate that 48 h after microinjection of 25 ng of antisense ζ-PKC RNA, a significant reduction (60% as confirmed by laser scanning densitometry) in the level of this protein is observed, with no effect on the immunoreactive band detected with the antibody specific for isotypes α, β, and γ. Therefore, by using this strategy we have been able to specifically deplete ζ-PKC levels in oocytes. Microinjection of water or control-sense RNA did not produce any effect on either PKC isotype (Fig. 5). Accordingly, oocytes incubated for 48 h with either sense or antisense ζ-PKC RNAs were microinjected with 25 μU of *B. cereus* PC-PLC or with 20 ng of transforming p21$^{v\text{-}H\text{-}ras}$ or they were incubated with insulin (1 μM) or progesterone (1 μM). Results from Fig. 6A clearly demonstrate that ζ-PKC-depleted oocytes responded less efficiently to activation with insulin, p21^{ras}, or PC-PLC, although they gave a full response to the addition of progesterone. This strongly suggests, again, that ζ-PKC is a specific critical step in maturation signal transduction in response to insulin-p21^{ras}-PC-PLC. The fact that the progesterone-activated maturation program is not affected by depletion of ζ-PKC is a good control of the specificity of this effect on the insulin pathway. Control oocytes microinjected with either water or sense

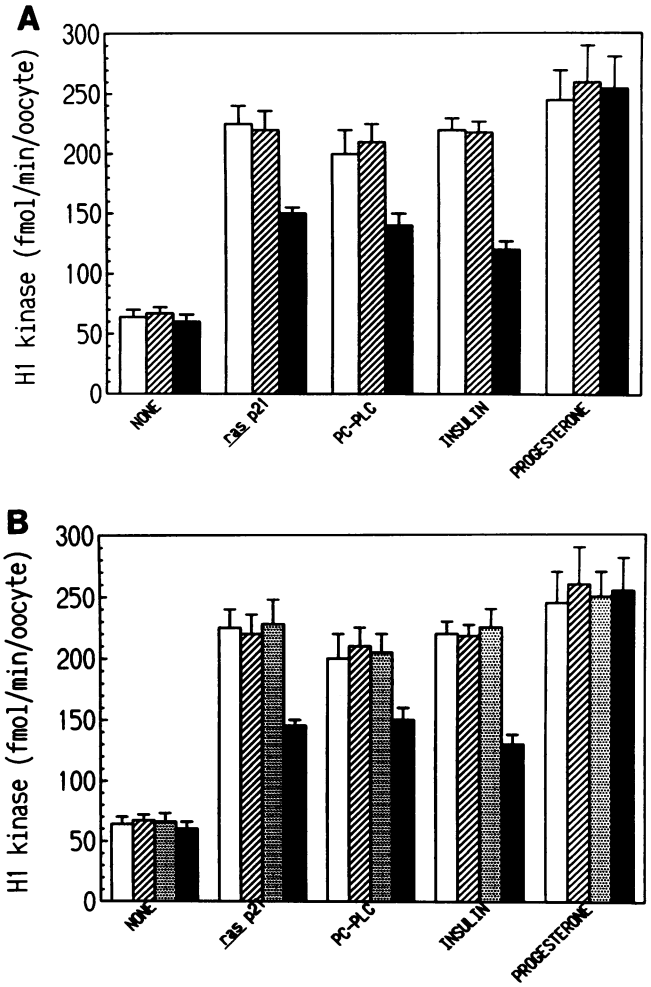

FIG. 6. Involvement of ζ-PKC in activation of H1 kinase in *Xenopus* oocytes. (A) Stage VI oocytes were microinjected with water (open bars) or with 25 ng of either sense (hatched bars) or antisense (solid bars) synthetic RNAs. (B) Another set of oocytes were microinjected with water (open bars) or with 150 ng per oocyte of sense (SZPKC; hatched bars), antisense (ASZPKC; solid bars), or nonsense (NSZPKC; stippled bars) oligonucleotides specific for ζ-PKC. Subsequently, oocytes were microinjected with 20 ng of transforming $p21^{v\text{-}H\text{-}ras}$ or with 25 μU of *B. cereus* PC-PLC or were incubated in the presence of insulin (1 μM) or progesterone (1 μM). Afterwards, H1 kinase activity was determined in the extracts, as described in the legend to Fig. 2 and in Materials and Methods, when oocytes displayed a 50% induction of GVBD. Results are mean ± standard deviation of three independent experiments, with incubations in duplicate.

RNA were microinjected in parallel with transforming v-H-*ras* or PC-PLC or were incubated with insulin or progesterone (Fig. 6A). Microinjection of water or ζ-PKC sense RNA did not affect the ability of these molecules to activate H1 kinase.

The use of small antisense oligonucleotides is a potential method utilized to inhibit the expression of proteins in oocytes (29). Therefore, as an independent strategy to decrease ζ-PKC levels in oocytes, we synthesized a 15-mer antisense oligonucleotide starting at the initiation codon of ζ-PKC as well as the corresponding sense and nonsense controls. To overcome degradation by nucleases, the oligonucleotides were modified on the backbone to phosphorothioates. Oocytes were microinjected with 150 ng of sense (SZPKC), antisense (ASZPKC), or nonsense (NSZPKC) oligonucleotides. A significant diminution of ζ-PKC levels was detected in Western blots of extracts from ASZPKC-microinjected oocytes, with no effect on the other PKC isotype (not shown). Microinjection of SZPKC or NSZPKC did not affect the levels of any PKC isotype present in oocytes. Therefore, oocytes were microinjected with 25 μU of *B. cereus* PC-PLC or with 20 ng of transforming $p21^{v\text{-}H\text{-}ras}$ or they were incubated with insulin (1 μM) or progesterone (1 μM). Results from Fig. 6B clearly demonstrate that the ability of ζ-PKC-depleted oocytes to respond to insulin, $p21^{ras}$, or PC-PLC was significantly impaired although they did respond normally to the addition of progesterone. Again, microinjection of sense or nonsense oligonucleotides did not affect activation of H1 kinase by any of the stimuli tested in this study.

TABLE 3. Maturation of *X. laevis* oocytes

Treatment[a]	GVBD (%) of oocytes[b]	
	Control	ζ-PKC-depleted
None	0	0
Insulin	65 ± 8	35 ± 2
Progesterone	89 ± 6	88 ± 9
$p21^{ras}$	80 ± 7	35 ± 5
PC-PLC	74 ± 9	38 ± 9

[a] Oocytes were incubated in the presence of insulin (1 μM) or progesterone (1 μM) or were microinjected with transforming $p21^{v\text{-}H\text{-}ras}$ (20 ng) or with *B. cereus* PC-PLC (25 μU), and induction of GVBD was determined 6 h thereafter.

[b] Results are mean ± standard deviation of three independent experiments, with incubations in duplicate.

Decreased ζ-PKC levels in *Xenopus* oocytes will conceivably lead to the inhibition of GVBD induction in response to insulin-$p21^{ras}$-PC-PLC but not to progesterone. Results from Table 3 indicate that this is actually the case. Thus, microinjection of transforming $p21^{v\text{-}H\text{-}ras}$ or *B. cereus* PC-PLC promotes a potent maturation response comparable to that produced by the addition of insulin or progesterone. Interestingly, depletion of ζ-PKC levels by microinjection of its antisense RNA significantly inhibited GVBD induction in response to microinjection of transforming $p21^{v\text{-}H\text{-}ras}$ or *B. cereus* PC-PLC or to the addition of insulin. Again, a good control of the specificity of this effect is that GVBD induction in response to progesterone was not affected by ζ-PKC depletion.

DISCUSSION

Great effort is being devoted by several groups to identify critical steps in mitogenic signal transduction pathways. Recently, evidence has accumulated showing that activation of PC degradation through a still poorly characterized PC-PLC is sufficient to mimic a significant portion of the PDGF mitogenic signal in fibroblasts (14). PC-PLC has also been shown to be stimulated by the product of the *ras* oncogene, $p21^{ras}$ (5, 12, 16, 24), whose role in mitogenic cascades has been demonstrated (26). More recent results with *Xenopus* oocytes show that PC-PLC activation is both required and sufficient for mitogenic signal transduction (9). Therefore, all these data permit one to suggest that PC-PLC activation could be critically involved in pathways controlling cell growth and tumor transformation.

Since PC hydrolysis generates diacylglycerol, a logical hypothesis should involve PKC as an important intermediary in mitogenic signalling through PC-PLC. From all the results shown here it appears that PKC isoenzymes α, β, and γ are not involved in the maturation pathway activated by insulin-p21^{ras}-PC-PLC. Neither the δ nor the ε PKC isotype appears to be involved, since no detectable amounts of these subspecies were found in stage VI oocytes. Consistent with these data is the fact that screening the oocyte cDNA library utilized to clone the *Xenopus* homolog of ζ-PKC with probes specific for isotype δ or ε reveals the complete absence of these PKC isotypes in *Xenopus* oocytes (6a). Furthermore, microinjection of peptide pseudosubstrate inhibitors corresponding to PKC subspecies δ and ε produced little or no effect on maturation or H1 kinase induction in response to any of the stimuli described in this study (not shown). The data presented here are consistent with our recent evidence that depletion by long-term incubation of fibroblasts with PMA of PKC isotypes that are recognized in blots with an antibody specific for α+β+γ does not affect the ability of PC-PLC to induce DNA synthesis (14) or the capability of p21^{ras}-PC-PLC to activate stromelysin gene expression (6).

On the other hand, it is shown here that PMA is unable to promote maturation in *Xenopus* oocytes unless a purified fraction of PKC from rat brain tissue has previously been microinjected. Immunoblot analysis of this PKC preparation reveals a band recognized by an antibody specific for isotypes α, β, and γ (not shown). Therefore, the ability of PMA to induce maturation under these conditions is most likely due to an overload of PKC isotypes α+β+γ. This is in agreement with previous data showing that expression of a cDNA corresponding to a permanently activated deletion mutant of αPKC activates at least some steps of the oocyte maturation program (17). The potential involvement of PKC in oocyte maturation has been addressed by a number of groups, and controversial data have been published (10, 25, 28, 31). Thus, whereas some authors claim that the oocyte PMA-sensitive PKC activity is critically involved in the induction of maturation (28), others suggest that, if anything, it is inhibitory (31). It has even been suggested that inhibition of the oocyte PMA-sensitive PKC activity leads by itself to maturation (31). The estimation of this parameter by the detection of a white spot in the animal pole, suggestive of the actual breakdown of the germinal vesicle (GVBD), can sometimes be misleading. Thus, it is very well known that PMA induces in the oocyte cortical granule exocytosis (1), cleavage furrow formation (1), and extreme mottling of the animal hemisphere (1, 25) while having an intact germinal vesicle (for a clear discussion on this matter, see reference 25). In order to obtain unequivocal data we chose to measure in the study reported here first the key biochemical event that regulates oocyte maturation: stimulation of p34-dependent H1 kinase activity (18). From these experiments, it is clear that neither PMA induces this parameter or GVBD nor do specific inhibitors of the classical PMA-sensitive PKC isotypes α, β, and γ affect oocyte maturation pathways controlled by p21^{ras}. However, it is clear that the oocyte PMA-sensitive PKC is effectively blocked not only in vitro but also in vivo since treatment of oocytes with either GF 109203X or peptide A but not with peptide Z inhibits the mottling of the animal hemisphere characteristic of PMA action (1). Although the concentration of inhibitors utilized in in vivo experiments is higher than those used in in vitro assays, this is a quite usual issue when analyzing the effects of drugs on functional parameters (see for example reference 30). That is why it is so important to carry out in parallel positive and negative controls. In the study reported here we have been especially careful on this matter. Thus, to investigate the PKC isotype involved in the activation of oocyte maturation in response to insulin-p21^{ras}-PC-PLC, two critical determinations were made: (i) effect of the inhibitory molecules on a reconstituted PMA-sensitive PKC pathway and (ii) effect on a completely independent route triggered by progesterone, which provides a good negative control of specificity and lack of toxicity.

Taken together, these results strongly suggest that no classical PMA-sensitive PKC present in *X. laevis* oocytes appears to be involved in oocyte maturation in response to PC-PLC-controlled mechanisms. A new distantly related member of the PKC family of isoenzymes (21), ζ-PKC, displays a number of interesting characteristics. Thus, a relatively detailed analysis of the biochemical properties of this PKC isotype shows that it does not bind PMA (21), is not stimulated by this pharmacological agent, and is resistant to down-regulation by phorbol esters (6a). In this regard, it is noteworthy that recent studies are beginning to characterize a PKC from *Saccharomyces cerevisiae* which, like ζ-PKC, is insensitive to PMA, and genetic studies strongly suggest that it may play a critical role in cell cycle control (15, 20). Remarkably, we show here that depletion of ζ-PKC subspecies or microinjection of a peptide inhibitor with a sequence specific for the pseudosubstrate region of that PKC isotype inhibited the ability of insulin-p21^{ras}-PC-PLC to activate oocyte maturation with no effect on the pathway activated by progesterone. Therefore, this enzyme seems to play a critical role in the control of proliferative cascades. Further work is undoubtedly necessary to clarify the precise mechanisms utilized by ζ-PKC to transmit the mitogenic signals triggered by insulin-p21^{ras}-PC-PLC that lead to the activation of maturation-promoting factor H1 kinase and GVBD. In any event, this is the first report demonstrating the requirement of this PKC isotype in mitogenic signalling.

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