Activation of protein kinase Cη triggers cortical granule exocytosis in *Xenopus* oocytes

Cameron B. Gundersen*, Sirus A. Kohan, Qian Chen, Joseph Iagnemma and Joy A. Umbach

Department of Molecular & Medical Pharmacology, UCLA School of Medicine, Los Angeles, CA 90095, USA

*Author for correspondence (e-mail: cgundersen@mednet.ucla.edu)

Accepted 12 December 2001

Journal of Cell Science 115, 1313-1320 (2002) © The Company of Biologists Ltd

Summary

Previous work has shown that phorbol esters or diacylglycerol trigger cortical granule exocytosis in *Xenopus* oocytes. We sought to identify the isoform(s) of protein kinase C (PKC) that mediate(s) this regulated secretory event. Because this process is initiated by lipid activators of PKC but is independent of calcium ions, we focused on the family of novel (calcium-independent) PKCs. Pharmacological investigations using Gö6976 and Gö6983 tended to exclude PKCδ, ε and μ as secretory triggers. Subcellular fractionation and immunoblot data revealed that these oocytes expressed all five members of the novel PKC family, but it was only PKCη that colocalized with cortical granules. Finally, expression of wild type or constitutively active forms of PKCδ and η strongly supported the conclusion that it is PKCη that initiates cortical granule exocytosis in these cells. These observations represent an important step in identifying the mechanism of secretory triggering in this system.

Key words: Secretion, Cortical reaction, protein kinase C

Introduction

The cortical reaction is an early response to fertilization in eggs of many species (Schuel, 1978). This process involves the exocytotic discharge of the contents of cortical granules, which produces a physical impediment to polyspermy (Grey et al., 1974; Grey et al., 1976; Epel and Johnson, 1976; Schuel, 1978). Because of their large size and relative ease of manipulation, eggs from amphibian species and sea urchins have received considerable attention in the study of the cortical reaction (Grey et al., 1974; Grey et al., 1976; Epel and Johnson, 1976; Schuel, 1978). Under physiological conditions, it appears that cortical granule exocytosis is a Ca^{2+}-dependent event that is initiated by binding of the sperm to the gamete surface (Whitaker and Steinhardt, 1980; Schmidt et al., 1982; Jaffe, 1983). Interestingly, Bement and Capco (Bement and Capco, 1989) reported that they could mimic this effect of sperm by treating *Xenopus* eggs, as well as follicle-free oocytes, with activators of protein kinase C (PKC). Thus, agents, such as phorbol 12-myristate 13-acetate (PMA) and 1-olyeoyl-2-acetyl-*sn*-glycerol, which are known to activate many isoforms of PKC (Blumberg, 1988; Stabel and Parker, 1991; Nishizuka, 1995), triggered cortical granule exocytosis in *Xenopus* eggs and oocytes (Bement and Capco, 1989).

As an important step toward clarifying the mechanism by which activators of PKC trigger cortical granule exocytosis in *Xenopus* oocytes, we sought to identify the isoform(s) of PKC that participate in this regulated secretory event. This endeavor was complicated by the fact that the family of PKC enzymes includes more than 10 members (Nishizuka, 1995; Jaken, 1996), many of which are expressed in oocytes and eggs of *Xenopus* (Chen et al., 1989; Sahara et al., 1992; Dominguez et al., 1992; Stith et al., 1997; Johnson and Capco, 1997). For instance, Johnson and Capco (Johnson and Capco, 1997) reported that *Xenopus* oocytes express six isoforms of PKC, including: (i) all four members (α, β_1, β_2 and γ) of the conventional PKC (cPKC) sub-family (whose members are activated by both Ca ions and diacylglycerol or phorbol esters) (Nishizuka, 1995); (ii) one member of the novel (or new) PKC family (δ, which is activated by diacylglycerol or phorbol esters, but not by Ca) (Nishizuka, 1995) and (iii) the zeta isoform of PKC which belongs to the atypical group of PKC isozymes which are insensitive to both Ca and phorbol esters (Nishizuka, 1995). To identify the PKC isoform (or isoforms) that regulates the cortical reaction in *Xenopus* oocytes, we used a combination of pharmacological, immunoblot and heterologous expression strategies. Our data indicate that the eta (η) isoform of PKC is centrally involved in triggering cortical granule exocytosis in these cells.

Materials and Methods

Isolation and culturing of *Xenopus* oocytes

Xenopus laevis oocytes were obtained by incubating fragments of ovary in Barth's solution (88 mM NaCl, 1 mM KCl, 24 mM $NaHCO_3$, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$ and 15 mM Hepes; pH 7.5 with 1 M NaOH to which is added the antibiotics gentamicin (0.1 mg/ml) and enrofloxacin (0.01 mg/ml)) with 3-5 mg/ml Type 1 collagenase (Sigma). After 2-4 hours at 20-22°C, the oocytes were rinsed 1-2 times with Barth's solution and incubated in 0.1 M potassium phosphate (pH 6.0) with 1mg/ml bovine serum albumin (Type V; Sigma). After 20-30 minutes at 20-22°C, the oocytes were rinsed at least three times with Barth's solution and stage V-VI oocytes (DuMont, 1972) that were completely devoid of follicle cell investments were collected and maintained at 17-18°C in Barth's solution plus antibiotics. The Barth's solution was replaced daily. For some experiments, stage II oocytes (Dumont, 1972) were collected by manual dissection using watchmaker forceps. These were early stage II oocytes (300-400 μm) with no pigment accumulation.

Assay of secretion

Protein secreted from oocytes in response to PMA (Sigma) was assayed using SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) by A_{280} or by the Bio-Rad dye-binding assay. For gel analysis (unless otherwise indicated), individual oocytes were incubated in 10-20 μl of Barth's solution (or OR-2 solution, see below) with PMA at the concentration specified. After 30-60 minutes, the solution was collected and mixed with 5× concentrated Laemmli (Laemmli, 1970) sample buffer (with 10-20 mM DTT as indicated) and incubated for 5 minutes at 70°C. Samples were resolved on 10-12.5% gels using molecular weight markers from Amersham-Pharmacia and either stained with rapid Coomassie stain (Diversified Biotech) or transferred to nitrocellulose for immunoblot analysis of cortical granule lectin (cgl) as described (Gundersen et al., 2001). To detect protein release using the Bio-Rad dye-binding assay, groups of 10-15 oocytes were incubated in 0.1-0.15 ml of OR-2 solution (83 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, pH 7.4 with NaOH) with PMA at the concentrations indicated. After 30-60 minutes, the fluid surrounding the oocytes was diluted into water for the protein assay using the Bio-Rad dye reagent and human IgG as a standard. Independent estimates of the amount of protein released from oocytes was made by incubating groups of 50 oocytes in 0.5 ml of OR-2 with 1 μM PMA for 30 minutes and reading the absorbance at 280 nm. For this assay we assumed that one A_{280} unit corresponded to 0.8 mg/ml protein. After the absorbance measurements, samples of this solution were also submitted to the Bio-Rad dye-binding assay and to electrophoretic detection of released protein. When PKC inhibitors (staurosprine, Gö6976, Gö6983 from Calbiochem) were used, they were prepared as 10 mM stocks in DMSO and used under low light conditions. Oocytes were preincubated (10-15 minutes) with these drugs prior to exposure to PMA (20 nM). IC_{50}s for these drugs were determined by densitometric analysis of Coomassie-stained cgl resolved on SDS-gels. Release of cgl from individual inhibitor-treated oocytes was normalized to the mean value for controls without inhibitor. Results are the mean±s.d. from at least 15 oocytes for each drug concentration.

Subcellular fractionation and immunoblot analysis

We used a modified fractionation protocol that effectively sediments cortical granules from triturated oocytes (Gundersen et al., 2001) (Fig. 2). Single oocytes were disrupted by repeated passage (8-12 times) through the orifice of a yellow pipet tip using 0.1 ml of HB (0.25 M sucrose, 10 mM Hepes, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM phenyl-methyl-sulfonylfluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, pH 7.4) followed by centrifugation for 1 minute at 14,000 ***g*** at 20-22ºC. The supernatant was recovered and the pellet was extracted with 0.1 ml of 10 mM Chaps (or 0.1% TritonX-100) in 0.1 M NaCl, 50 mM Tris, 1 mM EDTA (pH 7.4) by trituration and centrifugation (1 minute at 14,000 ***g***) to sediment yolk platelets and pigment granules that did not completely solubilize. Protein was recovered (Wessel and Flugge, 1984) separately from the supernatant and detergent extracts and subjected to immunoblot analysis (Gundersen et al., 2001) using PKC isoform-specific antibodies from Santa Cruz Biotechnology and ECL or ECL Plus (Amersham-Pharmacia) for detection. For these analyses, the secondary antibody was preadsorbed using oocyte acetone powder as described (Gundersen et al., 2001). As a positive control for the PKC antibodies, we used a *Xenopus* brain P_2 fraction prepared as in Gundersen et al. (Gundersen et al., 2001). As a negative control, the PKC isoform-specific antibodies were pre-adsorbed against the peptide immunogen following the supplier's protocol. Tests for expression of PKC isoforms in stage II oocytes used extracts of 20-30 oocytes homogenized directly in SDS sample buffer.

PKC constructs and expression of PKC isoforms in oocytes

Plasmid DNA containing inserts encoding wild type or constitutively active forms of PKCδ and η were graciously supplied by P. Parker (Imperial Cancer Research Fund, London). The wild-type and constitutively active PKCη cDNAs (Dekker et al., 1992) were in pKS-1 (a pUC derivative) and were linearized with *Xba*I for production of sense cRNA transcripts using T7 RNA polymerase and the mMessage Machine kit (Ambion). Wild-type and constitutively active PKCδ cDNAs that were originally in the pCO_2 vector (Schönwasser et al., 1998) had been sub-cloned into pBS such that linearization with *Bam*Hl allowed for production of sense cRNA using the T7 mMessage Machine (Ambion). RNA was recovered by LiCl precipitation and adjusted to about 1mg/ml in water for injection of oocytes. Individual oocytes were injected with 10-20 ng of cRNA and used for immunoblot analysis to detect expression of the PKC isoforms and for evaluation of cgl secretion. The latter was achieved by incubating oocytes in 20 μl of Barth's solution and recovering this fluid after 20-24 hours for gel electrophoresis and Coomassie staining of cgl. Oocytes were extracted (as above) for immunoblot analysis of the expressed PKC isoforms.

Autophosphorylation assay

Recombinant PKCη (25 ng; from Calbiochem) was incubated in the buffer in which it was provided with 5 μCi of ^{32}P-ATP for 10 minutes at 20-22°C and resolved electrophoretically for autoradiography. PKC antagonists were added 5 minutes before the ATP and the percentage inhibition of the autophosphorylation reaction was quantitated by densitometry.

Results

PMA-evoked secretion of protein from oocytes

The results of Fig. 1 confirm and extend the observations of Bement and Capco (Bement and Capco, 1989) that PMA triggers the secretion of protein from *Xenopus* oocytes. First, it is evident (Fig. 1) that in the absence of PMA, there is no detectable secretion of protein (as judged by Coomassie staining) or cgl (western) over the 30 minute collection period. However, the addition of PMA (1 μM) elicits secretion of protein and immunoreactive cgl from stage II and stage VI oocytes. Note that quantitatively, the results of Fig. 1 were obtained using single stage VI oocytes, whereas the data for the early stage II oocytes came from groups of 75 oocytes (0.3 to 0.40 mm in diameter). In the absence of the reducing agent DTT, most of the secreted protein remained as high mass species (>250 kDa) for both stage II and stage VI oocytes (Fig. 1; Coomassie staining). However, with DTT, the bulk of the secreted protein migrated as a broad band between 35 and 50 kDa with a lower intensity cluster of bands above 250 kDa (Fig. 1; Coomassie staining). Independently, immunoblot detection identified cgl immunoreactivity associated with material at about 100 kDa, 200 kDa and >250 kDa in unreduced samples of secreted protein from stage II or stage VI oocytes exposed to PMA. As expected from work of others (Nishihara et al., 1986; Chamow and Hedrick, 1986), treatment with DTT reduced much of the oligomeric cgl-immunoreactive complexes to a broad band between 35 and 50 kDa, which reflects the variable glycosylation of the cgl monomers. Note also that higher mass cgl immunoreactive material persisted even after treatment with DTT (Fig. 1). This broad smear of cgl immunoreactive material corresponds to the secreted proteins observed in the 250 kDa range on the Coomassie stained gel (Fig. 1). From these data we conclude that even at the earliest stage at which cortical granules are observed in

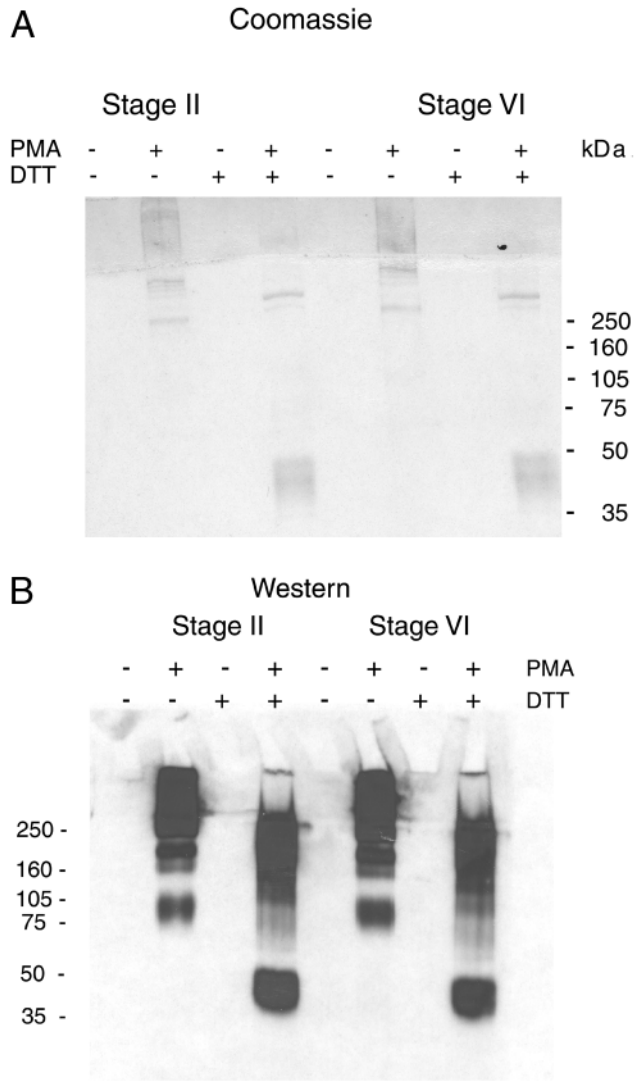

Fig. 1. PMA-induced secretion of protein and immunoreactive cortical granule lectin from *Xenopus* oocytes. Groups of 75 stage II or single stage VI oocytes were incubated without or with PMA (1 μM) as indicated. After 30 minutes, the fluid surrounding the oocytes was collected for analysis by SDS-polyacrylamide gel and Coomassie staining or by western blot using antibodies against cgl. Samples were treated with DTT, as indicated. Mobility of molecular weight standards is indicated in kDa.

Xenopus oocytes (namely in early stage II oocytes) (DuMont, 1972), it is possible to elicit the secretion of the contents of these organelles using PMA.

We sought independently to quantify protein secretion from oocytes exposed to PMA. As a first step in this direction, we were interested in the extent to which PMA induced cortical granule exocytosis in stage VI oocytes. The results of Fig. 2 show that while all of the detectable cgl immunoreactivity was retained in the supernatant and pellet fractions of a control oocyte, more than 90% of the cgl immunoreactivity was released from an oocyte exposed for 30 minutes to PMA (1 μM). In six separate trials, densitometry indicated that control oocytes secreted no detectable cgl, whereas PMA-treated oocytes released 80±10% of their total cgl immunoreactivity.

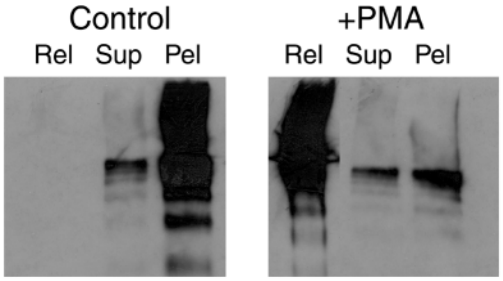

Fig. 2. Subcellular distribution of cortical granule lectin immunoreactivity before and after PMA-induced secretion. Single stage VI oocytes were incubated without or with PMA (1 μM) for 30 minutes as indicated. After recovering the fluid surrounding each oocyte (Rel), the oocytes were fractionated (see Materials and Methods) to generate supernatant (Sup) and pellet (Pel) fractions for immunoblot analysis using cgl antibody. Samples were not reduced with DTT (see Fig. 1). The +PMA example shown here reflects the most extensive release of cgl immunoreactivity observed in six trials.

Thus, PMA is an efficient trigger of cortical granule exocytosis in these cells. To quantify the amount of protein released, we used either the Bio-Rad protein assay or absorbance at 280 nm. In eight separate measurements using oocytes from three different frogs, A_{280} analyses indicated that PMA (1 μM for 30 minutes) evoked the secretion of 1.25±0.20 μg of protein per stage VI oocyte. Interestingly, when the same samples that had been submitted to A_{280} readings were subsequently analyzed using the Bio-Rad protein assay (with human IgG as the standard), protein release per stage VI oocyte was 0.70±0.06 μg. A possible explanation for the discrepancy in these estimates of protein secretion is addressed in the Discussion.

Pharmacological and ionic modulation of cortical granule exocytosis

As a first step toward identifying which isoform(s) of PKC participate in the PMA-triggered secretory process documented above (see also Bement and Capco, 1989), we conducted a series of experiments to investigate the impact of a variety of ionic and pharmacological manipulations.

Prior work by Bement and Capco (Bement and Capco, 1989; Bement and Capco, 1990) and Scheuner and Holz (Scheuner and Holz, 1994) strongly suggested that PMA-triggered cortical granule exocytosis was independent of Ca ions in stage V-VI oocytes. This is because the Ca ionophore A-23187, which promotes cortical granule exocytosis in *Xenopus* eggs, is ineffectual in oocytes (Bement and Capco, 1989). Similar results were obtained using ionomycin (Scheuner and Holz, 1994). Concomitantly, buffering of cytosolic Ca ions (to low nM concentrations) with BAPTA does not prevent cortical granule exocytosis in *Xenopus* oocytes treated with PMA (Bement and Capco, 1990). These results are significant because they imply that the four conventional PKC isoforms (α, β_1, β_2 and γ), which are Ca-dependent (Takai et al., 1979; for a review, see Nishizuka, 1995), are unlikely to participate in the activation of cortical granule exocytosis in these cells. To extend these observations we found that injection of oocytes with the Ca ion chelator EGTA (to a final concentration of 5-10 mM) did not block PMA-induced secretion of cgl (data not

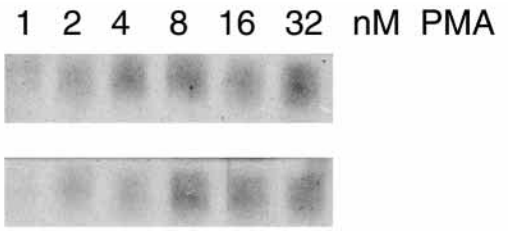

Fig. 3. Concentration-response study of PMA-induced secretion of cortical granule lectin. Single oocytes were incubated in increasing concentrations of PMA, as indicated. After 1 hour, the fluid surrounding each oocyte was collected for analysis by SDS-polyacrylamide gel and Coomassie staining. The results show the smear of cgl between 35 and 50 kDa (see also Fig. 1) for two separate sets of oocytes. Pilot experiments revealed that secretion of cgl induced by 10 nM PMA was complete within 1 hour.

shown). Independently, injection of oocytes with enough Ca ions to achieve a calculated concentration of 1-2mM (in the absence of buffering and assuming a cystolic volume of 0.5 μl) did not trigger cgl exocytosis nor was the Ca ionophore ionomycin (10 μM) an effective secretogogue (data not shown). Collectively, these data indicate that changes of Ca ion activity are not important for the exocytotic release of cgl. These results greatly diminish the likelihood that Ca-dependent isoforms of PKC are involved in cgl secretion in stage VI oocytes.

As a prelude to investigating the effects of more selective PKC inhibitors, we evaluated the threshold concentration for PMA to activate cgl secretion in *Xenopus* oocytes. (This proved to be important because pilot experiments using 1 μM PMA to evoke cgl secretion revealed no effect of inhibitors even at concentrations up to 20 μM.) As illustrated in the two examples of Fig. 3, low nM concentrations of PMA efficiently promoted the release of cgl. In eight experiments using oocytes from four different frogs, the EC_{50} (effective concentration for half-maximal secretion) was 6±2 nM PMA.

Over the last decade, two staurosporine derivatives (Gö6976 and Gö6983) were found to exhibit specificity in their inhibition of Ca-independent subtypes of PKC (Martiny-Baron et al., 1993; Gordge and Ryves, 1994; Gschwendt et al., 1995; Gschwendt et al., 1996). Thus, while Gö6976 inhibited PKCμ with an IC_{50} of 20 nM (Gschwendt et al., 1996), it had a negligible effect (no inhibition at 10 μm) on the activity of PKC δ or ε (Martiny-Baron et al., 1993). By comparison, Gö6983 inhibited PKC δ with an IC_{50} of 100 nM (Gschwendt et al., 1995), but much higher concentrations (>10 μM) were needed to inhibit PKCμ (Gschwendt et al., 1996). Because our pilot studies revealed that staurosporine inhibited PMA-induced secretion of cgl with an IC_{50} of about 1 μM, we inferred that the Gö compounds could provide insight into the PKC isoform(s) that initiate(s) this process. As indicated in Fig. 4, Gö6976 and Gö6983 inhibited PMA-evoked secretion of cgl. The IC_{50} for these agents was 15±5 μM and 1±0.2 μM, respectively. Given the pharmacological sensitivity of the PKC isoforms alluded to above, these results provisionally excluded PKCs δ, ε and μ as triggers of cgl secretion. As a final step in these pharmacological experiments, we determined the IC_{50} for Gö6976 or Gö6983 to block autophosphorylation of commercially available PKCη. For Gö6976 the IC_{50} was 7.5±1 μM, whereas for Gö6983 it was 200±50 nM. As reviewed in the Discussion, the fact that these drugs inhibit PKCη in vitro and antagonize PMA-induced secretion of cgl lend support to the hypothesis that PKCη plays a role in the cortical reaction.

Immunoblot investigations of PKC isoform distribution in *Xenopus* oocytes

The rationale for these experiments was that we could exclude certain PKC isoforms from being involved in the exocytotic release of cgl if these isoforms were not detectably present in oocytes. To this end, we obtained PKC-isoform-specific antibodies and first verified that these antibodies recognized a protein of the appropriate mass in an extract from *Xenopus* brain. Morever, since these were anti-peptide antibodies, we verified that pre-adsorption of the antibody with the peptide immunogen abolished the immunolabeling of the appropriate mass protein in *Xenopus* brains (data not shown). These antibodies were then used to probe soluble and particulate extracts from *Xenopus* oocytes that had been prepared as in Fig. 2. As shown previously (Gundersen et al., 2001) and in Fig. 2, cortical granules (as reflected by the presence of cgl) partition almost exclusively into the pellet in these experiments. Interestingly, all five of the novel isoforms (δ, ε, η, θ and μ) of PKC could be detected in these extracts (Fig. 5A). With the exception of PKCη, these PKC isoforms remained almost exclusively in the supernatant after the brief centrifugation of the oocyte homogenate (Fig. 5A). Since it is often observed that lipid activators of PKCs induce translocation of these enzymes to membranes (Nishizuka, 1995; Jaken, 1996), we investigated whether treatment of oocytes with PMA (1 μM for 30 minutes) would alter the distribution of PKC isoforms shown in Fig. 5A. In no instance did we detect any significant re-distribution of PKC isoform immunoreactivity in response to PMA (Fig. 5A). Thus, whereas the η isoform of PKC is present in a fraction of oocytes that is enriched in cortical granules, none of the other novel isoforms of PKC are significantly distributed in this fraction, even after treatment with PMA (Fig. 5A). The inference from these studies is that PKCη is the only isoform in oocytes that is associated with the structures that participate in the cortical reaction.

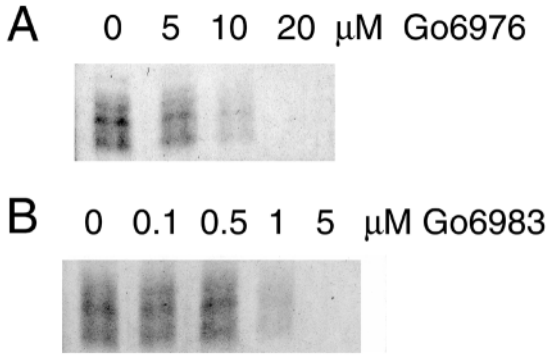

Fig. 4. Effect of Gö6976 (A) and Gö6983 (B) on PMA-induced secretion of cortical granule lectin. Oocytes were preincubated (10-15 minutes) with the indicated concentration of drug and then incubated for 1 hour with the addition of PMA (20 nM). Released cgl was detected as in Fig. 3.

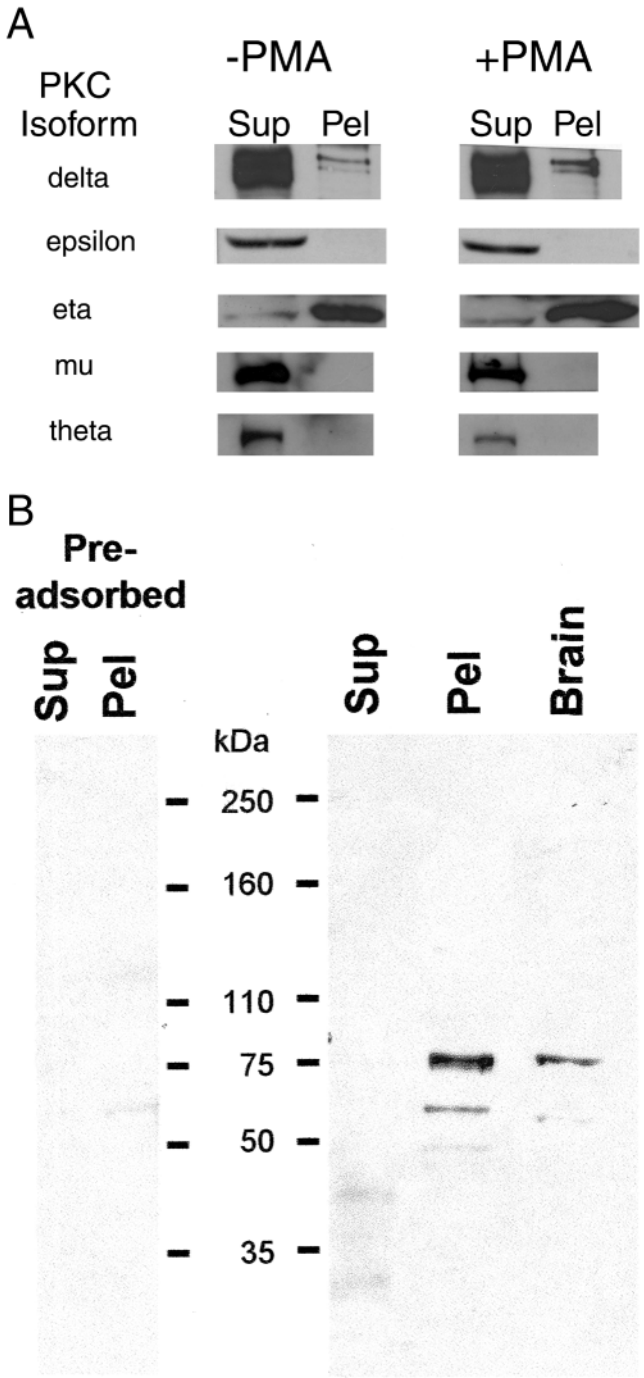

Fig. 5. Detection of PKC isoform immunoreactivity in oocyte fractions with or without exposure to PMA. (A) Single stage VI oocytes were fractionated into supernatant (Sup) and pellet (Pel) fractions as in Fig. 2 before (–PMA) or after treatment with PMA (1 μM for 30 minutes, +PMA). These fractions were tested for the presence of immunoreactivity for PKC isoforms using isoform-specific antibodies. The approximate mass (in kDa) of each isoform was: delta (75); epsilon (80); eta (76); mu (105); theta (79). Preadsorption of the primary antibody with peptide immunogen abolished these bands. (B) PKCγ immunoreactivity in *Xenopus* oocytes and the brain and effect of antibody preadsorption. The results show the immunoreactive bands that are detected in supernatant (sup) and pellet (pel) fractions of oocytes probed with PKCγ antibody with or without preadsorption of the antibody with peptide immunogen. Immunoreactivity in a sample of extract (approximately 25 μg protein) from *Xenopus* brain is shown as a control.

Results in Fig. 5B exemplify the criteria that were used to identify PKC immunoreactive species in oocytes (Fig. 5A). In this example PKCη antibodies identify a strongly immunoreactive species in *Xenopus* brain at 76 kDa and a weaker signal at about 60 kDa. In the oocyte pellet fraction, strong immunoreactivity is detected at 76 kDa, along with weaker signals at about 60 kDa and 52 kDa (Fig. 5B). Oocyte supernatant reveals weakly immunoreactive species at about 47 kDa and 33 kDa (Fig. 5B). Pre-adsorption of the PKCη antibody with the peptide immunogen eliminates all of these immunoreactive entities except for the signal at 52 kDa. Because the deduced mass of PKCη in most organisms is close to 75 kDa (Osada et al., 1990; Bacher et al., 1991; Nishizuka, 1995), we conclude that the prominent, 76 kDa species in frog brain and oocyte pellets is PKCη. Concomitantly, the identity of the other immunoreactive species, which could be due to cross-reactivity of these anti-peptide antibodies or to degradation products of PKCη remains to be clarified.

Because stage II oocytes have the machinery to release cgl in response to PMA (Fig. 1), we reasoned that the PKC isoform(s) responsible for transducing the PMA effect must also be expressed in these early stage oocytes. In this context, we note (data not shown) that stage II oocytes (we used extracts from 20-30 oocytes) detectably expressed immunoreactive PKCη using the same criteria (Fig. 5) for identification of this protein in stage VI oocytes. By the same token, we could also detect PKCδ and PKCε in these early stage oocytes, so this strategy did not enable us to exclude these PKC isoforms from playing a role in the cortical reaction in these cells. Nevertheless, these results verify that PKCη is present in oocytes at the stage when they first become competent to secrete cgl.

Expression of wild-type and constitutively active PKCδ and η in oocytes and their effect on cgl secretion

As addressed in the Discussion, both the pharmacological data and the immunoblot data were compatible with the hypothesis that the η isoform of PKC was important for initiating cgl secretion in this system. To obtain more direct evidence for this conclusion, we investigated whether expression of recombinant, constitutively active PKCη could trigger cgl secretion. As controls, we also over-expressed wild type and constitutively active PKCδ, as well as wild-type PKCη. Results in Fig. 6 show that oocytes expressing constitutively active PKCη secrete cgl. However, oocytes over-expressing (Fig. 6B) wild-type PKCδ or constitutively active PKCδ did not release detectable cgl (Fig. 6A). Although the results in Fig. 6 are representative of data from a total of 15-20 oocytes expressing each PKC isoform, it was interesting that in several oocytes over-expressing wild-type PKCη, there was a low level of cgl secretion (less than half of that obtained with the constitutively active isoform). These latter observations indicate that under some conditions, over-expression of wild-type PKCη is sufficient to trigger cortical granule exocytosis in these frog oocytes. Taken together, these data support the hypothesis that

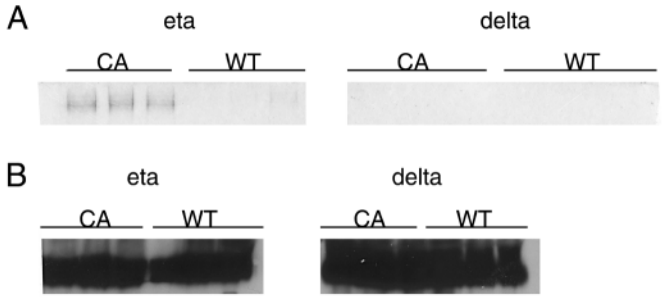

Fig. 6. Effect of over-expressing various PKC constructs on cortical granule lectin secretion from oocytes. Individual stage VI oocytes were injected with cRNA encoding wild-type (WT) or constitutively active (CA) PKC eta or delta. After incubation for 20 hours, the fluid surrounding each oocyte was collected for detection of released cgl (A). The oocytes were extracted for immunoblot analysis to verify the expression of PKC eta or delta (B). Under the conditions of immunoblot exposure in (B), endogenous expression of PKC eta or delta was essentially undetectable. Densitometric analysis revealed that injected oocytes expressed more than 50 times the endogenous level of each PKC isoform. Endogenous expression of these proteins is documented in Fig. 5.

PKCη is prominently involved in triggering cortical granule discharge in these cells.

Discussion

The goal of this investigation was to identify which isoform of PKC is activated by the phorbol ester PMA to trigger cortical granule exocytosis in *Xenopus* oocytes. These observations are of interest because there remains considerable debate about the identity of the proteins that trigger regulated secretion in cells as diverse as oocytes, neurons, pancreatic β cells and mast cells. For instance, synaptotagmin I has for some time been regarded as a likely 'Ca ion sensor' for exocytosis at nerve endings (Sudhof and Rizo, 1996), but it is only recently that compelling evidence has emerged that supports this proposal (Fernandez-Chacon et al., 2001). Independently, our data strongly support the conclusion that the η isoform of PKC is centrally involved in triggering cortical granule exocytosis in *Xenopus* oocytes. An important goal of future efforts is to identify the target(s) of PKCη that transduce its actions on the secretory machinery. These efforts will benefit from the fact that PKCη-dependent protein phosphorylation should produce a distinctive pattern of phosphorylated targets. This contrasts with synaptotagmin, whose downstream action, once it binds to calcium ions, remains conjectural (Fernandez-Chacon et al., 2001). Thus, PKCη-dependent protein phosphorylation holds considerable promise for characterizing downstream effectors in the regulated secretory cascade.

As outlined in the Introduction, the identified isoforms of PKC have been segregated into three categories that reflect the sensitivity of these enzymes to activation by Ca ions and lipid modulators, such diacylglycerol and phorbol esters (Stabel and Parker, 1991; Gordge and Ryves, 1994; Nishizuka, 1995; Jaken, 1996). Thus, the rate of substrate phosphorylation by conventional isoforms of PKC is regulated both by Ca ions and agents like PMA or diacylglycerol. Novel isoforms of PKC are insensitive to changes of Ca ion activity but show enhanced substrate phosphorylation in the presence of appropriate lipid activators. And, atypical PKCs are unaffected by either Ca ions or lipid activators. These criteria led us to focus on novel PKCs as the likely mediators of PMA-induced cortical granule discharge in *Xenopus* oocytes. This is because secretion of cgl, the major protein stored in cortical granules (Greve and Hedrick, 1978; Nishihara et al., 1986; Chamow and Hedrick, 1986) is elicited by low nM concentrations of PMA, but it is unaffected by treatments that alter Ca ion activity. For instance, use of BAPTA (Johnson and Capco, 1997) or EGTA to clamp cytosolic ionized Ca below the resting level did not interfere with PMA-evoked secretion of cgl. Moreover, neither injection of Ca ions nor use of the Ca ionophores A-23187 (Johnson and Capco, 1997) or ionomycin elicited cgl release. This lack of sensitivity to changes of Ca ion activity implies that the Ca-dependent family of PKCs is not centrally involved in secretory triggering in oocytes. Concomitantly, the fact that cgl secretion is initiated by PMA (or diacyglycerol) (Bement and Capco, 1989) excludes the atypical PKC isoforms, which are insensitive to these lipid mediators. By this process of elimination, the novel PKCs emerged as the most plausible targets of PMA action.

The efforts to identify pharmacological agents that selectively target specific PKC isoforms has culminated in the selection of several drugs that inhibit a subset of PKCs (Gordge and Ryves, 1994). Thus, derivatives of staurosporine (a broad spectrum protein kinase inhibitor, which blocked cgl secretion in the low micromolar range), like Gö6976 and Gö6983, appear to be selective antagonists of certain PKC isoforms (Martiny-Baron et al., 1993; Gordge and Ryves, 1994; Gschwendt et al., 1995; Gschwendt et al., 1996). In our experiments, Gö6976 significantly inhibited cgl secretion with an IC_{50} of 15 μM. Since in vitro kinase assays of PKC δ and ε revealed that these enzymes were unaffected by Gö6976 up to 10 μm ((Martiny-Baron et al., 1993); see also (Gschwendt et al., 1995) for conflicting results concerning PKCδ), these observations suggested that neither δ nor ε was involved in secretory triggering in oocytes. Similarly, Gö6983 was more potent than Gö6976 as an inhibitor of PMA-evoked cgl secretion in our experiments. Since in vitro phosphorylation assays revealed that Gö6976 was three orders of magnitude more potent than Gö6983 as an inhibitor of PKCμ (Gschwendt et al., 1996), our findings mitigated against a role for PKCμ in oocyte cgl secretion. Thus, by a process of elimination, the pharmacological data favored PKCη or θ as secretory triggers in oocytes. We independently verified that Gö6976 and Gö6983 blocked autophosphorylation activity of recombinant PKCη in a range compatible with the inhibitory effects of these drugs on cgl secretion. However, similar experiments were not performed for PKCθ (which is not available commercially), and this precluded us from drawing firm conclusions about the identity of the isoform of PKC that evokes secretion based on the pharmacology alone.

In our next set of experiments, we sought to determine which of the novel PKC isoforms were expressed in oocytes. For these experiments, we first ensured that the isoform-specific antibodies detected a protein of the appropriate mass in extracts from *Xenopus* brain and that this antibody detection was occluded by an excess of the immunogen. With these criteria of antibody specificity, it was interesting that we detected immunoreactivity corresponding to all five of the novel PKC isoforms in oocytes. In a previous study, PKCδ was detected

in oocyte extracts whereas PKCε and θ were not detectable (Johnson and Capco, 1997). The apparent discrepancy between our observations and this earlier report (Johnson and Capco, 1997) can almost certainly be attributed to the different antibodies used in the current experiments. Of greater interest was the fact that there was a distinctive subcellular distribution of the novel PKC isoforms. Using a simple sedimentation scheme, we found that almost all of the immunoreactivity corresponding to PKCs δ, ε, μ and θ remained in the supernatant. Under identical conditions, almost all of the PKCη immunoreactivity was found in the pellet. (As discussed in the review by Jaken (Jaken, 1996), it will be important to determine the basis for this differential subcellular distribution of PKC isoforms.) Interestingly, the pellet fraction that was obtained in these experiments was highly enriched in cortical granules (as judged by the presence of cgl) (Fig. 2) (Gundersen et al., 2001), and plasma membrane (on the basis of the presence of Na-K ATPase immunoreactivity; C.G., unpublished). Thus, PKCη colocalized with elements (cortical granules and plasma membrane) that are likely to harbor substrates of potential importance for triggering of cortical granule exocytosis. Moreover, we obtained no evidence that any of the other PKC isoforms redistributed into this fraction in a PMA-dependent fashion. Taken together, the fact that PKCη is found in a subcellular fraction enriched in cortical granules and plasma membrane and that none of the other novel PKCs are significantly present in this fraction supports the conclusion that PKCη participates in initiating cortical granule exocytosis in these cells.

The strongest support for the hypothesis that PKCη initiates cortical granule exocytosis is the observation that constitutely active PKCη triggers cgl secretion and that, even in some cases, over-expression of wild-type PKCη leads to detectable secretion of cgl. In contrast, high-level expression of wild-type or constitutively active PKCδ did not induce secretion of cgl. These latter results imply that there is sufficient substrate specificity between PKCδ and PKCη such that PKCδ does not initiate secretion in this system. Alternatively, it may be that PKCδ does not have access to the appropriate protein substrates, whereas PKCη does have access. In either case, these results indicate that activity of PKCη is sufficient to elicit the regulated secretion of cgl in these frog oocytes.

Relative to the seminal reports of PMA-induced cortical granule exocytosis in *Xenopus* oocytes (Bement and Capco, 1989; Bement and Capco, 1990) there are two issues worth noting. First, in our experience, this secretory event can be initiated using low nanomolar concentrations of PMA. In contrast, Bement and Capco (Bement and Capco 1989; Bement and Capco, 1990) used 0.1-3 μM PMA, which clearly triggers this exocytotic event, as well as other changes in these cells, such as cortical contraction and cleavage furrow formation. It remains to be determined whether PKCη also contributes to these other physiological events. A second issue is that we observed a considerable disparity between the levels of protein secretion from oocytes measured using absorbance at 280 nm relative to the Bio-Rad assay. Indeed, our A_{280} results were similar to those of Bement and Capco (Bement and Capco, 1989), who reported that single oocytes secreted about 1.5 μg of protein in response to PMA. (Here, it is noteworthy that for a cell as large as these oocytes, one can calculate that if cgl was packed in a hollow sphere 1 μm thick that was located immediately below the plasma membrane, this sphere could encapsulate approximately 5 μg of protein (this is obtained by subtracting the volume of a sphere of 1.298 mm from a sphere of 1.300mm and assuming that cgl has a density of 1.2 gm per cm^3); the fact that one detects secretion of 25-30% of this amount of protein, which ignores the glycosylation of cgl, indicates that these granules are very abundant in these cells.) A plausible explanation for our A_{280}-Bio-Rad disparity can be seen in the results in Fig. 1, where the overall intensity of Coomassie staining of secreted protein is higher in samples treated with DTT. Since the Bio-Rad dye-binding assay is incompatible with reducing agents, the lower apparent level of protein secretion is probably due to diminished binding of dye to un-reduced protein samples. Nevertheless, our electrophoretic data show that one can readily detect protein secreted from single stage VI oocytes (whereas Bement and Capco pooled exudates from 80 oocytes) and that even stage II oocytes are competent to secrete protein, the bulk of which is cgl.

A final comment concerns prior observations about PKCη, which was first identified as a novel PKC isoform whose mRNA was prominently expressed in lung, skin and heart (Osada et al., 1990; Bacher et al., 1991). Subsequent work with PKCη-specific antibodies indicated a high level of expression of this protein in cytosolic fractions of brain (Zang et al., 1994). Curiously, a later investigation revealed that PKCη was almost exclusively associated with membranes in brain and other tissues of mice (Frevet and Kahn, 1996). While this apparent discrepancy in the subcellular distribution of PKCη in the brain needs to be resolved, work using a mast cell line showed that over-expression of PKCη enhanced secretory responses of these cells (Chang et al., 1997). Thus, it will be interesting to assess further the role of PKCη in regulated secretory events in oocytes, mast cells and elsewhere.

We thank V. Niggli and H. Porzig (University of Bern) for providing reagents for the pharmacological studies. P. Parker (Imperial Cancer Research Fund, London) graciously supplied the PKC constructs and J. Hedrick (University of California, Davis) kindly supplied the cgl antibodies. This work was supported by NIH grants MH59938 (to Cameron Gundersen) and NS31934 (to Joy Umbach).

References

Bacher, N., Zisman, Y., Berent, E. and Livneh, E. (1991). Isolation and characterization of PKC-L, a new member of the Protein Kinase c-related gene family specifically expressed in lung, skin, and heart. *Mol. Cell. Biol.* **11**, 126-133.

Bement, W. M. and Capco, D. G. (1989). Activators of protein kinase c trigger cortical granule exocytosis, cortical contraction, and cleavage furrow formation in xenopus laevis oocytes and eggs. *J. Cell Biol.* **108**, 885-892.

Bement, W. M. and Capco, D. G. (1990). Protein kinase c acts downstream of calcium at entry into the first mitotic interphase of *Xenopus laevis*. *Cell Reg.* **1**, 315-326.

Blumberg, P. (1988). Protein kinase c as the receptor for the phorbol ester tumor promoters: sixth rhoads memorial award lecture. *Cancer Res.* **48**, 1-8.

Chamow, S. M. and Hedrick, J. L. (1986). Subunit structure of a cortical granule lectin involved in the block to polyspermy in *Xenopus laevis* eggs. *FEBS Lett.* **206**, 353-357.

Chang, E., Szallasi, Z., Acs, P., Raizada, V., Wolfe, P. C., Fewtrell, C., Blumberg, P. and Rivera, J. (1997). Finctional effects of overexpression of protein kinase C-α, -β, -δ, -ε, and -η in the mast cell line RBL-2H3. *J. Immunol.* **159**, 2624-2632.

Chen, K., Peng, Z., Lavu, S. and Kung, H. (1989) Molecular cloning and

sequence analysis of two distinct types of *Xenopus laevis* protein kinase C. *Second Messengers Phosphoproteins* **12**, 251-260.

Dekker, L.V., Parker, P. J. and McIntyre, P. (1992). Biochemical properties of rat protein kinase C-η expressed in COS cells. *FEBS Lett.* **312**, 195-199.

Dominguez, I., Diaz-Maco, M. T., Munico, M. M., Berra, E., Garcia de Herreros, A. G., Cornet, M. E., Sanz, L. and Moscat, J. (1992). Evidence for a role of protein kinase C ς subspecies in maturation of *Xenopus laevis* oocytes. *Mol. Cell Biol.* **12**, 3776-3783.

DuMont, J. N. (1972). Oogenesis in *Xenopus laevis* (Daudin). *J. Morphol.* **136**, 153-180.

Epel, D. and Johnson, D. J. (1976). Reorganization of the sea urchin egg surface at fertilization and its relevance to the activation of development. In J. S. Cook (Ed.): Biogenesis and Turnover of Membrane Macromolecules. (Raven, New York) pp. 105-120.

Fernandez-Chacon, R., Konigstorfer, A., Gerber, S. H., Garcia, J., Matos, M. F., Stevens, C. F., Brose, N., Rizo, J., Rosenmund, C. and Sudhof, T. C. (2001). Synaptotagmin I functions as a calcium regulator of release probability. *Nature* **410**, 41-49.

Frevet, E. U. and Kahn, B. B. (1996). Protein kinase C isoforms ε, η, δ and ς in murine adipocytes: expression, subcellular localization and tissue-specific regulation in insulin-resistant states. *Biochem. J.* **36**, 865-871.

Gordge, P. C. and Ryves, W. J. (1994). Inhibitors of protein kinase C. *Cell. Signal.* **6**, 871-882.

Greve, L. C. and Hedrick, J. L. (1978). An immunocytochemical localization of the cortical granule lectin in fertilized and unfertilized eggs of *Xenopus laevis*. *Gamete Res.* **1**, 13-18.

Grey, R. D., Wolf, D. P. and Hedrick, J. L. (1974). Formation and structure of the fertilization envelope in *Xenopus laevis*. *Dev. Biol.* **36**, 669-678.

Grey, R. D., Working, P. K. and Hedrick, J. L. (1976). Evidence that the fertilization envelope blocks sperm entry in eggs of *Xenopus laevis*: interaction of sperm with isolated envelopes. *Dev. Biol.* **54**, 52-60.

Gschwendt, M., Furstenberger, G., Liebersperger, H., Kittstein, W., Linder, D., Rudolph, C., Barth, H., Kleinschroth, J., Marme, D., Schaechtle, C. and Marks, F. (1995). Lack of an effect of novel inhibitors with high specificity for protein kinase C on the action of the phorbol ester 12-)-tetradecanoylphorbol-13-acetate on mouse skin in vivo. *Carcinogenesis* **16**, 107-111.

Gschwendt, M., Dieterich, S., Rennecke, J., Kittstein, W., Mueller, H. and Johannes, F. (1996). Inhibition of protein kinase C μ by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett.* **392**, 77-80.

Gundersen, C. B., Aguado, F., Sou, S., Coppola, T., Mastrogiacomo, A., Kornblum, H. I. and Umbach, J. A. (2001). Cysteine string proteins are associated with cortical granules of *Xenopus laevis* oocytes. *Cell Tiss. Res.* **303**, 211-219.

Jaffe, L. F. (1983). Sources of calcium in egg activation: a review and hypothesis. *Dev. Biol.* **99**, 265-276.

Jaken, S. (1996). Protein kinase C isozymes and substrates. *Curr. Opin. Cell Biol.* **8**, 168-173.

Johnson, J. and Capco, D. G. (1997). Progesterone acts through protein kinase C to remodel the cytoplasm as the amphibian oocyte becomes the fertilization-competent egg. *Mech. Dev.* **67**, 215-226.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D. and Schaechtele, C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. *J. Biol. Chem.* **268**, 9194-9197.

Nishihara, T., Wyrick, R. E., Working, P. K., Chen, Y.-H. and Hedrick, J. L. (1986). Isolation and charcterization of a lectin frm the cortical granules of *Xenopus laevis* eggs. *Biochemistry* **25**, 6013-6020.

Nishizuka, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **9**, 484-496.

Osada, S., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kuroki, T. and Ohno, S. (1990). A phorbol ester recpetor/protein kinase, nPKCη, a new member of the protein kinase C family predominantly expressed in lung and skin. *J. Biol. Chem.* **265**, 22434-22440.

Sahara, S., Sato, K., Aoto, M., Ohnishi, T., Kaise, H., Koide, H., Ogita, K. and Fukami, Y. (1992). Characterization of protein kinase C in *Xenopus* oocytes. *Biochem. Biophys. Res. Commun.* **182**, 105-114.

Scheuner, D. and Holz, R. W. (1994) Evidence that the ability to respond to a calcium stimulus in exocytosis is determined by the secretory granule membrane: comparison of exocytosis of injected chromaffin granule membranes and endogenous cortical granules in *Xenopus* oocytes. *Cell. Mol. Neurobiol.* **14**, 245-257.

Schmidt, T., Patton, C. and Epel, D. (1982). Is there a role for the Ca^{2+} influx during fertilization of the sea urchin egg? *Dev. Biol.* **90**, 284-290.

Schönwasser, D. C., Marais, R. M., Marshall, C. J. and Parker, P. J. (1998). Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol. Cell Biol.* **18**, 790-798.

Schuel, H. (1978). Secretory functions of egg cortical granules in fertilization and developent: a critical review. *Gamete Res.* **1**, 299-382.

Stabel, S. and Parker, P. J. (1991). Protein kinase C. *Pharmacol. Ther.* **51**, 71-95.

Stith, B. J., Woronoff, K., Espinoza, R. and Smart, T. (1997). Sn-1, 2-diacylglycerol and choline increase after fertilization in *Xenopus laevis*. *Mol. Biol. Cell* **8**, 755-765.

Sudhof, T. C. and Rizo, J. (1996). Synaptotagmins: C_2-domain proteins that regulate membrane traffic. *Neuron* **17**, 379-388.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979). Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* **254**, 3692-3695.

Wessel, D. and Flügge, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical Biochem.* **138**, 141-143.

Whittaker, M. S. and Steinhardt, R. A. (1980). Ionic regulation of egg activation. *Q. Rev. Biophys.* **15**, 593-666.

Zang, R., Mueller, H. J., Kielbassa, K., Marks, F. and Gschwendt, M. (1994). Partial purification of a type η protein kinase C from murine brain: separation from other protein kinase C isoenzymes and characterization. *Biochem. J.* **304**, 641-647.