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Original Article

Ca^{2+}-independent protein kinase C signalling in mouse eggs during the early phases of fertilization

CARLA TATONE*[,1], SIMONA DELLE MONACHE[1], ANTONELLA FRANCIONE[1], LUISA GIOIA[2], BARBARA BARBONI[2] and ROSELLA COLONNA[1]

[1]Dept. of Biomedical Sciences and Technologies,University of L'Aquila, Italy and [2]Dept. of Structures, Functions and Animal Pathologies, and Biotechnologies, University of Teramo, Italy

ABSTRACT Protein kinase C (PKC), an enzyme playing a central role in signal transduction pathways, is activated in fertilized mouse eggs downstream of the fertilization Ca^{2+} signal, to regulate different aspects of egg activation. Given the presence of Ca^{2+}-independent PKC isoforms within the egg, we investigated whether fertilization triggers PKC stimulation in mouse eggs by activating Ca^{2+}-independent signalling pathways. An increase in PKC activity was detected as early as 10 min after the beginning of insemination, when about 90% of eggs had fused with sperm and the first Ca^{2+} rise was evident in most of the eggs. A similar level of activity was found 20 min later, when about 60% of eggs had resumed meiosis. When the Ca^{2+} increase was buffered by an intracellular Ca^{2+} chelating agent, PKC stimulation was not blocked but only slightly reduced. Confocal microscopy analysis revealed that the increase in PKC activity at fertilization coincided with the translocation of PKCδ, a Ca^{2+}-independent and diacylglycerol-dependent PKC isoform, to the meiotic spindle. When, in the absence of the Ca^{2+} signal, metaphase-anaphase transition was inhibited, PKCδ moved to the meiotic spindle but still maintained a sustained cytoplasmic distribution. In summary, our results indicate that: 1) PKC activation is an early event of egg activation; 2) both Ca^{2+}-dependent and Ca^{2+}-independent pathways contribute to increased PKC activity at fertilization; 3) PKCδ is one of the isoforms participating in this signalling process.

KEY WORDS: *fertilization, egg activation, protein kinase C, PKCδ, meiotic spindle*

Introduction

The fertilization-competent mammalian egg is arrested at the metaphase of the second meiotic division (MII) with high MPF (M-phase promoting factor) activity, a condition maintained by the cytostatic factor (CSF) (Murray, 1995; Norbury and Nurse, 1992; Kubiak *et al.*, 1993). The fertilizing spermatozoon triggers egg activation events including early changes such as cortical granule exocytosis (CGE), MPF inactivation and meiosis resumption, as well as late changes such as inactivation of MAP kinase, a component of CSF, pronuclei formation and recruitment of maternal RNA (Ducibella, 1991; Verlhac *et al.*, 1994; Moos *et al.*, 1995; Schultz and Kopf, 1995). These cellular responses result from the action of signalling pathways that have characteristics of signal transduction events in somatic cells. In the "receptor" hypothesis the fertilizing sperm interacts with a specific egg surface receptor leading to signal transduction, while, in the "fusion" hypothesis, egg activation is triggered by a soluble sperm factor introduced into the egg cytoplasm upon fusion (as reviews, Evans and Kopf, 1998; Talmor-Cohen *et al.*; 2001). The first observable signal after gamete interaction is a rapid increase in intracellular Ca^{2+} concentration that in mammals takes the form of repetitive Ca^{2+} oscillations (Stricker, 1999 as a review). There is increasing evidence that sperm triggers phosphatidylinositol (PIP_2) hydrolysis in the egg. One of the PIP_2 cleavage products, inositol 1,4,5-triphosphate (IP_3), releases Ca^{2+} from intracellular stores generating the fertilization Ca^{2+} signal (Xu *et al.*, 1994; Jellerette *et al.*, 2000; Jones and Nixon, 2000), whereas the other product, diacylglycerol (DAG), activates the enzyme protein kinase C (PKC) (as reviews Capco, 2001; Eliyahu *et al.*, 2001). Numerous reports have identified PKC as one possible cytoplasmic signal acting downstream to the fertilization-induced Ca^{2+} rise and regulating different aspects of egg activation (Colonna and Tatone, 1993; Gallicano *et al.*, 1997; Raz and Shalgi, 1998; Fan *et al.*, 2002; Eliyahu and Shalgi, 2002). PKC is a family of phospholipid-dependent serine-threonine kinases

Abbreviations used in this paper: CSF, cytostatic factor; Indo-1 AM, Indo-1 acetoxymethyl ester; MPF, M-phase promoting factor; PKC, protein kinase C.

*Address correspondence to: Dr Carla Tatone. Dept. of Biomedical Sciences and Technologies, University of L'Aquila, Via Vetoio, Loc. Coppito, 67100 L'Aquila, Italy. Fax: +39-086-243-3433. e-mail: ctatone@univaq.it.

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playing a central role in signal transduction pathways. It is composed of at least 11 isoforms subdivided into three groups based on sequence homology as well as activator and cofactor requirements. These groups include the 'conventional' (cPKCα, -βI, -βII, and -γ), that are Ca^{2+}- and DAG-dependent, the 'novel' (nPKCδ, -ε, -θ, -μ and -η), that are Ca^{2+}-independent and DAG-dependent, and the 'atypical' (aPKCλ, and -ζ) isoforms, that are Ca^{2+}- and DAG-independent (Mellor and Parker, 1998). Differences were described among these isoforms with respect to the kinetics of their activity, their target proteins and their expression in the various tissues (Nishizuka, 1992; Dekker and Parker, 1994). In the fertilization-competent mammalian egg, PKC representatives from all three groups are expressed suggesting that they could be involved in different egg activation events and in early development. The isoforms present at the protein level are cPKC-α, -βI, -βII and -γ, nPKC-δ and -μ, and aPKC-λ and –ζ (Gangeswaran and Jones, 1997; Pauken and Capco, 2000; Luria *et al.*, 2000; Fan *et al.*, 2002; Eliyahu and Shalgi, 2002). In mouse eggs, activation of PKC at fertilization has been revealed by means of biochemical assays based on the use of a specific PKC substrate (Gallicano *et al.*, 1997), and immunocytochemistry. In many cell types, in fact, activation of PKC results in the translocation of the enzyme to new subcellular sites where it phosphorylates specific target proteins (Baron *et al.*, 1984). On the basis of immunocytochemistry studies, fertilization-associated PKC activity in mammalian eggs would involve isoforms of the conventional group that require both DAG and Ca^{2+} (Luria *et al.*, 2000; Fan *et al.*, 2002; Eliyahu and Shalgi, 2002). In mouse and rat eggs, some cPKC isoforms were shown to translocate to the periphery of the cell very early after the beginning of insemination (Luria *et al.*, 2000; Eliyahu and Shalgi, 2002), supporting the hypothesis that PKCs are Ca^{2+} effectors that may act very proximate to the fertilization Ca^{2+} signal. Nevertheless, no information exists regarding a possible recruitment of Ca^{2+}-independent PKC isoforms whose potential function would be that of being activated by DAG and of operating independently of the sperm-induced Ca^{2+} signal. A recent study reported that an increase in PKC activity during MI-to-MII transition was coincident with the translocation of nPKCδ to the meiotic spindle suggesting a role for this PKC isoform in the regulation of cell cycle progression during meiosis (Viveiros *et al.*, 2001).

In the present report, we focused on assessing PKC signalling during the initial phases of fertilization and egg activation. In particular, the main purpose of this study was to investigate whether fertilization in mouse eggs triggers a Ca^{2+}-independent signalling pathway leading to PKC stimulation, and to establish if nPKCδ could be one the PKCs participating in this process.

Results

Timing of Fertilization and Ca^{2+} Signalling

Prior to monitoring early PKC signalling at fertilization, we checked our *in vitro* fertilization system for synchronous fertilization and sperm-induced Ca^{2+} signalling. These parameters were monitored in both untreated eggs and eggs incubated with the Ca^{2+} chelator BAPTA-AM, prior to insemination, in order to clamp low the level of intracellular Ca^{2+}.

By using the Hoechst 33342 transfer technique we found that 10 min after insemination sperm fusion rates were elevated in both control and BAPTA-AM groups with about 90% of eggs showing at least one fused sperm (Table 1). Moreover, fertilization rates based on the presence of decondensed sperm heads in the cytoplasm after 30 min of insemination, were not significantly different when compared in the two groups of inseminated eggs. At this time, most of the fertilized control eggs had resumed meiosis, while, as previously reported (Kline and Kline, 1992), in the absence of the Ca^{2+} signal, fertilized eggs remained arrested at metaphase II (Table 1). Monitored rate of polar body emission in eggs inseminated for 30 min and examined 1 hr later was 86±7 (data not shown).

In our fertilization system, the initial transient increase in Ca^{2+} was quite synchronous within each group of eggs examined. This signal was observed 10.20 ± 3.30 (SD) min following sperm addition, it lasted 5.75 ± 2.36 min (SD) (peak ratio (405/490) 2.46±0.49) and was followed by shorter Ca^{2+} transients (total n. eggs examined=35, Fig. 1A). In accordance with Kline and Kline (1992), no Ca^{2+} increase was observed in eggs simultaneously loaded with Indo-1 AM and 5 μM BAPTA-AM and then inseminated (total n. eggs examined=31; Fig. 1B).

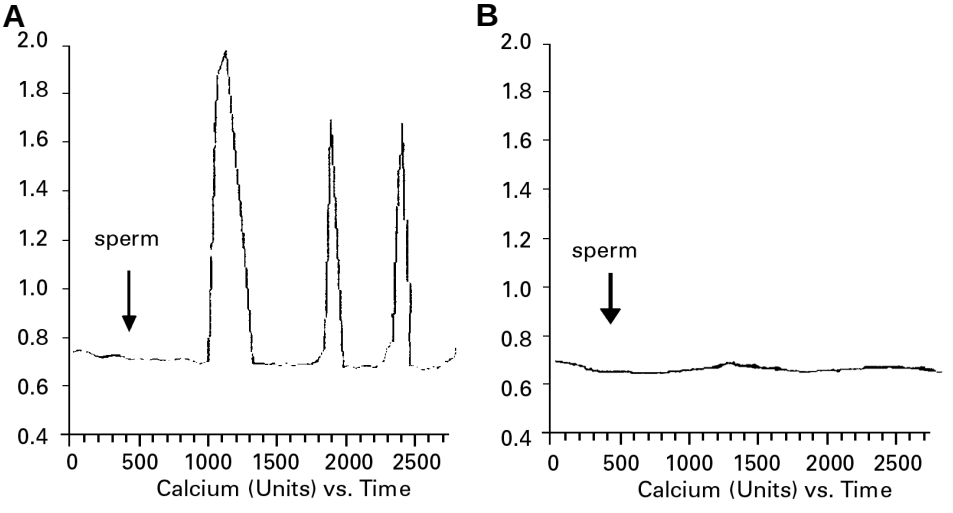

Fig. 1. Changes in intracellular Ca^{2+} levels induced by sperm *in untreated control eggs* **(A)** *or in eggs exposed to BAPTA-AM* **(B).** *The eggs were preloaded with Indo 1-AM for 30 min in the presence or absence of 5 μM BAPTA-AM and Ca^{2+} was continuously monitored for about 30 min after sperm addition. Shown are representative profiles.*

Changes in PKC Activity upon Fertilization in the Presence or Absence of the Ca^{2+} Signal

Until now, changes in the biochemical activity of PKC have been investigated during the lapse of time in which mouse eggs have already resumed meiosis and have progressed towards polar body emission and pronuclei formation (Gallicano *et al.*, 1997). Therefore, in the present study, we focused on assessing PKC signalling during the initial phases of fertilization and egg activation. Results from our biochemical assay revealed that an increase in PKC activity was detectable as early as 10 min after the beginning of

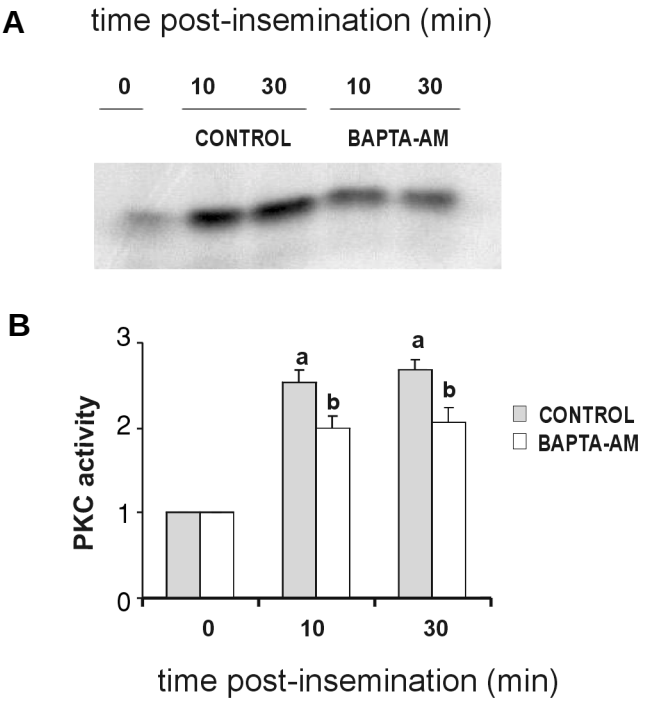

Fig. 2. PKC activity in mouse fertilized eggs in the presence (control) and in the absence (BAPTA-AM) of the Ca^{2+} signal. (A) R*epresentative autoradiogram showing typical results.* **(B)** *Histogram showing PKC activity in control and BAPTA-AM pretreated eggs at 0, 10 and 30 min after the beginning of insemination. Densitometric analysis was performed and data are expressed as mean ± SEM of ratios of band density at times 10 and 30 min post-insemination to that at time 0. Different superscripts indicate statistical difference at* P *< 0.05 level of significance.*

insemination (Fig. 2), when about 90% of eggs had fused with sperm and the fertilization Ca^{2+} signal could be observed in most of the eggs examined. Figure 2 also shows that a similar level of kinase activity was found 20 min later, when about 60% of inseminated eggs had resumed meiosis and were progressing through anaphase. In order to monitor PKC activity in the absence of the Ca^{2+} signal, eggs exposed to 5 μM BAPTA-AM were inseminated and collected over the same time interval as the untreated group to be processed for the biochemical assay. Our results revealed that egg interaction with sperm triggered PKC activation even when the intracellular Ca^{2+} concentration was clamped low. Similarly to the untreated group, under this condition

TABLE 1

***IN VITRO* FERTILIZATION PARAMETERS IN CONTROL AND BAPTA-AM TREATED EGGS**

	Control (n)*	BAPTA-AM (n)*
[a]Fusion rate %	94±1.7(78)	93±1.2(73)
[b]Fertilization rate %	90±1.2(65)	92±0.1(63)
[c]Fertilization index	1.4±0.3	1.6±0.5
[d]Meiosis resumption %	58±12(60)	0(58)

Data are expressed as mean±SEM; [a] %eggs showing at least one fused sperm; [b] % eggs showing at least one decondensed sperm head; [c] n. fused sperm/egg; [d] % eggs at anaphase stage after 30 min of insemination; * number of eggs examined.

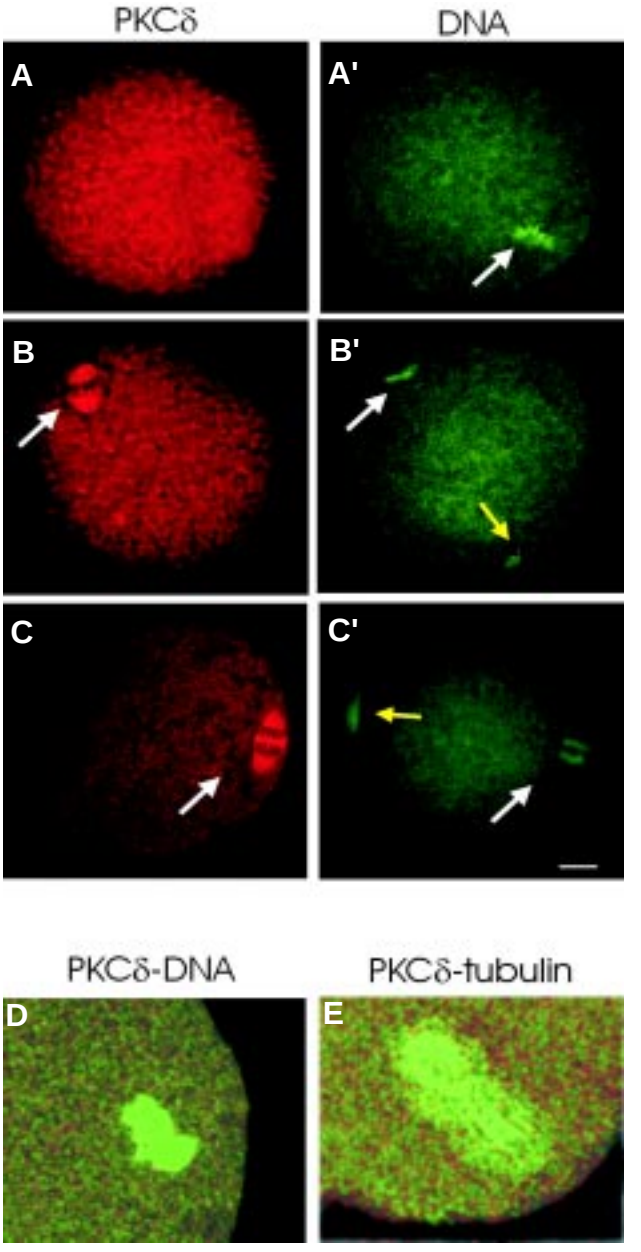

Fig. 3. Localization of PKCδ in mouse eggs during the metaphase II to anaphase II transition. *Metaphase II untreated eggs and eggs inseminated for 10 and 30 min were processed to analyze PKCδ (red) and DNA (green) and subjected to confocal analysis.* **(A, A')** *A metaphase II egg (arrow points to chromosomes on the metaphase plate).* **(B,B')** *Egg after 10 min of insemination (the white arrows point to PKCδ in the region of the spindle and to chromosomes still on the metaphase plate; the yellow arrow points to a condensed sperm head).* **(C,C')** *An egg after 30 min of insemination (white arrows point to PKCδ in the region of the spindle and to chromosomes in an anaphase configuration; the yellow arrow points to a decondensed sperm head). Metaphase II untreated eggs were processed to investigate colocalization of PKCδ with DNA or tubulin.* **(D)** *Chromosomes in a metaphase configuration of an egg double-stained for PKCδ (red) and DNA (green).* **(E)** *Region of the spindle of an egg double-stained for PKCδ (red) and tubulin (green). The absence of overlapping areas indicate that PKCδ is not localized either on the chromosomes or the spindle. Each experiment was repeated independently a minimun of three times, and for each experimental group about 20 eggs were observed. Bar, 10 μM.*

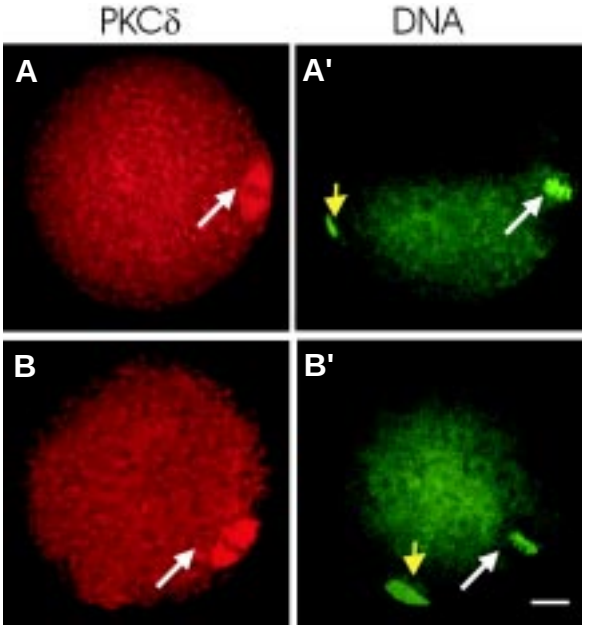

Fig. 4. Localisation of PKC-δ in mouse fertilized eggs in the absence of the Ca^{2+} signal. *Eggs preloded with BAPTA-AM were inseminated for 10 and 30 min and processed to analyze PKCδ (red) and DNA (green).* **(A,A')** *BAPTA-treated egg after 10 min of insemination (white arrows point to PKCδ in the region of the spindle and to chromosomes on the metaphase plate; the yellow arrow points to a condensed sperm head).* **(B,B')** *BAPTA-AM treated egg after 30 min of insemination (white arrows point to PKCδ in the region of the spindle and to chromosomes blocked in a metaphase configuration; yellow arrow points to a decondensed sperm head). Each experiment was repeated independently a minimun of three times, and for each experimental group about 20 eggs were observed. Bar, 10 μM.*

an increased level of PKC activity was detectable after 10 min of insemination and this level did not change during the next 20 min. Nevertheless, both values measured in the BAPTA-group were significantly lower than those in the untreated eggs where the fertilization Ca^{2+} signal was not prevented (Fig. 2).

Changes in Subcellular Distribution Pattern of PKCδ upon Fertilization

Experiments carried out in the second part of this study aimed at examining the hypothesis that PKC signalling at fertilization involves PKCδ, a Ca^{2+}-independent DAG- dependent isoform. In mouse eggs, PKCδ is expressed at the protein level (Pauken and Capco, 2000) and it was recently found to participate in the first meiotic division (Viveiros *et al.*, 2001). Since PKC activation can result in the translocation of the enzyme to new subcellular sites, we focused on assessing possible changes in the subcellular distribution pattern of PKCδ, upon fertilization, by inseminating and collecting the eggs at the same times as in the biochemical assay. Figures 3A and 3A' show that, when the plane of focus containing the chromosomes was monitored, the MII arrested egg exhibited diffuse cytoplasmic staining for PKCδ. More specifically, as shown below, PKCδ did not colocalize with either the chromosomes (Fig. 3D) or the spindle (Fig. 3E). Ten minutes after insemination, in the eggs showing at least one fertilizing sperm, cytoplasmic staining seemed slightly decreased with respect to the MII arrested eggs and intensive staining for PKCδ was detected in the region of the spindle in a pattern that suggested association with spindle microtubules. As shown in Fig. 3B, PKCδ localized on either side of the egg chromosomes that, at this early stage of fertilization, still appeared on the metaphase plate (Fig. 3B'). Thirty minutes after the beginning of insemination, in the eggs showing at least one decondensed sperm head and chromosomes at anaphase stage, the fluorescence in the cytoplam was markedly decreased and staining for PKCδ was present in the region between the two sets of chromosomes (probably the region of midzone microtubules) as well as in the regions between chromosomes and the pole of the spindle (Fig. 3C, 3C'). In inseminated eggs where the generation of the Ca^{2+} signal was prevented by preincubation with BAPTA-AM, 10 min after the beginning of insemination, the pattern of localization of PKCδ was similar to that observed in untreated inseminated eggs collected at the same time, that is, fluorescence was detected in the cytoplasm and on the spindle on either side of the chromosomes (Fig. 4A, 4A'). However, unlike the untreated group, in BAPTA-AM treated eggs such a distribution remained unchanged 30 min after the beginning of insemination when at least a decondensed sperm head was present in the cytoplasm but chromosomes were blocked on the metaphase plate (Fig. 4B, B').

Discussion

In the current study we provide evidence that sperm-egg interaction can result in the activation of both Ca^{2+}-dependent and Ca^{2+}-independent pathways leading to PKC stimulation. Moreover, we propose that PKCδ may be one of the isoforms participating in this signalling process.

Here, for the first time, we show that an increase in PKC activity is a very early signalling event of egg activation supporting previous evidence based on immunocytochemical studies (Luria *et al.,* 2000; Eliyahu and Shalgi, 2002). On the basis of our results, PKC activation occurs during the initial phases of fertilization, at about the time of the first Ca^{2+} transient, when sperm fusion has occurred and eggs are still at metaphase. Increased PKC activity can also be observed after 30 min of insemination when most eggs have undergone metaphase-anaphase transition. Moreover, we found that PKC is activated regardless of the sperm-induced Ca^{2+} rise, even though this signal seems to be necessary for full activation of the enzyme. This conclusion comes from the observation that when the sperm-induced Ca^{2+} increase is buffered by an intracellular Ca^{2+} chelating agent, the increase in PKC activity observed at fertilization isn't blocked but only slightly reduced. Thus, it can be suggested that sperm parallely activates Ca^{2+}-dependent and Ca^{2+}-independent pathways both of which are essential and act in synergy to trigger a full PKC signalling at fertilization. The fertilizing sperm could induce DAG production by introducing the sperm specific PLCζ into the ooplasm (Saunders *et al.,* 2002) and DAG, in turn, may activate PKC in a Ca^{2+}-independent manner, thereby, leading to specific recruitment of nPKC isoforms. Subsequent IP_3-released Ca^{2+} would be important for activation of cPKCs and for full activation of nPKCs due to DAG generated by the egg through a variety of Ca^{2+}-dependent pathways (e.g. PI-PLC, phosphatidylcholine-specific phospholipase C, or phospholipase D; Jones, 1998). Moreover, although the introduction of sperm component(s) into the ooplasm is necessary and sufficient for eliciting egg activation and embryo development, it cannot be excluded that a component of the PKC signalling network activated

by sperm could also involve an egg receptor-mediated mechanism (Evans and Kopf, 1998, for a review).

Our results show that the metaphase-anaphase transition, during meiosis II, is accompanied by translocation of PKCδ to the meiotic spindle along with an increase in PKC activity. However, in contrast to Viveiros *et al.* (2001), we found that in the fertilization-competent mouse egg, PKCδ exhibits a uniform cytoplasmic distribution and doesn't seem to be localized either on the chromosomes or the MII spindle. To explain this discrepancy, it is important to note that the LTXBO eggs analyzed in the above-mentioned study not only are they produced through a defective meiosis I but they also exhibit a high rate of spontaneous release of metaphase II arrest (Eppig *et al.*, 2000), a condition that may be associated with impaired regulation of signalling molecules in the MII arrested egg. Nevertheless, our finding that the intracellular distribution pattern of PKCδ changes upon fertilization supports the hypothesis that this PKC isoform participates in the signalling pathways activated in mouse eggs by the fertilizing sperm. We also observed that in the absence of the fertilization Ca^{2+} signal, that is, when fertilized eggs are not allowed to resume meiosis and fully activate PKC, PKCδ seems to move to the spindle 10 min after insemination. However, such an intracellular distribution pattern doesn't change over the next 20 min, as it does in the presence of the Ca^{2+} signal, and the eggs maintain a sustained cytoplasmic staining as though PKCδ was not allowed to complete translocation to the meiotic spindle. This observation suggests that Ca^{2+}-independent production of DAG is not sufficient for the full activation of this PKC isoform and/or that the complete association of PKCδ with the spindle requires transition to the anaphase stage.

Beyond these observations, the present findings might be relevant to the understanding of the role of PKC in mammalian egg activation. Regarding this issue, a few studies have demonstrated the possibility of different PKC isoforms being activated in eggs upon fertilization. In this regard, different kinds of evidence, including PKC translocation to the egg plasma membrane, support a possible role of cPKCs in the regulation of cortical granule exocytosis and later events of egg activation (Eliyahu and Shalgi, 2002). Here, we show that PKC activity increases in fertilized eggs when sperm are at fusion state and when a nPKC, PKCδ, translocates to the meiotic spindle, a major site for localisation of elements that control meiosis, such as MPF, MAP kinase, and Ca^{2+}-calmodulin protein kinase II (CaMKII) (Kubiak *et al.,* 1993; Hatch and Capco, 2001). Therefore, this study can be considered an initial step towards understanding the specific roles played by nPKC isoforms during egg activation. Present results lead us to hypothesize that PKCδ might be recruited at fertilization to phosphorylate spindle associated proteins in order to regulate the exit from M-phase. Mouse eggs are arrested at MII with high MPF activity, a condition maintained by the cytostatic factor (Kubiak *et al.*, 1993). The sperm-triggered Ca^{2+} oscillations are needed to promote MPF inactivation through cyclin degradation (Nixon *et al.*, 2002), an event that involves CaMKII (Lorca *et al.*, 1993; Winston and Maro, 1995; Tatone *et al.*, 2002). However, studies in somatic cells revealed that the activity of MPF can be influenced by PKC, as well, as through its interaction with regulatory factors (Livneh and Fishman, 1997, Black, 2000). Moreover, some evidence exists about PKC involvement in the exit from MII upon egg activation (Capco, 2001), and earlier work from this laboratory demonstrated that a PKC agonist can suppress MPF activity even in the absence of a Ca^{2+} signal (Colonna *et al.*, 1997). However, based on the observation that Ca^{2+}-independent PKC signalling in the absence of the Ca^{2+} rise, is not able to induce meiosis resumption, we can speculate that one of the possible roles exerted by PKC at fertilization may be to regulate exit from MII by providing signals that are essential but not sufficient for degradation of cyclin B, i.e. signals associated with the presence of an intact spindle (Winston *et al.*, 1995).

In summary, our results provide evidence that, even though the fertilization Ca^{2+} signal is the trigger for egg activation, parallel Ca^{2+}-independent signalling pathways may be stimulated and involved in the regulation of this event

Materials and Methods

Reagents

Pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Intervet (The Netherlands) and Serono (Italy), respectively. A23187 and ionomycin were obtained from Calbiochem (San Diego, CA, USA). Indo-1 acetoxymethyl ester (Indo-1 AM), Pluronic, and SYBR 14 were purchased from Molecular Probes (Eugene, OR, USA). Unless otherwise stated, products were obtained from Sigma (St Louis, MO, USA).

Egg Collection and Treatment

Random bred Swiss CD1 female mice (22-25 days old) were superovulated by intraperitoneal injection of 7.5 I.U. of pregnant mare's serum gonadotrophin (PMSG, Folligon) and of 7.5 I.U. of human chorionic gonadotrophin (hCG, Serono), 48 hr apart. MII arrested eggs were released at 15 h post-hCG from the oviducts into the medium M2 (Quinn *et al.,* 1982) buffered with Hepes, and cumulus cells were dispersed by a brief exposure to 0.3 mg/ml hyaluronidase. The zonae pellucidae were removed by treatment (approximately 1 min) with Tyrode's solution (Nicolson *et al.,* 1975) and zona-free eggs were cultured at 37°C, 5% CO_2 in M16 medium (Whittingham, 1971) for 1 h before their use in the experimental groups. In some experiments, the eggs were exposed to the Ca^{2+} chelator 1,2-bis-(o-aminophenoxy)ethane-N,N,N'-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) at 10 μM concentration for 30 min prior to insemination (Kline and Kline, 1992).

In Vitro *Fertilization and Sperm Fusion Assay*

Spermatozoa were obtained by excising the caudae epididymes from two adult CD1 males (3-5 month old, Charles River, Como, Italy) and by mincing the caudae in 1 ml T6 medium (Quinn *et al.*, 1982). After 5 min, tissue fragments were removed and the sperm were capacitated for 1.5 hr at approximately $5x10^6$ sperm/ml in T6. To induce the acrosome reaction, capacitated sperm were exposed to 10 μM calcium ionophore A23187 for 30 min (Boldt *et al.*, 1991).The sperm suspension was diluted to obtain 50 μl insemination drops containing 1-$2x10^5$ sperm/ml and about 25 eggs were incubated in each drop for 10 or 30 min. Inseminated eggs were freed from loosely associated sperm before the evaluation of the designated parameters. To visualize sperm-egg fusion after fertlization, we used the dye transfer technique (Conover and Gwatkin, 1988; Tatone *et al.*,1994). In this assay, eggs are preloaded with the DNA-staining dye Hoechst 33342 and, upon fusion, sperm nuclei become brightly fluorescent as the dye gains access and binds sperm DNA. In our experiments, eggs were loaded with Hoechst 33342 (0.1 μg/ml) by incubating 15 min, rinsed thoroughly in T6 medium and immediately incubated with sperm. Sperm-egg fusion rate (% of eggs with at least one fused sperm) and fertilization rate (% of eggs with at least one decondensed sperm head) were monitored in eggs collected after 10 and 30 min of insemination, respectively. Eggs fixed in 3.7% (w/v) paraformaldehyde in PBS were mounted on slides and scored for the presence of fused or decondensed sperm heads under a microscope fitted for epifluorescence (Leitz Dialux, Leitz, Wien, Austria).

Loading Eggs with Indo-1 AM and Measurement of Intracellular Ca^{2+} Changes

An Acas 570 System (Meridian Instrument Ltd, Okemos, MI) was used to evaluate the intracellular Ca^{2+} changes in the eggs after insemination. To this end, the eggs were preincubated at 37°C with 20 μM of the calcium-sensitive fluorescent dye Indo-1 AM and 0.02% Pluronic (w/v), for 30 min, in order to inhibit compartimentalization of the indicator, in the presence or in the absence of 5 μM BAPTA-AM. Eggs were then transferred on a polilysine (0.1mg/ml) precoated coverslip which formed the base of a chamber containing 1 ml of T6 medium supplemented with 20 mM HEPES (Quinn *et al.*, 1982). The assembly was positioned on the warming stage (37°C) of the laser confocal microscope equipped with filter sets required to detect the two emission wavelengths of Ca^{2+}-free (485 nm) and Ca^{2+}-bound (405 nm) Indo-1. After some basal scans taken at the equatorial level at 45s intervals, spermatozoa were added and the scans carried out for the subsequent 30-40 min. The Ca^{2+}signal was displayed as the fluorescence ratio for the two emissions after background subtraction. At the end of some experiments, 1 μM ionomycin was added to verify whether the dye was reporting in its dynamic range.

Protein Kinase C Assay

PKC activity was assayed using the protocol of Gallicano *et al.* (1997). For each reaction, groups of 10 eggs, collected at different times of insemination, were washed in collection buffer (phosphate buffered saline (PBS) containing 1mg/ml polyvinyl alcohol, 5 mM EDTA, 10 mM Na_3VO_4, 10 mM NaF), transferred to a centrifuge tube in 2 μl of collection buffer and immediately submerged in liquid N_2 to flash-freeze the eggs, followed by storage at –80°C until the kinase assay was performed. The frozen eggs were thawed in 10 μl PKC buffer which contained β–glycerophosphate (54 mM), para-nitrophenylphosphate (14.5 mM), MOPS (24 mM), $MgCl_2$ (14.5 mM), EGTA (14.5 mM), EDTA (0.12 mM), DTT (1 mM), leupeptin (1μg/ml), aprotinin (1 μg/ml), ML-9 (10 μM), genestein (75 μM), chimostatin (1μg/ml), tripsin-chimotripsin inhibitor (1 μg/ml), PKI (2.4 μM), 50 μCi/ml γ-[^{32}P]ATP(Amersham Pharmacia Biotech, Italy) and MARKS (2.5 mg/ml, BIOMOL, Plymouth Meeting, PA) as a specific substrate. After 30 min at 37°C, assays were stopped by adding tricine sample buffer (1:1) (Bio-Rad Laboratories, Hercules, CA). Samples were electrophoresed and the gel was subjected to autoradiography as previously described (Tatone *et al.*, 2002). Enzyme activity was semiquantified densitometrically using a Bio-rad GS-670 computerised imaging densitometer and Molecular Analyst software. The intensities of bands were quantified after background subtraction. For each autoradiogram, 2 replicates were performed and ratios of mean band density at times 10 and 30 min post-insemination to that at time 0 were evaluated. PKC activity was expressed as mean±SEM of ratios obtained from 4 autoradiograms.

Immunocytochemistry and Confocal Microscopy

The subcellular localization pattern of PKCδ was evaluated in mouse eggs at different times of insemination. In a single experiment, about 20 eggs from each experimental group were washed quickly with PBS-PVP and fixed for 30-60 min in 3.7% formaldehyde in PBS pH 7.4. The fixed cells were then permeabilized with Triton X-100 in PBS containing 3 mg/ml PVP (PBS-PVP), washed in blocking buffer (PBS-PVP, 0.1% BSA, 0.01% Tween20) for 30-60 min and incubated at room temperature for 2 hr with a specific primary antibody (rabbit polyclonal anti-PKCδ, Santa Cruz Technology, Santa Cruz, CA) diluted 1:250 in blocking buffer. Eggs were washed several times in blocking buffer, and stained with 1:800 dilution of a Cy-3 conjugated anti-rabbit IgG (Chemicon International, Temecula, CA) for 1 hour. For DNA staining, cells were then transferred to 1μM SYBR 14 (Molecular Probes, Eugene, OR) for 10 min and finally mounted on slides. In some experiments, for visualization of the spindle, a 1:500 dilution of a mouse monoclonal antibody (anti-α-Tubulin, clone DM1A, Sigma) and a 1:800 dilution of a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Chemicon International, Temecula, CA) were used as a primary antibody and a secondary antibody, respectively. In each experiment, negative control samples, in which the primary antibody was omitted, were also evaluated. Conventional immunofluorescence microscopy was performed using a Bio-Rad laser scanning confocal microscope (Radiance 2000 K-2) equipped with a krypton-argon ion laser for the simultaneous excitation of SYBR, FITC (excitation wavelength 488 nm) and Cy-3 (excitation wavelength 568 nm). This system was mounted on an inverted microscope Zeiss Axiovert equipped with x63 oil immersion objective.

Statistical Analyses

Each group of experiments was repeated at least three times and data are presented as mean±SEM, unless stated otherwise. Multiple comparison of PKC activity values were analysed using Student-Newman-Keul's test (SigmaStat software; Jandel Scientific Software Corporation, San Rafael, CA). Differences associated with a *P* value lower than 0.05 were considered statistically significant.

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