Profile of protein kinase C isozymes and their possible role in mammalian egg activation

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Abstract Western blot analysis was used to investigate protein kinase C (PKC) profile of rat eggs. The presence of eight PKC isozymes was demonstrated: conventional PKC α, β and γ; novel PKC δ, ε and μ; atypical PKC ζ and λ. PKC α was detected by RT-PCR as well. PKC translocation from the cytosol to the plasma membrane served as a marker for enzyme activation. Immunofluorescence confocal microscopy demonstrated a relatively uniform distribution of PKC α, βI, and βII throughout the cytosol of metaphase II arrested eggs. PKC accumulation at the plasma membrane was detected 5 min after exposure to 12-*O*-tetradecanoyl phorbol-13-acetate and increased with time, thus demonstrating activation of these PKCs.

Key words: Cortical granule; Egg activation; Fertilization; Protein kinase C; Rat egg

1. Introduction

At fertilization, the mammalian egg is activated by the sperm either by binding to a sperm receptor, or directly by introduction of a soluble sperm protein [1,2]. Egg activation involves phosphatidylinositol turnover. One cleavage product, inositol 1,4,5-trisphosphate (IP_3), leads to the release of Ca^{2+} from intracellular stores, whereas the other product, diacylglycerol (DAG), activates the enzyme protein kinase C (PKC). Egg activation triggers early events, such as the cortical reaction , thus leading to modification of the zona pellucida (ZP) and block to polyspermy. Egg activation also leads to later events, such as resumption of meiosis and completion of the first cell cycle [3–5].

PKC, a serine/threonine kinase family, is a key regulatory enzyme in signal transduction pathways that governs various cellular responses such as exocytosis. There are currently at least 12 known subspecies of PKC that are classified into three main groups based on their cofactor requirements. Conventional PKCs require Ca^{2+} and DAG for their activity (cPKC α, βI, βII, γ). Novel PKCs are Ca^{2+} independent but require DAG for their activity (nPKC δ, θ, ε, η) as do the PKCs μ and D. Atypical PKCs require neither Ca^{2+} nor DAG for their activity (aPKC λ, ζ, τ) [6,7]. In many cell types, activation of PKC results in an enzyme shift of the enzyme from the cytosol to the plasma membrane, a process referred to as translocation [8].

The role of PKC during sperm-induced egg activation is still controversial. Activating PKC by biologically active phorbol esters and DAG analogues has been shown to induce the cortical reaction, facilitating ZP_2 to ZP_2f modification and block to polyspermy [9–12]. However, PKC inhibitors failed to block fertilization-induced cortical reaction [13]. Other studies demonstrated cell cycle resumption in eggs treated with PKC [14–17]. Very few studies have actually analyzed the presence of different PKC isozymes and their biological activity in eggs. In *Xenopus* eggs PKC α, βI, βII, γ, δ, and ζ were identified, while in mouse eggs only PKC δ and λ were detected [18,19].

The present study was conducted to identify which members of the PKC family are present in rat eggs and to examine a possible involvement in egg activation, using enzyme translocation to the plasma membrane as a marker for PKC activation.

2. Materials and methods

2.1. Egg collection and Western blotting

Eggs arrested at metaphase II (MII) were isolated from immature superovulated rats as previously described [12]. Samples of 200 MII eggs were collected in a minimal volume of medium (5–10 μl) and a lysis buffer was added as described earlier [20]. The extracts were thawed and refrozen twice and stored at −70°C. SDS-PAGE was performed on 10% polyacrylamide minigel and transferred to nitrocellulose membranes [20]. Approximate molecular masses were determined by comparison with the migration of prestained protein standards (Sigma, St. Louis, MO). Western blots were blocked for 2 h with 5% low fat milk powder in 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.1% Tween-20, followed by 2 h incubation with anti-PKC monoclonal antibodies (Mabs; Transduction Laboratories, Kentucky). Proteins were detected by incubating the membranes with the secondary antibody anti-mouse IgG peroxidase for 1 h (Sigma) in 1:5000 dilution followed by an ECL detection system (Amersham, UK). Positive controls, Jurkat or macrophage cells (5 μg protein per lane; Transduction Laboratories), were run for each experiment.

2.2. Isolation of total RNA and RT-PCR

Total RNA of 150 MII zona -ree oocytes was extracted with Trireagent (TR 118, Molecular Research Center, Ohio), after the addition of 7.5 U yeast tRNA as a carrier. Oligonucleotide primers (Biotechnology General, Israel) were designed for the C_2 domain of PKC α. The 5′ primer was designed for the V_2 sequence (positions 709–730) including *Bam*HI and *Eco*RI cloning sites (GCGGGATCCGAATTCTGAAGGCAGAGGTCACAGATG). The 3′ primer was designed for the V_3 sequence (positions 1081–1105) including *Sal*I and *Hin*dIII cloning sites (ACGCGTCGACAAGCTTCATCTCCTTCTGGAATGGGCAC). Reverse transcription and amplification were performed using access RT-PCR introduction system (cat. no. A1260, Promega, Madison, WI). The first cycle of reverse transcription was carried for 45 min at 48°C. Inactivation was for 2 min at 94°C. Second strand cDNA synthesis and PCR amplification were carried out during 40 cycles of denaturing, annealing and extension steps optimized for 30 s at 94°C; 1 min at 60°C and 2 min at 68°C, with a final extension step for 7 min at 68°C. A negative control was made without reverse transcriptase in the reaction mixture. RT-PCR fragments were analyzed by electrophoresis on 1% TAE agarose gel and DNA was visualized by ethidium bromide staining.

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2.3. Egg activation and immunohistochemistry

MII eggs were parthenogenetically activated by incubation with 50 ng/ml of the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA, Sigma), which can induce a full cortical reaction in rat eggs [12]. Eggs were incubated with TPA for 5, 15, or 15 min followed by an additional 30 min incubation in fresh medium lacking the activator. Following parthenogenetic activation eggs were fixed in a mixture of 3% paraformaldehyde and 0.01% glutaraldehyde for 10 min. Zonae pellucidae were removed by 0.02% pronase, and egg membranes were permeabilized by 0.05% NP-40. Eggs were incubated for 2 h in the presence of 3% fetal calf serum and polyclonal anti-PKC α, βI, or βII antibodies in 1:500 dilution (Santa Cruz Biotechnology, Santa Crux, CA). Primary antibodies were detected using fluorescent-labeled Cy-3 secondary antibody (Jackson Immunoresearch Laboratories, Pennsylvania) in 1:1000 dilution. Localization of PKC was visualized using an inverted laser scanning confocal microscope (Zeiss 410, Oberkochen, Germany) equipped with a 25 mM krypton-argon laser. Specificity of staining was demonstrated by incubating each primary antibody for 1 h with an excess of the appropriate peptide (Santa Cruz). Each egg was scanned through the *Z* axis to perform the section plan at the equator of the egg. Densitometric analysis was performed on the confocal micrographs of 2–4 eggs for each experimental day and the intensity of staining was measured using corrected mean density values obtained by the NIH image software. PKC activation was evaluated by calculating the ratio between the signal at the egg membrane and that of the cytosol.

3. Results and discussion

The presence of PKC isozymes in rat eggs was investigated using Western blot analysis. We have detected cPKC α (84 kDa), β (84 kDa), and γ (84 kDa); nPKC δ (80 kDa), ε (87 kDa) and μ (112 kDa); aPKC ζ (77 kDa) and λ (78 kDa; Fig. 1A–C). We were unable to detect PKC θ and τ using the appropriate Mabs at 1:250 concentration. All Mabs used recognized the PKC isozymes corresponding to the positive controls for each isozyme. The relatively faint signal of PKC β and ε observed could result either from the low amount of these isozymes in the eggs, or from low affinity of the Mabs used. PKC α was detected by an alternative strategy of RT-PCR as well. Our primers gave a RT-PCR product of the predicted size for PKC α, thus supporting the existence of

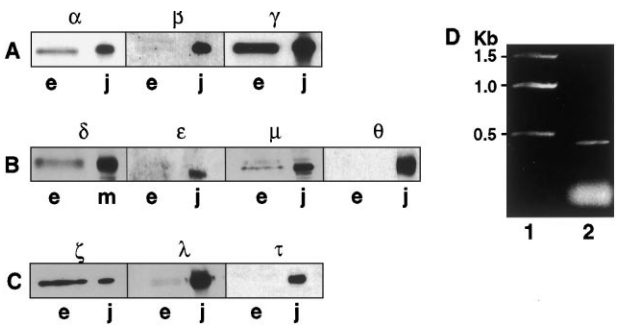

Fig. 1. Identification of PKC isozymes in rat eggs. Proteins (200 eggs per lane, representing approximately 7 μg protein) were separated by SDS-PAGE and immunoblotted with anti-PKC Mabs (Eggs, e). Jurkat cells (J) or macrophage cells (m) served as positive controls for each antibody. A secondary antibody, anti-mouse IgG peroxidase, was used in 1:5000 dilution followed by an ECL detection system. A: cPKC anti-PKC α (1:250), anti-PKC β (1:250), and anti-PKC γ (1:500). B: nPKC anti-PKC δ (1:1000), anti-PKC ε (1:250) and anti-PKC μ (1:1000). No signal was detected using anti-PKC θ (1:250). C: aPKC anti-PKC ζ (1:500) and anti-PKC λ (1:250). No signal was detected using anti-PKC τ (1:250). D: Detection of PKC α by RT-PCR using specific primers. Lane 1, markers; lane 2, RT-PCR.

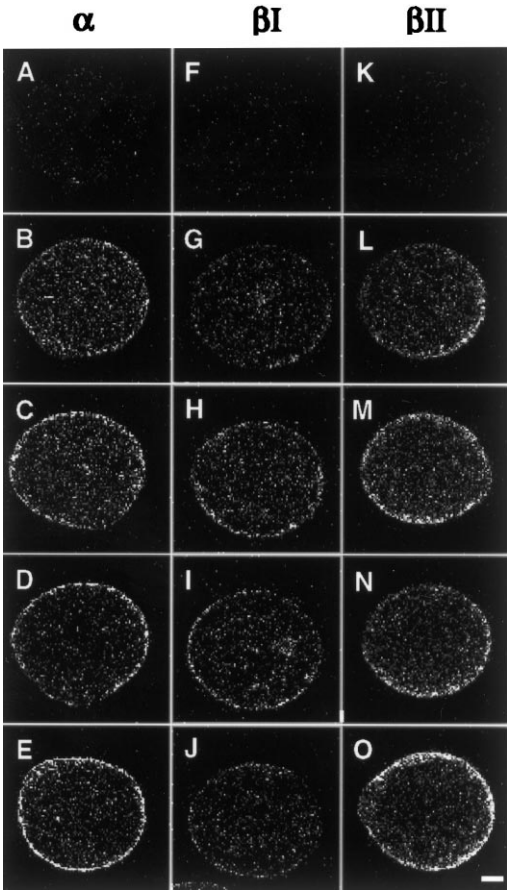

Fig. 2. Subcellular localization of PKC α, βI and βII following activation by TPA. Eggs were fixed at the MII stage (B,G,L) or after incubation with TPA for 5 min (C,H,M), 15 min (D,I,N) or 15 min followed by an additional 30 min incubation in fresh medium lacking the activator (E,J,O). Eggs were labeled with primary anti-PKC polyclonal antibodies (1:500). As controls, eggs were labeled with the primary antibody which has been previously incubated for 1 h with 2 μg/ml of the specific peptide (A,F,K). Localization of the antibodies was imaged using a second antibody Cy-3 (1:1000) and laser scanning confocal microscopy. PKC α (A–E), PKC βI (F–J), and PKC βII (K–O). Each image was taken at the equator of the egg. Bar = 10 μm.

PKC α in rat eggs at both protein and mRNA levels (Fig. 1D).

Our study demonstrates, for the first time, the presence of eight PKC isozymes in mammalian eggs, including members of the cPKC, nPKC and aPKC groups. A recent report by Gangeswaran and Jones [19] demonstrated the presence of PKC δ and λ in mouse eggs, while members of the cPKC group or other PKC isozymes were not detected. This difference may be explained by blotting conditions, low sensitivity of the assays used, or by a species-specific differences. Our inability to detect PKC θ and τ does not necessarily exclude their presence in rat eggs. Further studies using other approaches, such as antibodies generated against different regions of the proteins, are required. The band obtained for PKC γ was very distinct and much stronger than all other isoforms used. PKC γ was also detected by immunohistochemistry (data not shown). The presence of many PKC isozymes in the egg, including PKC γ, as already demonstrated in *Xenopus* [17], is not surprising, since the egg is a totipotential

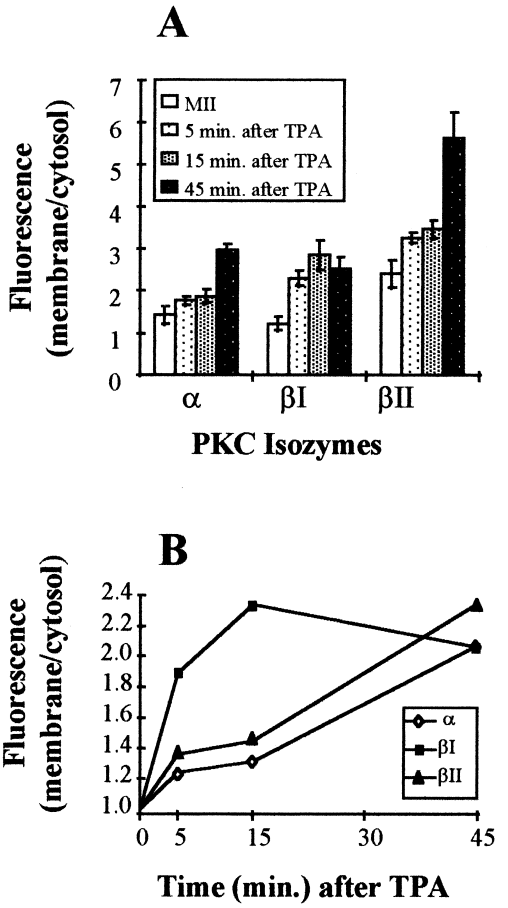

Fig. 3. Activation of PKC α, βI and βII. Ratio calculated between the signal measured at the egg plasma membrane and that of the cytosol. Eggs at the MII stage or 5, 15, or 45 min after incubation with TPA were fixed and labeled with primary anti-PKC polyclonal antibodies (1:500). Localization of the antibodies was imaged using a second antibody Cy-3 (1:1000) and laser scanning confocal microscopy. Densitometric analysis was performed on the confocal micrographs of 3–4 eggs for each experimental day and the intensity of staining was measured using corrected mean density values obtained by the NIH image software. At least three independent experiments were performed for each PKC isozyme. A: Membrane to cytosol ratio ± S.E.M. of a typical representative experiment. B: Membrane to cytosol ratio expressed relative to that obtained for MII eggs, which was arbitrary set at 1.0.

cell which may contain various PKC isozymes involved in different developmental processes. However, since PKC γ is known to be brain-specific in the adult, its role in this early stage of development should be further elaborated.

An early cellular event observed upon sperm-egg interaction is an increase in intracellular calcium concentration ($[Ca^{2+}]_i$). Several studies demonstrated a $[Ca^{2+}]_i$ rise as well as PKC activation and assigned them as possible mediators of the cortical reaction [11,12,21]. Using general reporter dyes of PKC, enzyme activation was demonstrated upon fertilization or egg activation in sea urchin and mouse eggs [16,22,23]. In *Xenopus*, activation of PKC α and β during fertilization was shown [24]. Since the cortical reaction is known as a Ca^{2+}-dependent exocytotic process, and since the Ca^{2+}-dependent cPKC α and β were demonstrated as exocytosis mediators in other systems [25,26], we chose to further study activation of these PKCs in the egg. Translocation of PKC isozymes from the cytosol to the plasma membrane served as a marker for activation. Immunohistochemistry and confocal microscopy presented a relatively uniform distribution of PKC α, βI, and βII throughout the cytosol of MII arrested eggs. In all eggs examined, the mean ratio calculated between the signal at the membrane and that of the cytosol was in the range of 1.2–2.7, for the three isozymes. PKC accumulation at the plasma membrane was already detected 5 min after exposure to TPA. Both PKC α and βII presented similar kinetics of translocation to the plasma membrane, demonstrating an increase in membrane to cytosol ratio which continued to rise even at 45 min. PKC βI presented a more rapid kinetics of translocation, the maximum occurring at 15 min, followed by a decrease at 45 min. In addition, the total signal detected for PKC βI decreased at 45 min as compared to earlier time points (5 and 15 min). A representative experiment is demonstrated in Figs. 2 and 3. Specificity of each antibody was demonstrated by its ability to abolish labeling following incubation of each primary antibody with its appropriate peptide.

The detection of PKC β by immunohistochemistry, as opposed to the relatively faint signal obtained by Western blotting, could be explained by the different experimental conditions (i.e. alterations of the protein conformation during electrophoresis and Western blotting), thus leading to modifications of the specific antibody binding site, or by the different affinity of the antibodies used.

Our results demonstrate for the first time activation of different cPKC isozymes during activation of the mammalian egg. All three isozymes examined presented translocation to the plasma membrane within 5 min following exposure to TPA. Electrophysiological data demonstrate that cortical granules exocytosis in hamster eggs begins within 4 s of the first Ca^{2+} rise, which is initiated rapidly following sperm binding. However, the dispersal of the cortical granular material over the cell surface is relatively slow [27,28]. Cortical granule exocytosis can also be induced by TPA, although in our hands a minimum of 5 min exposure was required, probably due to permeability of TPA through the egg plasma membrane [12]. The dynamics described for the cortical reaction induced by TPA implies a possible involvement of PKC α and/or βI or βII in initiating the cortical reaction. PKCα and βII might also be involved in later stages of egg activation, probably after induction of the cortical reaction, but other than resumption of the cell cycle, which is not triggered by TPA [12].

Identification of PKC binding proteins indicates the involvement of PKC in linking intracellular membranes and/or the plasma membrane with cytoskeletal structures. Thus, an important role of PKC signaling appears to be regulation of membrane cytoskeletal interactions [6,7]. Actin microfilaments are required for the cortical reaction in hamster and porcine eggs [29,30]. In other species, the arrangement of the cortical actin was demonstrated to be PKC-dependent [18,31,32]. In view of the aforementioned data we suggest a possible function of the cPKCs α, βI and βII in cortical reaction induction and egg activation. However, since ascribing a pathway based solely on the use of a parthenogenetic agent does not strictly recapitulate the physiological events, prior to any definitive conclusions, PKC activation following sperm-induced egg activation should be assessed.

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