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Subcellular Distribution of Protein Kinase Cα and βI in Bovine Spermatozoa, and Their Regulation by Calcium and Phorbol Esters[1]

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ABSTRACT

Protein kinase C (PKC), the major cell target for tumor-promoting phorbol esters, is central to many signal transduction pathways. Previously we have demonstrated the presence of PKC in ram and bovine spermatozoa. However, the relative distribution of various PKC isozymes in the cytosolic and membrane fractions and their regulation by calcium and phorbol esters have not been elucidated. Immunocytochemical studies and Western blotting with antibodies specific for individual isoforms revealed that at least two PKC isoforms, cPKC$_\alpha$ and cPKC$_\beta$I, are found in bovine sperm cells. We demonstrate, by Western blotting analysis, that both PKC isozymes were predominantly localized in the cytosol when subcellular fractionation was carried out in the presence of EGTA. When cell lysis was carried out in the presence of Ca^{2+}, most PKC$_\alpha$ and PKC$_\beta$I redistributed to the particulate fraction. Treatment of sperm cells with the biologically active phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) resulted in a rapid and extensive translocation of cytosolic PKC$_\alpha$ and cytosolic PKC$_\beta$I to the membrane fraction within 1 min. Furthermore, PKC's total activity was measured as a calcium- and phospholipid-dependent phosphorylation of a synthetic peptide in the cytosolic and membrane fractions derived from control and TPA-treated spermatozoa. TPA evoked a decrease in cytosolic PKC activity, accompanied by an increase in the activity associated with the plasma membrane fraction. This translocation of PKC enzymes may ensure their binding to intracellular receptor proteins ("RACKs") and the phosphorylation of specific substrates, which appears to determine their physiological function. The presence of RACK in the membrane fraction of bovine sperm cells was confirmed with use of an antibody directed against the RACK protein. Previously we demonstrated the involvement of PKC in sperm acrosomal exocytosis, a process induced by signal transduction events. Thus, our results suggest that the rapid association of PKC$_\alpha$ and PKC$_\beta$I with the sperm plasma membrane, as shown in the present work for the first time, may be an early event in sperm cell regulation, leading to acrosomal exocytosis and fertilization.

INTRODUCTION

Protein kinase C (PKC) is a multigene family of serine/threonine kinases that are central to many signal transduction pathways [1, 2]. The PKC isozyme family comprises at least 11 different kinases. They can be divided into three major groups, namely, classical (cPKC$_\alpha$, βI, βII, and γ [3]), novel (nPKCδ, ε, η, θ, and μ [4–7]), and atypical (aPKC$_\zeta$ and i [8, 9]).

Classical PKCs and novel PKCs translocate from the cytosol to membranous sites upon activation with diacylglycerol (DAG) [10]. Nevertheless, a notable difference between them is their sensitivity to Ca^{2+}: cPKCs are dependent on Ca^{2+} for activity [11], whereas the other PKCs are not [2]. Phorbol esters such as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) can substitute for DAG in the activation of PKC [12, 13]. Because of TPA's potency and in vivo stability, it is able to irreversibly insert PKC into the lipid bilayer, thereby causing a cumulative and long-term stimulation of the enzyme [14]. Membrane association of PKC causes proximity with its substrates and may serve to ensure preferential and rapid phosphorylation of these substrates.

The presence and involvement of PKC in the mammalian sperm acrosome reaction and motility has been previously demonstrated by us [15] and others [16–19]. Immunocytochemical analysis with type-specific PKC antibodies revealed staining in the postacrosomal region and the apical region of the bovine sperm head [15]. In human sperm, PKC$_\alpha$ and PKC$_\beta$II were localized in the equatorial segment [17], and PKC$_\beta$I and PKC$_\epsilon$ were present in the principal piece of the tail [20]. However, the relative distribution of PKC isoforms in the cytosolic and membrane fractions has not been elucidated. In this study we demonstrate, by two different methods, changes in PKC distribution and activity in bovine spermatozoa as a result of treatment with the phorbol ester TPA or with Ca^{2+}. TPA and Ca^{2+} evoked a rapid decrease in cytosolic PKC$_\alpha$ and PKC$_\beta$I, accompanied by a significant increase in their membrane association. It is well established that bovine sperm cells are able to undergo acrosomal exocytosis in the presence of Ca^{2+} [21], and other studies have suggested that TPA treatment may also influence this reaction [15, 20]. Hence, the present results suggest that the rapid association of PKC$_\alpha$ and PKC$_\beta$I with the plasma membrane may be an early event in sperm cell regulation, leading to acrosomal exocytosis and fertilization.

MATERIALS AND METHODS

Sperm Preparation

Bovine sperm (Hasherut Breeder Service, Hafetz Haim, Israel) were collected by an artificial vagina and diluted (1:1, v:v) in NKM medium containing NaCl (110 mM), KCl (5 mM), and 3-[*N*-morpholino]propanesulfonic acid (10 mM), pH 7.4. The cells were washed by three centrifugations at 780 × *g* for 10 min, and the final pellets were resuspended in NKM, with the sperm concentration adjusted to 1–3 × 10^9 cells/ml.

Whole Cell Lysates

Proteins of washed sperm cells (10^9 cells) were solubilized in SDS-lysis buffer containing 125 mM Tris (pH 7.5), 4% SDS, 1 mM sodium orthovanadate, 1 mM benzamidine, and 1 mM PMSF added just before use. Cells were lysed for 10 min at room temperature and centrifuged at 12 930 × *g* for 5 min at 4°C. The supernatant was supplemented with 0.05% bromophenol blue, 5% glycerol, and 2% β-mercaptoethanol and boiled for 5 min.

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Subcellular Fractionation for the Cytosolic and Membrane Fractions

Sperm cells (10^8 cells/ml) were left untreated or were treated with 0.1–100 ng/ml TPA for 0.5–5 min. TPA was added to the medium in dimethylsulfoxide (Me_2SO) to give a final concentration of 0.1% Me_2SO. After each incubation period, cells were fractionated for cytosolic and membrane proteins. For protein and enzyme assays, the cells were centrifuged at 4300 × *g* for 10 min at 4°C (Sorvall RC2-B; Sorvall Inc., Norwalk, CT) and resuspended in 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 1 mM benzamidine, 1 mM Na_3VO_4, 10% glycerol, 25 μg/ml leupeptin, 4 μg/ml aprotinin, and 1 mM PMSF (homogenization buffer). For lysis in Ca^{2+}, 3 mM $CaCl_2$ was added to the homogenization buffer instead of EGTA and EDTA. Cell suspensions were then sonicated (30-sec pulse, power setting 4) with a Vibra cell, Sonics (Sonics & Materials Inc., Danbury, CT) material sonicator. The homogenate was centrifuged for 10 min at 10 000 × *g* for pelleted cell debris. The resulting supernatant was centrifuged at 100 000 × *g* (60 min, 4°C) for recovery of the cytosolic fraction (supernatant) and the membrane fraction (pellet). The cytosolic fraction was concentrated to at least 1/10 of the original volume using a microconcentrator 30 (Amicon, Lexington, MA). The membrane fraction was resuspended in the homogenization buffer supplemented with 0.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [22]. The membrane and cytosolic fractions were kept on ice until assayed for PKC activity. Protein concentration was determined by Bradford's method [23] using Bio-Rad (Richmond, CA) reagents. For immunoblot analysis, the cell lysates were boiled for 5 min in SDS-PAGE sample buffer two times [24] and separated on a 7.5% SDS-polyacrylamide gel.

Immunoblot Analysis

For immunoblotting, proteins of equivalent cell amounts were separated on 7.5% SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes (200 mAmp; 1 h), using a buffer composed of 25 mM Tris (pH 8.2), 192 mM glycine, and 20% methanol. For Western blotting, nitrocellulose membranes were blocked with 5% BSA in TRIS-buffered saline, pH 7.6, containing 0.1% Tween 20 (TBST), for 30 min at room temperature. The various PKC isoforms and the RACK protein were immunodetected using the following antibodies: rabbit polyclonal anti-PKC_α, anti-PKC_βI, anti-PKC_βII, anti-PKC_δ, anti-PKC_ϵ, and anti-PKC_ξ (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:3000, and mouse monoclonal anti-RACK (Transduction Laboratories, Lexington, KY) diluted 1:400. The membranes were incubated overnight at 4°C in the appropriate primary antibody. Next, the membranes were washed three times with TBST and incubated for 1 h at room temperature with specific horseradish peroxidase (HRP)-linked secondary antibody (Jackson Laboratories, West Grove, PA) diluted 1:5000 in TBST. The membranes were washed three times with TBST and visualized by enhanced chemiluminescence (Amersham, Little Chalfont, UK). Specificity of the PKC antibodies was determined by preabsorbing the antibodies with 10 μg of their peptide antigens for 1 h before incubating the antibodies with the membrane. Quantitation of the Western blots was performed using a laser densitometer.

PKC Activity Assay

PKC activity was measured in the cytosolic and membrane fractions using a commercially available assay (Amersham), which is based on methodology described by Hannun et al. [25]. Cytosolic and membrane fractions (20–50 μg of protein) were incubated in 50 mM Tris-HCl (pH 7.5) containing 300 mM synthetic peptide substrate, 4 mM $CaCl_2$, 2.6 mol% phosphatidylserine (PS), 8 μg/ml TPA, 10 mM dithiothreitol, 15 mM magnesium acetate, 50 μM ATP, and 0.05% (w:v) sodium azide. [γ-^{32}P]ATP (Amersham) was added to a final concentration of 10 μCi/ml. Reactions were terminated after 15 min at 25°C by addition of acidic stop solution, 125-μl aliquots were pipetted onto binding papers and washed twice with 5% (v:v) acetic acid, and the ^{32}P incorporated was measured in a Liquid Scintillation Analyser, (Packard 1600 TR, Packard Inst. Co., Downers Grove, IL). Phosphorylation of the synthetic peptide was calculated as picomoles of phosphate transferred per minute; Ca^{2+}- and phospholipid-independent activity (basal, unstimulated PKC activity) was measured in the presence of 4 mM EGTA instead of activators (PS, TPA, Ca^{2+}).

Immunocytochemistry

Sperm (10^6 cells) were spread on glass coverslips and then fixed and permeabilized with cold methanol (30 sec). Nonspecific reactive sites were blocked with 10% nonimmune goat serum for 10 min at room temperature. The cells were incubated for 2 h at room temperature with the appropriate primary PKC isoform-specific antibody diluted 1:10 in TBS. Between antibody incubations, the cells were washed three times (5 min) in TBS. Primary antibodies were detected by a biotin-conjugated donkey anti-rabbit IgG antibody (10 min), followed by an extravidin-HRP enzyme conjugate and by the aminoethyl carbazole chromogen. Cells were counterstained with hematoxylin, and mounting was performed with aqueous mounting solution, GVA-mount (Zymed, San Francisco, CA). The cells were then examined with an Olympus photomicroscope (Vanox AHBT3; Olympus Corporation, Lake Success, NY) and photographed using Kodak 100 ASA film (Eastman Kodak, Rochester, NY) and a ×40 objective. Nonspecific staining, determined by incubation without primary antibody or in the presence of antigenic peptide (10 μg), was negligible (results not shown).

RESULTS

Expression of PKC_α and PKC_βI in Bovine Spermatozoa and Antibody Specificity

To confirm the presence of PKC isozymes in bovine sperm cells, SDS-extracted cells were subjected to Western immunoblot analysis with isozyme-specific antipeptide antibodies. We detected high levels of PKC_α and PKC_βI (Fig. 1), while other PKC isoforms, βII, δ, ϵ, and ξ, were not detectable (data not shown). The antibodies directed against PKC_α and PKC_βI specifically recognized a single protein of approximately 80 kDa. The specificity of these antibodies was demonstrated by their preabsorption with the specific peptide antigens, which completely prevented immunodetection of the 80-kDa protein band.

Immunocytochemical Studies of PKC_α and PKC_βI

The PKC isozyme-specific antibodies were used to examine the cellular localization of PKC_α and PKC_βI through

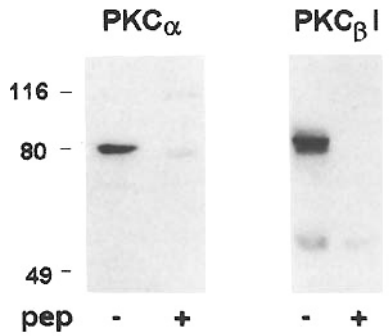

FIG. 1. Expression of bovine sperm PKC_α and $PKC_\beta I$. Protein immunoblots showing PKC_α and $PKC_\beta I$ in bovine sperm. Total protein was extracted with SDS-lysis buffer, separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with PKC-specific polyclonal antibodies in the absence (−) or presence (+) of a specific antigenic peptide (pep), as described in *Materials and Methods*. Molecular weights of prestained high-range marker proteins are indicated (× 10^{-3}). The blot shown is representative of three separate experiments.

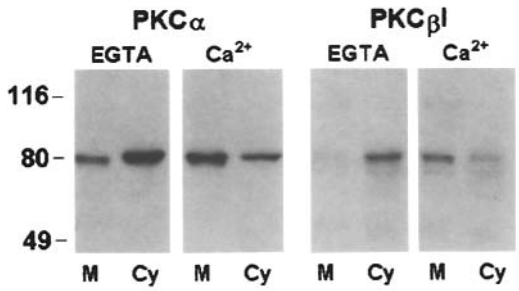

FIG. 3. Subcellular distribution of PKC_α and $PKC_\beta I$ in response to Ca^{2+}. Protein immunoblots showing the levels of PKC_α and $PKC_\beta I$ in subcellular fractions of the cells. Cell extracts were partitioned into cytosol (Cy) and membrane (M) fractions, using either EGTA- or Ca^{2+}-containing buffers, as described in *Materials and Methods*. Cytosol and membrane samples, for both extraction conditions, were analyzed on the same gel; immunodetections are depicted separately for the sake of presentation. The immunoreactive bands for PKC_α (~80 kDa) and $PKC_\beta I$ (~82 kDa) were visualized as described in *Materials and Methods*.

immunocytochemical studies (Fig. 2). Intense staining of the postacrosomal region was observed with the PKC_α-specific antibody (Fig. 2, left). Staining was also observed along the tail. In contrast, labeling with the $PKC_\beta I$-specific antibody revealed prominent staining only in the acrosomal region (Fig. 2, right). Preabsorption of the PKC isozyme-specific antibodies with the immunizing peptides abrogated immunostaining in both cases (data not shown).

Subcellular Distribution of PKC_α and $PKC_\beta I$ in Response to Ca^{2+}

In order to determine the subcellular distribution of PKC_α and $PKC_\beta I$ and their sensitivity to Ca^{2+}-dependent membrane association, bovine sperm cells were fractionated in buffers containing either EGTA or Ca^{2+}. The cytosolic and the membrane fractions were analyzed for the amount of immunoreactive PKC_α and $PKC_\beta I$. We found that when extraction was performed in the presence of EGTA, most of PKC_α (77%) resided in the cytosol, and a minor fraction (23%) was membrane bound (Fig. 3). On the other hand, subcellular fractionation in the presence of Ca^{2+} revealed that most of the PKC_α (60%) was located in the membrane fraction, with a smaller amount (40%) in the cytosolic fraction (Fig. 3).

The subcellular localization of $PKC_\beta I$ was also dependent on the presence of Ca^{2+} in the fractionation buffer, in the same manner as with PKC_α (Fig. 3). These results demonstrate that the subcellular distribution of PKC_α as well as of $PKC_\beta I$ is sensitive to Ca^{2+}. This is consistent with the fact that the cPKCs are dependent on Ca^{2+}, obviously due to their Ca^{2+}-binding domain.

Subcellular Distribution of PKC_α and $PKC_\beta I$ in Response to TPA

Previous reports have demonstrated that TPA modulates PKC subcellular distribution from a predominately cytosolic location to a membrane-associated site during stimulation [26]. To study the effect of TPA-induced activation of PKC_α and $PKC_\beta I$, experiments were performed on untreated and TPA-treated sperm cells. PKC_α and $PKC_\beta I$ were present in abundant amounts in the cytosolic fractions of untreated cells, while in TPA-treated cells, PKC isozymes were translocated to the membrane fraction (Fig. 4).

We observed that the increase in membrane-associated PKCs, as well as their decrease in the cytosol, was TPA concentration dependent. For example, 100 ng/ml TPA caused a 2- to 3-fold increase in membrane-associated PKC_α and $PKC_\beta I$ over the control value (as estimated by densitometric scanning) within 5 min (Fig. 4). Furthermore, the translocation was time dependent; a rapid and extensive increase in membrane-bound PKC_α could be seen during the first 5 min of incubation (Fig. 5). Densitometric analysis revealed that at 5 min, the membrane-bound PKC_α increased from 17% to 64% in comparison to values obtained in basal conditions. Further incubation with TPA (up to 20 min) had no significant effect on the amount of membrane-associated PKC_α. Similar results were obtained for $PKC_\beta I$ (data not shown).

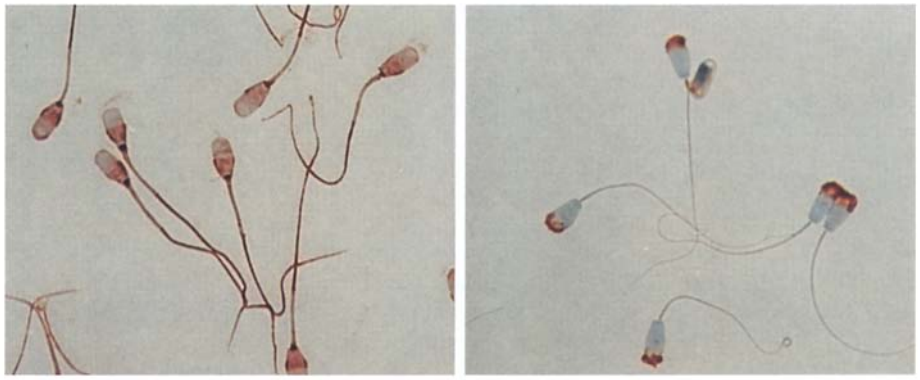

FIG. 2. Immunocytochemical localization of PKC isoforms in sperm cells. Bovine sperm cells were fixed and stained, as described in *Materials and Methods*, with the corresponding isoform-specific antibodies. **Left**) Immunostaining with anti-PKC_α antibody; staining can be observed in the postacrosomal region. **Right**) Immunostaining with anti-$PKC_\beta I$ antibody; staining can be observed in the acrosomal region.

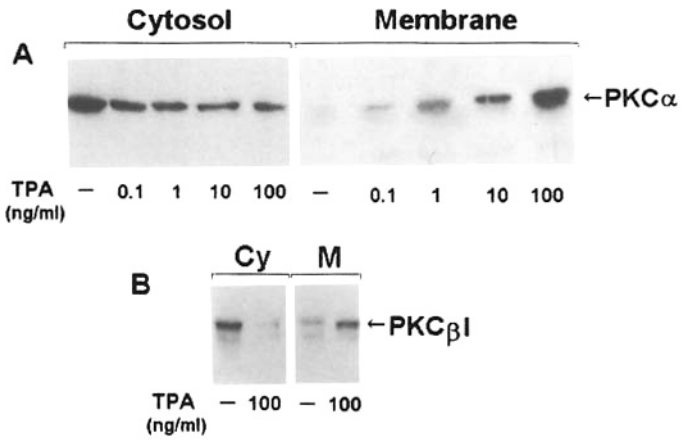

FIG. 4. Subcellular distribution of PKC_α and $PKC_\beta I$ in response to TPA; protein immunoblots of PKC_α (**A**) and $PKC_\beta I$ (**B**). Sperm cells were homogenized and fractionated into cytosol (Cy) and membrane (M) fractions as described in *Materials and Methods*. The two fractions were obtained from untreated sperm cells (−) or cells treated with 0.1, 1, 10, or 100 ng/ml TPA for 5 min. PKC isozymes in these fractions were immunodetected as described in the legend to Figure 1.

Effect of TPA on PKC Activity

In order to ascertain that the translocation of PKC_α and $PKC_\beta I$ to the membrane fraction in response to TPA was accompanied by an increase in membrane PKC activity, an independent assay was employed. We measured calcium- and phospholipid-dependent phosphorylation in the cytosolic and membrane fractions derived from untreated and TPA (100 ng/ml)-treated sperm cells (Fig. 6). It was found that the PKC activity was predominately cytosolic in untreated cells, while in cells treated with TPA, both an increase in the membrane activity and a decrease in cytosolic activity were observed. These data indicate that translocation of PKC to the membrane fraction by TPA is indeed accompanied by an increase in membrane PKC activity. Although a 3-fold increase in PKC in the membrane fraction was observed when the extraction was performed in the presence of either Ca^{2+} or TPA, the amount of PKC activity lost from the cytosolic fraction was not quantitatively recovered in the membrane fraction. TPA(−) membranes displayed 15% of the detected PKC activity, whereas the TPA(+) membranes displayed 40% of the detected PKC activity. It may be that membrane-bound PKC did not effectively phosphorylate the exogenous synthetic substrate peptide because of competition by endogenous proteins.

Identification of Intracellular Receptor Proteins for Activated PKC (RACK)

A group of proteins collectively termed RACKs have been identified as proteins that bind PKC in the particulate fraction [27]. By sequence homology, RACK1 appears to belong to a superfamily that includes the β subunit of G proteins [28]. To determine whether RACK1 is also present in bovine sperm cells, the particulate and cytosolic fractions of fractionated sperm were treated with a RACK1-specific antipeptide antibody. It can be observed that only in the membrane fraction did the antibody recognize a single band of the expected apparent mass (~36 kDa) (Fig. 7). These results demonstrate, for the first time, that membranes of bovine sperm cells also express receptors for activated kinase C.

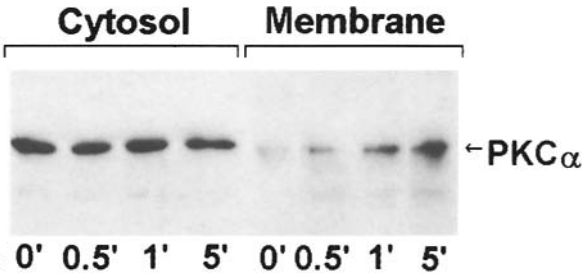

FIG. 5. Time course of TPA-induced PKC_α translocation. Sperm cells were incubated with 100 ng/ml TPA for 0.5, 1, or 5 min. Cells were homogenized and fractionated into cytosol and membrane fractions as described in *Materials and Methods*. Immunodetection was as described in the legend to Figure 1.

DISCUSSION

PKC is a well-characterized example of a cellular kinase that is translocated in response to stimulation (reviewed by Mochly-Rosen [29]). We have analyzed the Ca^{2+}-sensitive subcellular distribution as well as the phorbol ester-induced translocation of PKC isozymes in bovine sperm cells. These cells appeared to express only two classical Ca^{2+}-dependent isozymes, PKC_α and $PKC_\beta I$, in significant amounts (Figs. 1 and 2). In bovine sperm homogenized in the presence of the Ca^{2+} chelator EGTA, PKC_α and $PKC_\beta I$ were enriched in the cytosolic fraction. Homogenization in the presence of millimolar Ca^{2+} revealed a quantitative decrease of the two isozymes in the cytosol and an increase in the particulate fraction (Fig. 3). These data may suggest an important role for intraspermatozoal free Ca^{2+} concentration in regulating PKC translocation to the plasma membrane. It is

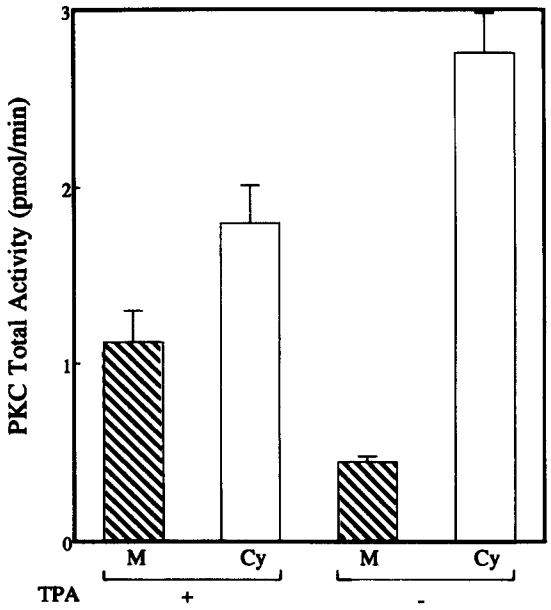

FIG. 6. PKC activity in cytosolic and membrane extracts of sperm cells. TPA-dependent translocation of PKC total activity to the membrane fraction. Sperm cells were incubated in the presence or absence of 100 ng/ml TPA for 5 min. Cells were homogenized and fractionated as described in *Materials and Methods*. Total PKC activity was assayed in the cytosolic (Cy) and membrane (M) fractions. The complete reaction system contained TPA, phosphatidylserine, Ca^{2+} or EGTA, and a peptide as a substrate. The values reflect total PKC activity minus basal PKC activity measured in the presence of EGTA and the absence of TPA, PS, and Ca^{2+}. Data are presented as mean ± SD from a representative experiment assayed in triplicate and repeated three times.

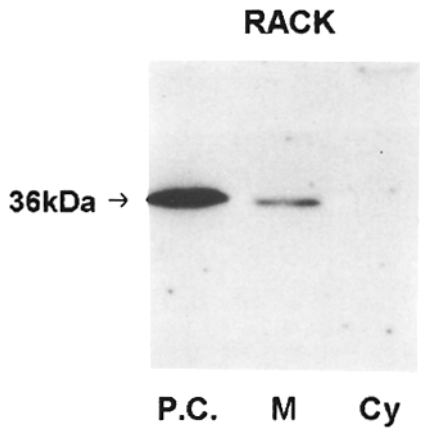

FIG. 7. Identification of RACK proteins. Protein immunoblots showing the level of RACK in subcellular fractions. Bovine sperm membrane fraction (M) and cytosolic fraction (Cy) were prepared as described in *Materials and Methods*. RACK was detected using anti-RACK antibody. The antibody reacted with a ~36-kDa protein that corresponds to RACK. For positive control we used a partially purified extract of RACK from mouse NIH-3T3 fibroblasts (P.C.).

widely recognized that this translocation is a necessary step in PKC activation [10]. Previously it has been reported that the cellular localization of the cPKC isozymes in other cell types, such as rat fibroblasts, is also dependent on the Ca^{2+} content in the homogenization buffer [30]. This property of the cPKC is probably attributable to the presence of a Ca^{2+}-binding domain in the C2 region of these enzymes. We also demonstrate the translocation of cytosolic PKC_α and $PKC_\beta I$ to the plasma membrane as a result of TPA treatment (Fig. 4). The decrease in cytosolic PKC_α and $PKC_\beta I$, and the increase in the amount of PKC_α and $PKC_\beta I$ associated with the membrane fraction, were dependent on time and on the TPA concentration (Figs. 4 and 5). Moreover, the present results indicate that TPA treatment evoked a marked reduction in the calcium- and phospholipid-dependent phosphorylation of cytosolic PKC activity, together with an increase in membrane PKC activity (Fig. 6). These results indicate, for the first time, that TPA triggered a new compartmentalization for PKC in spermatozoa. This was assessed by two independent methods: Western immunoblot analysis employing PKC isozyme-specific antibodies, and measurement of PKC's total activity. Our data correlate with findings from similar investigations concerning other cell types [26, 31, 32]. For example, treatment of fibroblasts with TPA resulted in the translocation of different PKC isozymes from the cytosol to distinct subcellular structures, including the membrane fraction [30, 33].

In the present work, PKC_α was localized in the head, mainly to the postacrosomal region, and along the tail, whereas $PKC_\beta I$ was prominently localized in the acrosomal region (Fig. 2). The fact that the PKC isozymes are differentially located may suggest that different subspecies of PKC may be involved in modulating acrosomal exocytosis or sperm motility. Indeed, PKC_α localization along the tail of bovine sperm may indicate its involvement in sperm motility. Conversely, in the studies reported by Rotem et al. [16, 17] and by Kalina et al. [34], PKC_α and $PKC_\beta II$ were localized in the equatorial segment of human sperm cells, and staining for $PKC_\beta I$ and PKC_ζ was observed in the principal piece of the flagellum, suggesting their involvement in human sperm motility. Moreover, the localization of $PKC_\beta I$ in the acrosomal region of bovine sperm may suggest a possible role for this isozyme in the acrosomal exocytosis.

Prior to fertilization, mammalian spermatozoa must go through a species-dependent period of time during which they undergo a series of changes collectively referred to as "capacitation" [21]. After binding to the egg zona pellucida, sperm cells undergo the acrosome reaction, an exocytotic event that allows the cell to penetrate the zona pellucida and to fuse with the oocyte plasma membrane [21]. Recently, we have described the involvement of PKC in the bovine sperm acrosome reaction [15]. We found that only in capacitated bovine sperm was TPA able to stimulate acrosomal exocytosis, while preexposure to a very low concentration of the PKC inhibitor staurosporine [35] blocked TPA-triggered exocytosis [15]. However, the functional significance of TPA-dependent PKC_α translocation is not yet clear, since it occurred in uncapacitated (Figs. 3–5) as well as in capacitated sperm cells (data not shown). Nevertheless, it may be suggested that translocation of both PKC isozymes from the cytosol to the plasma membrane upon activation by Ca^{2+} or by TPA presumably brings the activated enzymes into proximity with their specific substrates. A group of membrane PKC-binding proteins, collectively termed RACKs (receptors for activated C-kinase), have been identified [27, 36]. RACKs of 30–36 kDa were purified from the cell particulate fraction of rat heart and brain [27]. Recently, Ron et al. [28] cloned RACK1, a gene encoding for a 36-kDa homologue of the β subunit of G proteins. RACK1 is neither a PKC substrate nor an inhibitor. Rather, it increases PKC phosphorylation of substrates presumably by stabilizing the active form of PKC [28]. Our studies are the first to demonstrate that bovine sperm cells express RACK in the membrane fraction (Fig. 7). It is likely that these proteins provide anchorage sites for the PKC isozymes close to their physiological substrates. Thus, it is possible that this translocation may determine PKC_α and $PKC_\beta I$ physiological functions, and it may ensure rapid phosphorylation of specific substrates. In mouse epididymal sperm, PKC_β was located in the acrosomal region, close to three of its specific substrates [37]. Furthermore, in human sperm, PKC was colocalized with various cytoskeletal and other structural elements such as myosin, vimentin, and actin. The codistribution of PKC with the elements mentioned above suggests that these proteins are potential substrates for human sperm PKC [34]. Thus it seems that PKC-dependent phosphorylation plays a role in sperm cell function. However, further investigations are necessary to determine and identify specific proteins phosphorylated by PKC, and to establish the role they may have in acrosome reaction and subsequent fertilization.

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REFERENCES

1. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 1992; 258:607–614.
2. Hug H, Sarre TF. Protein kinase C isozymes: divergence in signal transduction? Biochem J 1993; 291:329–343.
3. Knopf JL, Lee M, Sultzmann LA, Kritz RW, Loomis CR, Hewick RM, Bell RM. Cloning and expression of multiple protein kinase C cDNAs. Cell 1986; 46:491–502.
4. Johannes FJ, Prestle J, Eis S, Oberhagemann P, Pfizenmaier K. PKC mu is a novel, atypical member of the protein kinase C family. J Biol Chem 1994; 269:6140–6148.

5. Ono Y, Fujii T, Ogita K, Kikkawa U, Igarashi K, Nishizuka Y. The structure, expression, and properties of additional members of the protein kinase C family. J Biol Chem 1988; 263:6927–6932.
6. Osada S, Mizuno K, Saido TC, Akita Y, Suzuki K, Kuroki T, Ohno S. A phorbol ester receptor/protein kinase, nPKC eta, a new member of the protein kinase C family predominantly expressed in lung and skin. J Biol Chem 1990; 265:22434–22440.
7. Osada S, Mizuno K, Saido TC, Suzuki K, Kuroki T, Ohno S. A new member of the protein kinase C family, nPKC theta, predominantly expressed in skeletal muscle. Mol Cell Biol 1992; 12:3930–3938.
8. Ono Y, Fujii T, Ogita K, Kikkawa U, Igarashi K, Nishizuka Y. Protein kinase C ζ subspecies from rat brain: its structure, expression, and properties. Proc Natl Acad Sci USA 1989; 86:3099–3103.
9. Selbie LA, Schmitz-Peiffer C, Sheng Y, Biden TJ. Molecular cloning and characterization of PKC_2, an atypical isoform of protein kinase C derived from insulin-secreting cells. J Biol Chem 1993; 268:24296–24302.
10. Nishizuka Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 1988; 334:661–665.
11. Marais RM, Parker PJ. Purification and characterization of bovine protein kinase C isotypes alpha, beta and gamma. Eur J Biochem 1989; 182:129–137.
12. Castagna M, Takai Y, Kabuchi K, Kikkawa U, Nishizuka Y. Direct activation of calcium-activated phospholipid dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 1982; 257:7847–7851.
13. Weinstein IB. The origins of human cancer: molecular mechanisms of carcinogenesis and their implications for cancer prevention and treatment—twenty-seventh G.H.A. clowes memorial award lecture. Cancer Res 1988; 48:4135–4143.
14. Anderson WB, Estival A, Tapiovaara H, Gopalakrishna R. Altered subcellular distribution of protein kinase C (a phorbol ester receptor). Possible role in tumor promotion and the regulation of cell growth: relationship to changes in adenylate cyclase activity. In: Cooper DMF, Seamon KB (eds.), Advances in Cyclic Nucleotide and Protein Phosphorylation Research. New York: Raven Press; 1985: 287–306.
15. Breitbart H, Lax Y, Rotem R, Naor Z. Role of protein kinase C in the acrosome reaction of mammalian spermatozoa. Biochem J 1992; 281: 473–476.
16. Rotem R, Paz GF, Homonnai ZT, Kalina M, Naor Z. Further studies on the involvement of protein kinase C in human sperm flagellar motility. Endocrinology 1990; 127:2571–2577.
17. Rotem R, Paz GF, Homonnai ZT, Kalina M, Naor Z. Protein kinase C is present in human sperm: possible role in flagellar motility. Proc Natl Acad Sci USA 1990; 87:7305–7308.
18. De Jonge CJ, Han H-L, Mack SR, Zaneveld LJD. Effect of phorbol diesters, synthetic diacylglycerols, and a protein kinase C inhibitor on the human sperm acrosome reaction. J Androl 1991; 12:62–70.
19. Chaudhry PS, Casillas ER. Isotypes of protein kinase C in bovine sperm. Arch Biochem Biophys 1992; 295:268–272.
20. Rotem R, Paz GF, Homonnai ZT, Kalina M, Lax Y, Breitbart H, Naor Z. Calcium-independent induction of acrosome reaction by protein kinase C in human sperm. Endocrinology 1992; 131:2235–2243.
21. Yanagimachi R, Knobie E, Neil JD (eds.). Mammalian Fertilization in the Physiology of Reproduction. New York: Raven Press; 1994: 189–318.
22. Rush JS, Klein J, Fanti P, Bhat NR, Waechter CJ. Direct assay of membrane-associated protein kinase C activity in B lymphocytes in the presence of brij 58. Anal Biochem 1992; 207:304–310.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–254.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.
25. Hannun YA, Loomis CR, Bell RM. Activation of protein kinase C by triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. J Biol Chem 1985; 260:10039–10043.
26. Kraft AS, Anderson WB. Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. Nature 1983; 301:621–623.
27. Mochly-Rosen D, Khaner H, Lopez J. Identification of intracellular receptor proteins for activated protein kinase C. Proc Natl Acad Sci USA 1991; 88:3997–4000.
28. Ron D, Chen C-H, Caldwell J, Jamieson L, Orr E, Mochly-Rosen D. Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G proteins. Proc Natl Acad Sci USA 1994; 91: 839–843.
29. Mochly-Rosen D. Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science 1995; 268:247–251.
30. Borner C, Guadagno SN, Fabbro D, Weinstein IB. Expression of four protein kinase C isoforms in rat fibroblasts. Distinct subcellular distribution and regulation by calcium and phorbol esters. J Biol Chem 1992; 267:12892–12899.
31. Kraft AS, Anderson WB, Cooper HL, Sando JJ. Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. J Biol Chem 1982; 257:13193–13196.
32. Disatnik MH, Buraggi G, Mochly-Rosen O. Localization of protein kinase C isozymes in cardiac myocytes. Exp Cell Res 1994; 210:287–297.
33. Goodnight J, Mischak H, Kolch W, Mushinski F. Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. Isoform-specific association with microfilaments, golgi, endoplasmic reticulum, and nuclear and cell membranes. J Biol Chem 1995; 270:9991–10001.
34. Kalina M, Socher R, Rotem R, Naor Z. Ultrastructural localization of protein kinase C in human sperm. J Histochem Cytochem 1995; 43: 439–445.
35. Tamaoki T, Namoto H, Takahashi I, Kato Y, Marimoto M, Tomita F. Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. Biochem Biophys Res Commun 1986; 135:397–402.
36. Ron D, Luo J, Mochly-Rosen D. C2 region-derived peptides inhibit translocation and function of b protein kinase C *in vivo*. J Biol Chem 1995; 270:24180–24187.
37. Furuya S, Endo Y, Osumi K, Oba M, Suzuki S. Effects of modulators of protein kinase C on human sperm capacitation. Fertil Steril 1993; 59:1285–1290.