Protein kinase C activation during progesterone-stimulated acrosomal exocytosis in human spermatozoa

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The involvement of protein kinase C (PKC) in exocytosis of the mammalian sperm acrosome is still a controversial issue. Work carried out thus far has failed to provide direct evidence for the activation of this enzyme upon stimulation with natural agonists of acrosomal exocytosis. We have therefore used progesterone stimulation of the acrosome reaction in human spermatozoa to clarify this issue. In spermatozoa preincubated under conditions known to support capacitation and fertilization in vitro, treatment with progesterone caused a time-dependent stimulation of phosphorylation of at least eight proteins ranging in size from ~20–220 kDa. The inclusion of the PKC inhibitors chelerythrine chloride or calphostin C reduced the observed phosphorylation in a concentration-dependent manner. Exogenously supplied phorbol 12-myristate-13-acetate (PMA) or the permeant diacylglycerol 1-oleoyl-2-acetyl-sn-glycerol (OAG), synthetic activators of PKC, also stimulated phosphorylation in preincubated spermatozoa, but inclusion of calphostin C diminished the response. Furthermore, the prior inclusion of the 1,4-dihydropyridine Ca²⁺ channel antagonist nifedipine also inhibited phosphorylation, suggesting that PKC is activated downstream of Ca²⁺ channel opening. Exocytosis triggered by progesterone was significantly inhibited by chelerythrine chloride or calphostin C. Both PMA and OAG triggered exocytosis, but the inclusion of chelerythrine chloride significantly inhibited the response; exocytotic responses were seen only in capacitated cells. These results provide the first direct evidence that PKC activation plays a role in the signal transduction pathway underlying acrosomal exocytosis in progesterone-stimulated capacitated spermatozoa.

Key words: Ca²⁺ channels/diacylglycerol/fertilization/membrane fusion/phorbol ester

Introduction

The mammalian sperm acrosome is analogous to a secretory granule and the ‘acrosome reaction’, an exocytotic process whereby acrosomal contents are released from the spermatozoon during interaction with the oocyte, is a prerequisite for successful fertilization (Yanagimachi, 1994). Stimulation of spermatozoa to undergo exocytosis causes an increase in intracellular Ca²⁺ concentrations, due to influx from the extracellular space, and the activation of an array of signal transduction pathways important for the sequence ending in membrane fusion (Fénichel and Parinaud, 1995). One important component in this chain of events could be the activation of protein kinase C (PKC) because, in somatic cells, this enzyme appears to play a modulatory role during exocytotic responses (Kikkawa and Nishizuka, 1986; Cheek, 1993). However, an unequivocal role for PKC during the acrosome reaction has yet to be demonstrated.

The presence of detectable PKC activity in spermatozoa has been disputed (Kimura et al., 1984; Roldan and Harrison, 1988; Rotem et al., 1990a,b; Breibart et al., 1992). It now seems clear that PKC activity in mammalian spermatozoa is extremely low, ~3% of that seen in the rat brain enzyme, or ~20% of that found in PC12 cells or in rat pituitary cells (based on μg protein) (Rotem et al., 1990a,b; Breibart et al., 1992). Furthermore, when activity is compared taking into account the number of cells used to obtain enzyme preparations, the activity of sperm PKC is only 0.4–1.3% of that in PC12 or in pituitary cells (O'Toole et al., 1990a). Several studies employing either PKC antagonists or exogenous synthetic activators of PKC have provided indirect (i.e. circumstantial) evidence that PKC may function during acrosomal exocytosis (DeJonge et al., 1991; Breibart et al., 1992; Rotem et al., 1992). However, the major problem with these studies is that they have relied on the use of PKC activators (e.g. phorbol esters) that may have other effects (Ahmed et al., 1993; Areces et al., 1994), and non-selective inhibitors such as H7 or staurosporine (see Wilkinson and Hallam, 1994, for review). Therefore, the results presented so far do not allow one to draw any firm conclusion on the likely role of PKC in intact spermatozoa. Moreover, no evidence of protein phosphorylation in response to either activators of PKC or natural agonists of acrosomal exocytosis has been presented.

In somatic cells, a series of PKC isoforms is regulated by Ca²⁺ and diacylglycerol (DAG) (Asaoka et al., 1992; Dekker and Parker, 1994). We have previously demonstrated that DAG is produced in human spermatozoa undergoing acrosomal exocytosis in response to progesterone treatment (O'Toole
et al., 1996b), and that DAG production, in fact, depends on a prior activation of Ca\(^{2+}\) channels (O'Toole et al., 1996a). The inclusion of nifedipine, a 1,4-dihydropyridine Ca\(^{2+}\) channel antagonist, either at time zero or just prior to addition of progesterone, significantly inhibited DAG production. Furthermore, the same treatment inhibited acrosomal exocytosis as determined by chlorotetracycline fluorescence (O'Toole et al., 1996b).

Progesterone is known to be synthesized and secreted by the cumulus cells surrounding the oocyte (Meizel, 1995). Acting via a non-genomic receptor (reviewed by Revelli et al., 1994), it has been shown to stimulate specific signal transduction pathways that result in the triggering of acrosomal exocytosis (Meizel, 1995). In the present study we demonstrate that stimulation of spermatozoa with progesterone results in phosphorylation of endogenous proteins, this response being elicited also by synthetic activators of PKC and inhibited by specific inhibitors of PKC. Acrosomal exocytosis could be stimulated both by progesterone and by various synthetic activators of PKC, and inhibited by the same inhibitors of PKC. Taken together, these results strongly indicate that PKC activation plays an important role in the signal transduction pathway underlying acrosomal exocytosis in spermatozoa.

Materials and methods

Medium and reagents

The standard medium used was Earle’s medium (TCN Flow, High Wycombe, Bucks, UK) with added benzylpenicillin (100 IU/ml, Sigma, Poole, Dorset, UK) and human serum albumin (HSA; Sigma) at 4 mg/ml; this medium contains 1.8 \(\mu\)M CaCl\(_2\). Percoll was obtained from Pharmacia LKB (Uppsala, Sweden), \([^{32}\text{P}]\text{Pi}\) (specific activity 370 MBq/ml, \(\sim 10\) mCi/ml) from Amersham International (Amersham, UK), and both chelerythrine chloride and calphostin C were obtained from Calbiochem (Nottingham, UK). All other reagents were purchased from Sigma.

Preparation of sperm suspensions

Semen obtained by masturbation was provided by volunteer research donors at King’s College London. The use of human semen for this research has received ethical approval from the King’s College Research Ethics Committee. Motile cells were prepared using mini-Percoll (Ord et al., 1990) gradients (300 \(\mu\)l each of 95, 70 and 50% v/v solutions prepared from a substock containing 90 ml of 100% Percoll, 10 ml 10X Earle’s concentrate, 0.37 ml sodium lactate, 2 ml HEPES buffer, 3 mg sodium pyruvate and HSA at 4 mg/ml). After centrifugation at 600 g for 5 min all the supernatant was removed and the pelleted cells were resuspended in Earle’s medium, centrifuged again at 600 g for 5 min and resuspended in fresh medium. The sperm concentration was estimated using a haemocytometer and adjusted to 5\(\times\)10^6 cells/ml. The percentage of motile cells in a sample was estimated microscopically; usually >90% exhibited progressive motility. All incubations were carried out in an atmosphere of 5% CO\(_2\)-5% O\(_2\)-90% N\(_2\). The medium and conditions used for experimental incubations are similar to those frequently used in successful human in-vitro fertilization (Stock et al., 1989).

In all experiments, sperm suspensions were preincubated for a total of 5 h before exocytosis was stimulated with 10 \(\mu\)g/ml progesterone (31.8 \(\mu\)M). A stock solution of progesterone was prepared in dimethylsulphoxide (DMSO), diluted 10-fold in Earle’s medium and then added at 1/100 to suspensions (final concentration of DMSO was 0.1%).

Assessment of acrosomal exocytosis

The vital dye Hoechst bis-benzimide 33258 was used to assess cell vitality and the functional state of sperm was assessed using a chlorotetracycline (CTC) fluorescence assay (DasGupta et al., 1993). Cells were assessed first for live/dead status using ultraviolet light. The Hg excitation beam was passed through a 334 nm band pass filter and fluorescence emission was observed through a DM 400 dichroic mirror. The same cells were then assessed using violet light (the Hg excitation beam was passed through a 405 nm band pass filter and fluorescence emission observed through a DM 455 dichroic mirror) and classified as expressing one of the three main CTC staining patterns. These patterns are: F, with uniform bright fluorescence over the entire head, which is characteristic of uncapacitated, acrosome-intact cells; A, with a fluorescence-free band in the post-acrosomal region, which is characteristic of capacitated, acrosome-intact cells; AR, with dull or no fluorescence in the head region, which is characteristic of capacitated, acrosome-reacted cells. An earlier study (DasGupta et al., 1993) ascertained that cells assessed as expressing the AR pattern had undergone exocytosis.

The Percoll-prepared samples used had >90% motile cells and the proportion of dead (Hoechst-positive) cells was similar to that of immotile cells. As the numbers of dead cells were low and randomly distributed among the assessment categories, all our CTC data are for live (Hoechst-negative) cells only (200 assessed per replicate).

Labelling of intact cells

A modified method of Roldan and Harrison (1989) was used to label intact sperm cells with \([^{32}\text{P}]\)Pi. Sperm suspensions were preincubated for 3 h 45 min at 37°C, \([^{32}\text{P}]\)Pi, (carrier free, 10 mCi/ml) was added to give a final concentration of 500 \(\mu\)Ci/ml and the suspensions were incubated in a water bath at 37°C for a further 1 h. Samples were then centrifuged at 600 g for 5 min, the supernatant was discarded and the pellet was resuspended to its original volume in Earle’s medium. After stimulation ± inhibitors (see individual experiments) all suspensions were centrifuged at 2600 g for 5 min. The supernatant was discarded and each pellet was resuspended in 50 ml Earle’s medium + 50 ml sodium dodecyl sulphate (SDS) loading buffer [containing 2.7% SDS, 13.5% glycerol, 0.14 \(\mu\)M dithiothreitol (DTT), 0.1 \(\mu\)M Tris pH 6.8 and 0.03% Bromphenol Blue]; the samples were boiled for 5 min and centrifuged at 10\(\times\)500 g for 5 min to sediment DNA before applying 15 \(\mu\)l (40 \(\mu\)g protein) to each lane for electrophoresis.

Electrophoresis and autoradiography

SDS–polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) was carried out using a Mighty Small II electrophoresis system (Hoefer Pharmacia, St Albans, Herts, UK). Proteins were separated using either a 7.5–12.5% linear gradient or a non-gradient 10% acrylamide resolving gel, with a 4% acrylamide stacking gel; \([^{14}\text{C}]\) methylated protein high molecular weight standards (Amersham International) were run on each gel and electrophoresis was carried out at 20 mA per gel for ~2 h. After electrophoresis gels were stained with Coomassie R250. Gels were then dried, exposed to HRX film (Fujifilm) for up to 14 days at ~70°C, and developed.

Statistical analysis

CTC results were analysed using Cochran’s modification of the \(\chi^2\) test (Snedecor and Cochran, 1980). This is a stringent test that compares responses within replicates; significant differences indicate that responses were both consistent and of a reasonable magnitude in all replicates.
Figure 1. Autoradiographic analysis of human sperm phosphoproteins following sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Sperm suspensions were preincubated for 4 h in Earle’s medium ± nifedipine (final concentration 100 nM), then labelled with [32P]Pi for 1 h. After centrifugation the cells were resuspended in fresh medium ± nifedipine and then exposed to 31.8 μM progesterone for 20 min. Sperm proteins were solubilized in SDS loading buffer and separated using either (A) a 10% non-gradient or (B) a 7.5–12.5% linear gradient resolving gel with a 4% stacking gel. The bands were visualized after up to 14 days exposure of the gel to a Fuji HRX X-ray film at −70°C. Lanes show: a, control cells (not exposed to progesterone); b, progesterone-only treated cells; c, nifedipine-only treated cells and d, cells treated with nifedipine and progesterone. In this figure, the molecular weight standards are shown in the far left lane (S). In this and Figures 2 and 3, the phosphoproteins consistently seen are indicated by arrowheads.

Results

Phosphorylation patterns in progesterone-stimulated suspensions

Sperm suspensions preincubated under capacitating conditions were labelled with [32P]Pi, washed and then exposed to 10 μg progesterone/ml. Phosphorylation patterns were evaluated after 10, 20 and 30 min. We observed a time-dependent stimulation in phosphorylation of an array of proteins, with the maximal response at 20 min (Figure 1). A total of at least eight phosphoproteins, of ~20, 24, 29, 38, 46, 64, 98, and 220 kDa, could be detected. In order to evaluate low and high molecular weight proteins adequately, we used both non-gradient 10% (Figure 1A) and 7.5–12.5% linear gradient acrylamide gels (Figure 1B). The 10% gels have the advantage of showing the full range of phosphoproteins in terms of size, but the disadvantage of relatively poor resolution of the high molecular weight phosphoproteins. As these are the most heavily-labelled proteins, we have included Figure 1B to demonstrate clearly the presence of the ~98 and 220 kDa phosphoproteins. Under the conditions used, the smaller and relatively less heavily-labelled proteins are not visible.

To investigate the possible involvement of Ca2+ influx via Ca2+ channels in the activation of PKC, we included nifedipine, a 1,4-dihydropyridine Ca2+ channel antagonist, at time 0. Nifedipine inhibited the phosphorylation observed at 5 h in response to progesterone. Little change over the 30 min assessment period was detected in either the control suspensions (no added progesterone) or those receiving nifedipine only, but the maximal response to progesterone was observed at 20 min (Figure 1). In subsequent experiments, suspensions were incubated for 20 min following addition of progesterone or PKC activators and then evaluated for phosphorylation. Coomassie-stained gels indicated that equal amounts of protein were added to each lane; proteins of sizes similar to several (~29, 38, 46, 220 kDa) but not all of the phosphoproteins could be identified (not shown).

In a second series we examined the effects of two PKC inhibitors, chelerythrine chloride (which interacts with the catalytic domain; Ko et al., 1990) and calphostin C (which interacts with the regulatory domain; Kobayashi et al., 1989), on progesterone-induced stimulation of phosphorylation. Again, progesterone stimulated phosphorylation of an array of proteins (Figure 2), but the inclusion of either chelerythrine chloride (0.6 or 1.8 μM) or calphostin C (0.15 μM) 15 min before the addition of progesterone inhibited the response; all phosphoproteins appeared to be equally affected. Thus PKC activity would appear to be responsible for the observed phosphorylation.

Phosphorylation patterns in PMA- or OAG-stimulated spermatozoa

Since PKC activity in a wide range of cells is stimulated by permeant DAG and phorbol esters, we then investigated the effects of 1-oleoyl-2-acetyl-sn-glycerol (OAG), phorbol-12-myristate-13-acetate (PMA), and the inactive phorbol ester (Neidel and Blackshear, 1986) 4α-phorbol-12, 13-didecanoate (4α-PDD) (Figure 3). In spermatozoa preincubated and labelled as indicated above, exposure to 1 μM PMA or 10 μM OAG stimulated phosphorylation, but 1 μM 4α-PDD had little effect. The inclusion of calphostin C (0.3 μM) inhibited the response to PMA, again indicating that the phosphorylation events observed involved PKC activity.
Figure 2. The effect of protein kinase C (PKC) inhibitors on progesterone-stimulated phosphorylation. Sperm suspensions were preincubated for 3 h 45 min in Earle's medium, then labelled with [32P]P for 1 h. After centrifugation, cells were resuspended in fresh medium and the PKC inhibitors chelerythrine chloride and calphostin C were added to three samples. At 5 h these samples were exposed to 31.8 µM progesterone for 20 min. Sperm proteins were solubilized in SDS loading buffer and separated using a 10% resolving gel with a 4% acrylamide stacking gel. Lanes show: (a) control cells (not exposed to progesterone); (b) progesterone-treated cells; (c and d) cells treated with chelerythrine chloride (0.6 and 1.8 µM respectively) plus progesterone; (e) blank (some overspill from adjacent lanes is evident); and (f) cells treated with calphostin C (0.15 µM) plus progesterone.

Effects of PKC modulators on exocytosis

In order to establish links between progesterone-stimulated phosphorylation and exocytosis, we first investigated the effects of the PKC inhibitors chelerythrine chloride and calphostin C on progesterone-stimulated acrosomal exocytosis. Inhibitor was added to cells that had been preincubated for 4 h 45 min. Progesterone at 10 µg/ml (31.8 µM) was added 15 min later, cells were incubated for 30 min and then assessed using CTC fluorescence. CTC analysis has the advantage that it permits evaluation of the capacitation state as well as the occurrence of exocytosis (DasGupta et al., 1993).

Consistent with other studies (Osman et al., 1989; DasGupta et al., 1994), progesterone significantly stimulated exocytosis (Figure 4); as reported earlier (DasGupta et al., 1994), it also stimulated capacitation, as evidenced by a significant decrease in the proportion of uncapacitated F pattern cells. The prior inclusion of either chelerythrine chloride (0.6 or 1.8 µM) or calphostin C (0.05 or 0.15 µM) significantly inhibited exocytosis; consequently, there were more capacitated acrosome-intact B pattern cells in the inhibitor plus progesterone-treated suspensions than in progesterone-only treated ones. No effects of the inhibitors on capacitation per se were observed, i.e. progesterone stimulation of the F→B transition occurred in the presence as well as the absence of the inhibitors.

We next investigated the effect of PMA (1 µM) in the absence and presence of calphostin C (0.05 or 0.15 µM). PMA significantly stimulated exocytosis in capacitated (B pattern) cells only, resulting in fewer B pattern and more AR pattern cells (Figure 5A). The prior inclusion of the PKC inhibitor significantly inhibited exocytosis. When 4α-PDD, an inactive phorbol ester, was used, no significant stimulation of exocytosis was observed.

In that same experimental series, the effects of permeant synthetic diacylglycerols that can and cannot activate PKC were also evaluated. The active compound 1,2-dioctanoyl-sn-glycerol (1,2-Dag), used at 10 µM, significantly stimulated exocytosis in the capacitated subpopulation of cells (Figure 5B), a response similar to that observed with PMA. In contrast, the inactive 1,3-dioctanoylglycerol (1,3-Dag) had no significant stimulatory effect and an equal mix of the active 1,2- and inactive 1,3- compounds had an intermediate stimulatory effect, essentially half-way between the values obtained with the two compounds when used individually.

Discussion

Protein kinase C (PKC) comprises a family of serine- and threonine-specific protein kinases that share common enzymatic or structural features (Stabel and Parker, 1993). These enzymes, in general, can be activated by DAG, one of the
is now recognized that both phorbol esters and DAGs have hence may not mimic normal endogenous events. Moreover, it appears to function during fertilization (Yanagimachi, 1994) and effectively circumvent the signal transduction pathways that synthetic DAGs have been reported capable of initiating the exocytotic pathway has remained equivocal, with available of PKC in both the head and tail. However, a role for PKC in 1995) indicate the presence of at least some isoforms et al., chemical analyses of human spermatozoa using both light compartment (Roldan and Harrison, 1989). Immunohistochemical analyses of human spermatozoa using both light and hence phosphorylation, does not take place due to the absence of a rise in DAG, the endogenous activator of PKC (Rando, 1988). If this is the case, then exogenously added DAG should cause phosphorylation and this proved to be the case: a permeant synthetic DAG (OAG) and a tumour-promoting phorbol ester (PMA), known to activate PKC, stimulated phosphorylation of the same proteins, and (ii) a phorbol ester which is incapable of activating PKC did not stimulate phosphorylation; (iii) both progesterone- and PMA-stimulated phosphorylation was inhibited by inclusion of two different and highly specific PKC inhibitors.

To explore the role of Ca\(^{2+}\) in the activation of PKC we examined the pattern of protein phosphorylation in spermatozoa exposed to nifedipine, a dihydropyridine Ca\(^{2+}\) channel antagonist, before stimulation with progesterone. Inclusion of nifedipine inhibited the phosphorylation response elicited by progesterone, indicating that an influx of Ca\(^{2+}\) from the extracellular compartment is required for the activation of PKC. However, the fact that treatment with nifedipine also blocks DAG formation and acrosomal exocytosis (O’Toole et al., 1996a) raises the question of which mechanism is actually being inhibited. It is possible that PKC activation, and hence phosphorylation, does not take place due to the absence of a rise in DAG, the endogenous activator of PKC (Rando, 1988). If this is the case, then exogenously added DAG should cause phosphorylation and this proved to be the case: a permeant synthetic DAG (OAG) stimulated this response, as did PMA. These results then raise the question of whether an elevation in intracellular Ca\(^{2+}\) is required in of whether an elevation in intracellular Ca\(^{2+}\) is required in products of phosphatidylinositol turnover. This suggests a role for PKC in the signal transduction pathway involving activation of polyphosphoinositide-specific phosphoinositidase C (PIC) and subsequent hydrolysis of polyphosphoinositides to generate inositol 1,4,5-trisphosphate and DAG. Tumour-promoting phorbol esters can also activate PKC, suggesting that the enzyme may be important in both normal and abnormal cell metabolism (Nishizuka, 1984; Martelly and Castagna, 1989).

Activation of PIC and PKC has been demonstrated in a wide variety of somatic cell systems where Ca\(^{2+}\)-dependent exocytosis occurs (Kikkawa and Nishizuka, 1986; Cheek, 1993). The mammalian sperm acrosome reaction is a Ca\(^{2+}\)-dependent exocytic event and activation of PIC is known to be an early response to Ca\(^{2+}\) entry from the extracellular compartment (Roldan and Harrison, 1989). Immunohistochemical analyses of human spermatozoa using both light (Rotem et al., 1990a,b; 1992) and electron microscopy (Kalina et al., 1995) indicate the presence of at least some isoforms of PKC in both the head and tail. However, a role for PKC in the exocytotic pathway has remained equivocal, with available evidence being indirect in nature. Phorbol esters and permeant synthetic DAGs have been reported capable of initiating acrosomal exocytosis in capacitated human sperm suspensions (DeJonge et al., 1991; Rotem et al., 1992), but such treatments effectively circumvent the signal transduction pathways that appear to function during fertilization (Yanagimachi, 1994) and hence may not mimic normal endogenous events. Moreover, it is now recognized that both phorbol esters and DAGs have other targets and, therefore, may be acting via mechanisms not involving PKC (Wilkinson and Hallam, 1994).

As stated earlier, human spermatozoa stimulated to undergo acrosomal exocytosis are known to generate DAG, with both PIC and phospholipase C contributing to the overall pool of DAG detected (Roldan and Harrison, 1989; O’Toole et al., 1996b); no evidence for phospholipase D activity in human spermatozoa has been obtained (O’Toole et al., 1996b). Therefore, it is possible that this endogenously-generated DAG might activate PKC; the experimental evidence obtained in this study supports a role for PKC in acrosomal exocytosis.

Direct evidence for PKC activation in response to a natural agonist of exocytosis was obtained by investigating phosphorylation of endogenous proteins in response to progesterone. This agonist interacts with the sperm plasma membrane and activates a receptor-mediated signal transduction pathway leading to membrane fusion (Meizel et al., 1990; Meizel, 1995; Roldan, 1995). The human sperm response to progesterone resulted in a time-dependent increase in protein phosphorylation. At least eight phosphoproteins, ranging in size from ~20 to 220 kDa, could be detected in stimulated cells; proteins of a size similar to four of these phosphoproteins (~29, 38, 46 and 220 kDa) could be seen on Coomassie Blue-stained gels (data not shown), suggesting that some of the protein substrates may be reasonably abundant. Several results suggest that phosphorylation of these proteins in response to progesterone is due to PKC: (i) a permeant synthetic DAG (OAG) and a tumour-promoting phorbol ester (PMA), known to activate PKC, stimulated phosphorylation of the same proteins; and (ii) a phorbol ester which is incapable of activating PKC did not stimulate phosphorylation; (iii) both progesterone- and PMA-stimulated phosphorylation was inhibited by inclusion of two different and highly specific PKC inhibitors.
Figure 5. Stimulation of acrosomal exocytosis by a phorbol ester and a synthetic diacylglycerol and inhibition by inclusion of a protein kinase C (PKC) inhibitor. (A) Sperm suspensions were preincubated for 4 h 45 min in Earle's medium and the PKC inhibitor calphostin C was then added to two samples. At 5 h, phorbol 12-myristate-13-acetate (PMA) (final concentration 1 μM) was added to the inhibitor-treated samples plus one other; a fourth sample was exposed to an inactive form of PMA, 4α-phorbol-12,13-didecanoate (4α-PDD) (final concentration 1 μM). All samples were incubated for 30 min, then stained with chlorotetracycline (CTC) and assessed. Results are mean ± SE (n = 8). Histograms show: (a) untreated control cells; (b) PMA-treated cells; (c and d) cells treated with calphostin C (0.05 and 0.15 μM respectively) plus PMA; (e) 4α-PDD-treated cells. ■ = F-uncapacitated acrosome-intact; ■ = -capacitated acrosome-intact; ■ = AR-capacitated acrosome-reacted. *P <0.05, **P <0.001 compared with untreated controls; +P <0.01 compared with PMA-treated cells. (B) Sperm suspensions preincubated for 5 h in Earle's medium were incubated in the presence of: (a) no added compounds (control cells); (b) a synthetic active form of diacylglycerol (DAG), 1,2-dioctanoyl-sn-glycerol (final concentration 10 μM); (c) a 1:1 mix of 1,2-dioctanoyl-sn-glycerol and 1,3-dioctanoylglycerol (final concentration 10 μM); (d) an inactive DAG, 1,3-dioctanoylglycerol (final concentration 10 μM) for 30 min. All samples were then stained with CTC and assessed. Results are mean ± SE (n = 8). *P <0.05, **P <0.01, ***P <0.001 compared with untreated controls.

et al., 1981). Since either OAG or phorbol esters alone can stimulate capacitated spermatozoa to complete the sequence leading to membrane fusion (DeJonge et al., 1991; Rotem et al., 1992; this study), a process which requires an intracellular Ca\(^{2+}\) rise (Roldan and Harrison, 1989; Roldan and Fragio, 1993), some mechanism presumably provides the required Ca\(^{2+}\). One possible explanation is that the rise in intracellular Ca\(^{2+}\) occurring during capacitation (Florman, 1994; Suarez and Dai, 1995) provides sufficient Ca\(^{2+}\) to support events occurring downstream of PKC activation. For example, mouse spermatozoa preincubated for 30 min (insufficient time for complete capacitation) in reduced Ca\(^{2+}\)-containing medium (0.09 μM; sufficient to support capacitation but not spontaneous acrosomal exocytosis) did not undergo exocytosis in response to A23187 (Fraser and McDermott, 1992), but this same treatment applied to 120 min preincubated capacitated cells triggered a significant exocytotic response (Forsdike and Fraser, 1996). This altered response suggests a capacitation-dependent rise in intracellular Ca\(^{2+}\), resulting in cells able to respond to ionophore despite the low extracellular Ca\(^{2+}\). Therefore capacitated cells might be equally responsive to activators of PKC.

Whether PKC itself might control additional changes in intracellular Ca\(^{2+}\) is unclear at present. Foresta et al. (1995) reported that inclusion of two non-specific inhibitors of PKC (H-7 and staurosporine) inhibited the progesterone-stimulated Ca\(^{2+}\) rise in human spermatozoa, whereas Bonaccorsi et al. (1995) found that a different inhibitor, sangivamycin, did not. Further research is required to address this specific point.

A clear link between PKC-mediated phosphorylation of a series of sperm proteins and exocytosis was evident. Conditions that stimulated phosphorylation (e.g. treatment with progesterone or with either PMA or exogenous DAG, both well known activators of PKC) also resulted in exocytosis in capacitated cells, whereas the use of reagents incapable of activating PKC caused neither. Also, inhibition of progesterone and phorbol ester-induced phosphorylation was paralleled by inhibition of exocytosis. These results are consistent with an essential role for PKC in the exocytic pathway.

As noted in an earlier study, progesterone treatment of preincubated suspensions not only triggered exocytosis in capacitated cells but also stimulated capacitation, as determined by the significant decrease in the proportion of F pattern (uncapacitated) cells, even after a short (30 min) exposure to progesterone. Interestingly, the presence of either PKC inhibitor had no inhibitory effect on this response to progesterone, suggesting that PKC may not play a role in capacitation itself but only during exocytosis. Furthermore, treatment of preincubated suspensions with PMA or OAG only caused a transition from the B (capacitated) to the AR pattern, but not from the F (uncapacitated) to the B pattern; consistent with this, an earlier study demonstrated that uncapacitated suspensions were unable to undergo exocytosis in response to OAG (O'Toole et al., 1996b). The present data indicate that PKC can only be activated in capacitated cells, suggesting that there are capacitation-related changes in the availability of PKC for activation, e.g. alterations in PKC association with the sperm plasma membrane.

In conclusion, the evidence gathered in this study demonstrates a fundamental role for PKC activation, with its con-
sequent phosphorylation of endogenous proteins, during progesterone-induced acrosomal exocytosis in human spermatozoa and therefore during fertilization.

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