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Role of protein kinase C in the acrosome reaction of mammalian spermatozoa

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Mammalian spermatozoa undergo a Ca^{2+}-dependent exocytotic event before fertilization which is known as the acrosome reaction. The process of exocytosis in several cell systems is mediated by a protein kinase C (PKC)-catalysed phosphorylation. Addition of phorbol 12-myristate-13-acetate or the membrane-permeant diacylglycerol analogue 1-oleoyl-2-acetylglycerol, which are potent activators of PKC, to bovine spermatozoa resulted in stimulation of the acrosome reaction. This stimulation was inhibited by low concentrations (50% inhibition at 0.7 nM) of the PKC inhibitor staurosporine. PKC specific activity in bovine spermatozoa is extremely low in comparison with other cells; however, it is comparable with the activity found in human spermatozoa. Immunohistochemical analysis using anti-PKC antibodies revealed staining in the equatorial segment, the post-acrosomal region and the upper region of the head. We propose that PKC is involved in the mammalian sperm acrosome reaction.

INTRODUCTION

Mammalian spermatozoa must undergo an exocytotic process known as the acrosome reaction, at the time of fertilization, in order to penetrate the oocyte vestments. In the female reproductive tract, an agonist triggers a series of events which culminate in the fusion of the outer acrosomal membrane and the overlying plasma membrane, thereby releasing the contents of the acrosome [1,2]. Several lines of research on the mechanism of the acrosomal reaction have suggested the involvement of receptor-mediated signal transduction in this process (see [3] for review). Protein kinase C (PKC), which is a key regulatory enzyme in signal transduction mechanisms [4], has been implicated in several cell systems as being involved in cellular exocytosis [5–7]. It has been reported that diacylglycerol and phorbol esters, which are known to stimulate PKC activity, enhance the onset of the early stages of the acrosome reaction in mouse spermatozoa [8]. It has also been demonstrated that, during the acrosome reaction of ram spermatozoa, there is a rapid breakdown of phosphoinositides, which results in the release of diacylglycerol, and which seems to be linked with subsequent exocytosis [9,10]. It has been reported that very little PKC activity, which was lost after DEAE-cellulose chromatography, is present in pig spermatozoa [11]. Also PKC activity was detected in human spermatozoa [12], but not in ram sperm [13], although ram sperm produce inositol 1,4,5-trisphosphate, which is a component of the signal-transduction mechanism [14]. Here we demonstrate, for the first time, the presence of PKC in ram and bull spermatozoa, and evidence is presented for the involvement of PKC in the mechanism of the sperm acrosome reaction.

MATERIALS AND METHODS

Materials

Phosphatidylserine (bovine brain), calf thymus histone (type III-S), phorbol 12-myristate 13-acetate (PMA), leupeptin, phenylmethanesulphonyl fluoride, 1-oleoyl-2-acetylglycerol (OAG) and diolein were purchased from Sigma. [γ-^{32}P]ATP (> 5 mCi/mmol; 1 Ci = 37 GBq) was obtained from Amersham. Staurosporine and 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) were purchased from Kyowa, Tokyo, Japan. A23187 (free acid) was from Calbiochem. A23187 and PMA were dissolved in ethanol, and the final concentration of ethanol in the assay was 0.1%. The solvent was added to the control tubes.

Methods

Sperm preparations. Frozen bull sperm cells were thawed at 37 °C, in medium comprising 150 mM-NaCl and 10 mM-histidine, pH 7.4. The cells were washed by three centrifugations at 780 *g* for 10 min. For preparation of ram sperm, semen was collected from a ram by electric induction. The fresh semen was immediately diluted (1:1, v/v) with medium containing 110 mM-NaCl, 5 mM-KCl and 10 mM-Mops (pH 7.4). The cells were washed by three centrifugations at 780 *g* for 10 min. Only those samples with a minimum of 70% motile spermatozoa were used for experimentation.

Determination of acrosome reaction. Washed cells (10^8 cells/ml) were capacitated for 4 h at 37 °C in modified Tyrode's medium (TALP medium [15]) containing 20 μg of heparin/ml [16]. PMA was then added for another 20 min of incubation. At the end of the incubation, the cells were spun down by centrifugation (7500 *g* for 20 min), and the occurrence of the acrosomal reaction was determined by measuring the activity of the released acrosin in the supernatant as described by us previously [17]. Briefly, the supernatant was adjusted to pH 3.0 with 1 M-HCl, and the acrosin activity was determined by the esterolytic assay with the substrate benzoylarginine ethyl ester (BAEE), by recording the increase in A_{259} with time. The molar absorption coefficient was taken as 1150. The occurrence of the acrosome reaction was confirmed by observing thin sections of spermatozoa in the transmission electron microscope [17].

PKC activity studies. Washed cells [$(2–3) \times 10^8$ cells] were transferred to the homogenization buffer [20 mM-Tris/HCl (pH 7.5)/0.25 M-sucrose/10 mM-EGTA/2 mM-EDTA/1 mM-phenylmethanesulphonyl fluoride/leupeptin (20 μg/ml). The cells were homogenized and then sonicated for 60 s at 4 °C; the homogenate was then centrifuged for 60 min at 100000 *g*. The particulate fraction was resuspended in the homogenization buffer, homogenized, sonicated and incubated with Triton X-100 (0.5%) for 30 min at 4 °C to release the membrane-associated

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetylglycerol; BAEE, benzoylarginine ethyl ester; IC_{50}, concn. giving 50% inhibition.

PKC. The particulate fraction was centrifuged for 30 min at 12000 *g*. The two supernatants were applied to mini-columns of DEAE-cellulose DE-52 equilibrated with 20 mM-Tris/HCl (pH 7.5) containing 0.5 mM-EGTA, 0.5 mM-EDTA and 10 mM-2-mercaptoethanol (buffer A). The column was washed with 4 vol. of buffer A, and then with 4 vol. of buffer A containing 20 mM-NaCl. The PKC was eluted with 4 vol. of buffer A containing 120 mM-NaCl as recently described [18]. Samples (30 μl; 1 μg of protein) were taken for assay of PKC activity as described previously [19]. The reaction mixture (0.25 ml) contained 5 μmol of Tris/HCl, pH 7.5, 1.25 μmol of magnesium acetate, 50 μg of histone, 2.5 nmol of [γ-^{32}P]ATP [(5–15) $\times$ 10^5 c.p.m./nmol], 8 μg of phosphatidylserine/ml, 0.8 μg of diolein/ml, 0.3 mM-$CaCl_2$ and enzyme preparation. Basal activity was measured in the presence of 0.3 mM-Ca^{2+} and was subtracted from the total activity. The assay was carried out for 20 min at 30 °C. The reaction was then stopped by addition of 25% (w/v) trichloroacetic acid and the acid precipitate was collected on membrane filters (0.45 μm pore size). The samples were counted for radioactivity by the Čerenkov method.

Immunocytochemistry studies. Sperm (2×10^6 cells) were collected on glass slides in a cytocentrifuge (600 rev./min). The cells were fixed and permeabilized with cold methanol (10 min), followed by cold acetone (10 min). The cells were treated with the respective antibodies (diluted 1:10) for 18 h at 4 °C. The immunostaining procedure was performed by the avidin/biotin technique [20].

The antibodies were kindly provided by Professor Y. Nishizuka (Department of Biochemistry, Kobe Medical School, Kobe, Japan).

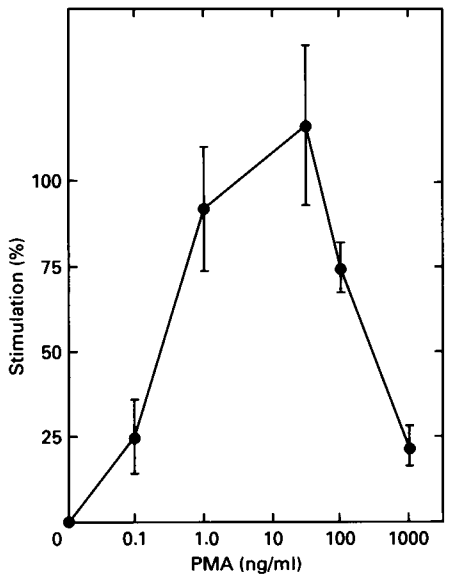

Fig. 1. Effect of PMA on the acrosome reaction

Bovine sperm cells (10^8/ml) were incubated in 1 ml of TALP medium containing heparin for 4 h; then increased concentrations of PMA were added and the cells were incubated for another 20 min. Acrosin released from the cells was determined at the end of the incubation time as described in the Materials and methods section; 100% stimulation of acrosin activity represents 150±35 nmol of BAEE/10^8 cells. This activity represents 40% acrosome-reacted cells, as revealed in the electron microscope. The acrosin activity in the absence of Ca^{2+} (with 2 mM-EGTA) was subtracted from each measurement. Under these conditions, only 5% of the cells are acrosome-reacted. Each point represents the mean ± S.E.M. of duplicates from at least three experiments. For 1 and 50 ng of PMA/ml, $n = 10$ (1 ng of PMA/ml = 1.62 nM).

RESULTS

The acrosome reaction in bovine sperm was determined by measuring the release of acrosin from the cells [17]. Addition of the tumour promoter PMA, a known PKC activator [4], resulted in a dose-dependent increase in acrosin release (Fig. 1), with a maximal effect observed at 50 ng of PMA/ml (81 nM). However, at higher concentrations of PMA inhibition was observed. Observation of cells by transmission electron microscopy revealed that 58% or 35% of cells had undergone the acrosome reaction after treatment with A23187 or 1 mg of PMA/ml respectively. Very weak stimulation (approx. 20%) of acrosin release was detected after addition of the inactive phorbol ester analogue 4α-phorbol 12,13-didecanoate (1 ng/ml) to the incubated cells.

Addition of the membrane-permeant diacylglycerol analogue OAG, a specific PKC activator, to the incubated cells, resulted in an increase in acrosin release (Table 1). The stimulatory effect of PMA or OAG on the sperm acrosome reaction was inhibited by the known PKC inhibitors 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) and staurosporine ([21–23]; Fig. 2 and Table 1). These two inhibitors were also active when the acrosome reaction was induced by the Ca^{2+} ionophore A23187 (Table 1). The calculated IC_{50} values (concn. giving 50% inhibition) for staurosporine are 0.7 nM and ~ 10 nM for the acrosome reaction induced by 1 ng of PMA/ml (1.62 nM) or 2 μM-A23187 respectively.

The activity of PKC in ram and bull spermatozoa was

Table 1. Effect of PKC inhibitors on acrosome reaction induced by A23187, PMA or OAG

Bovine sperm cells (10^8/ml) were incubated as described in the legend to Fig. 1. Cells were incubated for 4 h in heparin-containing TALP medium, and then the inhibitor was added for another 10 min and then the inducer was added for another 20 min of incubation. The activity of the acrosin released from the cells was determined at the end of the incubation time as described under 'Methods'. The acrosin activity in the absence of Ca^{2+} but in the presence of A23187 or PMA (104±23 nmol of BAEE/10^8 cells) was subtracted from each measurement. This activity is not inhibited by the PKC inhibitor. No detectable acrosin activity above the baseline (without Ca^{2+}) was found in Ca^{2+}-containing medium in the absence of the inducer. Abbreviation: STP, staurosporine.

	2 μM-A23187			50 ng of PMA/ml (81 nM)			3 μM-OAG		
Inhibitor	*n*	Activity (nmol of BAEE/10^8 cells)	Inhibition (%)	*n*	Activity (nmol of BAEE/10^8 cells)	Inhibition (%)	*n*	Activity (nmol of BAEE/10^8 cells)	Inhibition (%)
None	8	211±52	–	18	167±39	–	3	128.6±12	–
10 nM-STP	4	133±35	37	11	28.4±7	83	3	6.4±2	95
100 nM-STP	4	27.5±9	87	7	26.7±6	84	–	–	–
10 μM-H-7	2	102±18	52	4	30.1±8	82	–	–	–

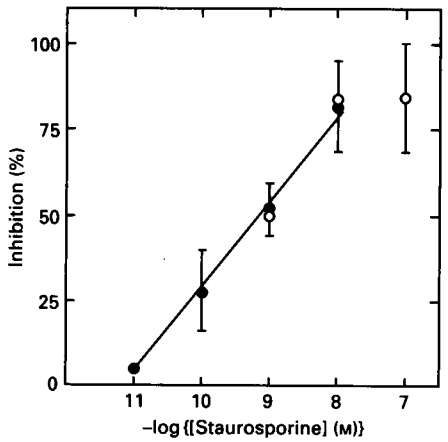

Fig. 2. Effect of the PKC inhibitor staurosporine on PMA-induced acrosome reaction

Bovine sperm cells (10^8/ml) were incubated under conditions similar to those described in Fig. 1. Staurosporine was added 10 min before PMA [●, 1 ng/ml (1.62 nM); ○, 50 ng/ml (81 nM)] and incubation was continued for another 20 min. The 100% acrosin activity in the absence of staurosporine was 163 ± 39 nmol of BAEE/10^8 cells. Each point represents the mean ± S.E.M. of duplicates from three experiments.

determined by measuring the phosphorylation of histone III-S with [γ-^{32}P]ATP as the phosphate donor. Approx. 80% (0.40 pmol of ^{32}P/min per μg of protein) of the PKC activity of bull sperm was found in the soluble fraction and only 20% (0.10 pmol of ^{32}P/min per μg of protein) was located in the particulate fraction. Ram sperm show a similar distribution of the PKC activity between the soluble and the particulate fractions. The PKC activity in the ram was 0.88 pmol of ^{32}P/min per μg of protein in the soluble fraction and 0.28 pmol of ^{32}P/min per μg of protein in the particulate fraction. It is noteworthy that different results were obtained for human sperm, in which 55% and 45% of the PKC activity was located in the particulate and the soluble fractions respectively. Bull and ram sperm PKC activity was very weak, and represented approx. 3% of the specific activity found in rat brain enzyme [19]. These results might explain the lack of information on sperm PKC and the report on the absence of PKC in ram spermatozoa [13].

For location of the PKC, monoclonal antibodies (CKm CIβ-a [19,20,24]) reactive with rat brain α, β and γ PKC subspecies were used. PKC in bull sperm is localized mainly in the post-acrosomal region of the sperm, and also in the upper region of the acrosome (Fig. 3*a*). For ram sperm the picture is similar; the post-acrosomal region shows less staining and the upper region of the acrosome was stained more strongly (results not shown).

The specificity of the immunostaining was demonstrated by pretreatment of the antibodies with purified rat brain PKC [19,24], which almost completely blocked the immunostaining (Fig. 3*b*). Furthermore, no staining was observed when non-immune rabbit or mouse serum was used (results not shown).

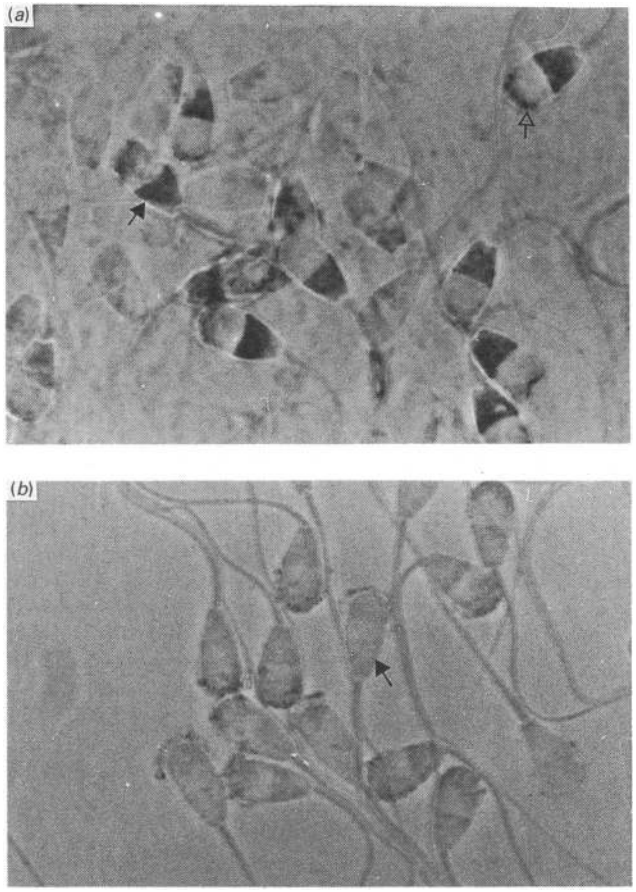

Fig. 3. Localization of PKC in bovine sperm

Sperm cells (2×10^6) were collected on glass slides by cytocentrifuge and stained for PKC. (*a*) Cells treated with the monoclonal antibody CKm CIβ-a, which is reactive to the rat brain PKC subspecies (α, β and γ). Note the region of the acrosome (white arrow) and the post-acrosomal region (black arrow). (*b*) Cells treated with CKm CIβ-a antibody, which was pretreated with rat brain PKC for 1 h at 37 °C. Note the absence of staining in the post-acrosomal region (see the arrow). Magnification × 1000.

DISCUSSION

The presence of signal-transduction molecules in mammalian spermatozoa has been supported by several experimental techniques. This concept is supported by the observation that mouse and bovine sperm contain a protein with properties similar to the inhibitory guanine-nucleotide-binding regulatory protein G_i [25,26]. This protein appears to mediate a signal-transduction mechanism distal to the binding of cell-surface receptors by their respective ligands in somatic cells [27]. Functional inactivation of the G_i-like protein in mouse sperm by pertussis toxin inhibits the zona-induced acrosome reaction [28]. A G-protein has been found to be involved in the increases in internal Ca^{2+} and pH that mediate bovine sperm acrosomal exocytosis [29]. Our findings of PKC in bull and ram spermatozoa are in accordance with other reports showing that phorbol esters and diacylglycerol enhanced the onset of the early stages of the acrosome reaction in mouse sperm [8]. Furthermore it has been reported that treatment of human sperm with Ca^{2+} and the Ca^{2+} ionophore A23187 resulted in accumulation of diacylglycerol and phosphatidic acid [10], and that these cells contain phosphatidylinositol-specific phospholipase C activity [30]. A recent publication described PKC activity in human sperm and the involvement of the enzyme in regulation of cell motility [12]. In the present study, we describe for the first time PKC activity in bull and ram spermatozoa, and we propose that the enzyme is involved in the regulation of the acrosome reaction.

The presence of PKC is supported by the data showing Ca^{2+}/phospholipid-dependent phosphorylation of histone III-S, and the staining by specific anti-PKC monoclonal antibodies of specific compartments of the sperm. The stimulation of the acrosome reaction by PMA and OAG, its inhibition by low concentrations of staurosporine and the localization of PKC in

the sperm head further support the notion that PKC is involved in the regulation of the acrosome reaction. The distribution of PKC between the soluble and the particulate fractions, and the IC_{50} for staurosporine, further reveal a typical behaviour of PKC that has been found in a large number of somatic cells [4,21–23]. The lower IC_{50} value observed for staurosporine when the acrosome reaction was induced by PMA (0.7 nM) as opposed to A23187 (approx. 10 nM) suggests the involvement of other protein kinases, such as Ca^{2+}/calmodulin-dependent kinase, in the A23187-treated cells.

Ca^{2+} and cyclic AMP are thought to be the two pivotal regulators of the sperm acrosome reaction [31]. These two second messengers exert their biological effects by means of activation of a respective protein kinase and phosphorylation of key proteins involved in the acrosome reaction [32]. Since most biological systems that are controlled by protein phosphorylation are now recognized to be under the complex interaction of cytosolic Ca^{2+}, guanine and cyclic nucleotides and PKC [4], it is not surprising that PKC also plays a crucial role in regulating the sperm acrosome reaction along with cyclic AMP and Ca^{2+} by means of phosphorylation of endogenous proteins.

Initiation of the mammalian sperm acrosomal reaction is thought to be induced by increases in intracellular pH and Ca^{2+} [29]. The increase in intracellular Ca^{2+} may be mediated by a voltage-dependent Ca^{2+} channel, which is present in the plasma membrane of mammalian spermatozoa [33,34]. Since PKC has been shown to regulate Ca^{2+} channels [35], this may be one possible mechanism in which PKC is involved in the regulation of the acrosome reaction. Alternatively, or in addition, PKC is known to activate Na^+/H^+ exchange [36], and the resulting increase in pH might mediate the acrosome reaction, in concert with the elevated intracellular Ca^{2+}.

The Ca^{2+} channel and/or the Na^+/H^+ exchanger or the PKC itself might be translocated during the capacitation period towards the upper region of the head, in which the acrosome reaction takes place. This can explain our observation that the bulk of the PKC is localized in the post-acrosomal region (bull and ram) or in the equatorial segment (human; see [12]). One can postulate that before capacitation the Ca^{2+} channel and/or the Na^+/H^+ exchanger are localized in the post-acrosomal region, and after being phosphorylated by PKC they are translocated to the upper region of the head and initiate the acrosome reaction. We do not think that this is true, since the PKC stimulator PMA is added for a period of 20 min only after 4 h of capacitation. Thus there is no need for PKC activation during the capacitation time, and at the end of this period of time the ion carriers or PKC are already localized in their functional location. Alternatively, we suggest that PKC can be translocated during capacitation from the post-acrosomal region to the upper region of the head in order to phosphorylate proteins which were involved in the occurrence of acrosome reaction.

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