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Protein kinase C is present in human sperm: Possible role in flagellar motility

(immunohistochemistry/phorbol ester)

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ABSTRACT **We report the presence of protein kinase C (PKC) in ejaculated human sperm as revealed by enzymatic activity assay and indirect immunohistochemistry. PKC is localized in the equatorial segment and in the principal piece of the tail. Addition of phorbol 12-myristate 13-acetate resulted in increased flagellar motility that was blocked by known PKC inhibitors such as sphingosine, staurosporine, and 1-(5-isoquinoylinylsulfonyl)-2-methylpiperazine. A very good correlation ($r = 0.9$, $P < 0.001$) was found between the percentage of PKC-stained sperm cells and motility. We propose that PKC is involved in the regulation of flagellar motility in human sperm.**

Protein kinase C (PKC) is a key regulatory enzyme in signal transduction mechanisms governing cellular responses (1). Nevertheless, its presence and role in human sperm have not been reported. PKC is present in rat seminiferous tubules and Leydig cells (2, 3). However, very little PKC activity was detected in the cytosol of mature spermatozoa isolated from pig epididymis, and the residual activity was lost after DEAE-cellulose chromatography (2). The two above-mentioned reports (2, 3) suggested the presence of very low levels of PKC in rat and pig germ cells. The present report demonstrates the presence of PKC in human sperm and evidence is presented that PKC is involved in human sperm flagellar motility.

MATERIALS AND METHODS

Materials. Phosphatidylserine (bovine brain), calf thymus histone (type III-S), phorbol 12-myristate 13-acetate (PMA), leupeptin, phenylmethylsulfonyl fluoride (PMSF), 1-oleoyl-2-acetylglycerol (OAG), and diolein were from Sigma. [τ-^{32}P]ATP (>5000 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham.

Methods. Frozen human sperm from healthy donors (21–27 years old) was thawed and washed twice with Ham's F-10 medium to remove the seminal plasma and the cryoprotectant. Cells (2–3 × 10^8 per tube) were then transferred to homogenization buffer (20 mM Tris·HCl, pH 7.5/0.25 M sucrose/10 mM EGTA/2 mM EDTA/2 mM PMSF/40 μg of leupeptin per ml). The cells were homogenized and then sonicated for 60 s at 4°C; the homogenate was then centrifuged for 60 min at 100,000 × g. The particulate fraction was resuspended in homogenization buffer, homogenized, sonicated, and incubated with Triton X-100 (0.5%) for 30 min at 4°C to release the membrane-associated PKC. The particulate fraction was centrifuged for 30 min at 12,000 × g. The two supernatants were applied to minicolumns of 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 washed with 4 vol of buffer A containing 20 mM NaCl. PKC was eluted with 4 vol of buffer A containing 120 mM NaCl as described (4). Samples of 30 μl (≈1 μg of protein) were subjected to assay of PKC activity as described (5). 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 (III-S), 2.5 nmol of [τ-^{32}P]ATP (5–15 × 10^5 cpm/nmol), 8 μg of phosphatidylserine per ml, 0.8 μg of diolein per ml, 0.3 mM Ca^{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 stopped by addition of 25% trichloroacetic acid and collection of the acid precipitate on membrane filters (0.45 μm); this was followed by assaying the samples for radioactivity using the Cerenkov method.

Sperm motility studies. The sperm was washed as described above and incubated in Ham's F-10 medium with the stimulants at 33°C. Motility was counted under a microscope using an A. R. Horwell counting chamber (London; ref. 6).

Immunohistochemistry studies. Human sperm (2 × 10^6) was collected on glass slides by cytospin (600 rpm). The cells were fixed and permeabilized by cold methanol (10 min) followed by cold acetone (10 min). The cells were treated with the respective antibodies (all of them diluted 1:10) for 18 hr at 4°C. The immunostaining procedure utilized the avidin-biotin technique (7).

Preparation of antibodies. Preparation of monoclonal antibodies CKI-97 (type I, τ-specific) and CKII-90 (type II, β-specific) has been described by Kitano *et al.* (8). CKmCIβ-a (a monoclonal antibody) and CKpCIβ-a (a polyclonal antibody), both reactive to the rat brain PKC subspecies (α, β, and τ), have been described (9, 10). Polyclonal peptide antibodies against βI and βII PKC subspecies (CKpV5βI-a and CKpV5βII-a) were described by Ase *et al.* (11). All of the above antibodies were kindly provided by Y. Nishizuka (Department of Biochemistry, Kobe Medical School, Kobe, Japan).

RESULTS

PKC activity in human sperm was determined by measuring the transfer of the phosphate group from [τ-^{32}P]ATP to histone III-s as described (4, 5). Whereas most of the PKC activity was found in the soluble fraction of the majority of the cells examined (1), the opposite was found in human sperm, where the enzyme is distributed in the particulate (0.22 pmol of ^{32}P per min per μg of protein, representing 55%

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Abbreviations: PKC, Ca^{2+}-activated phospholipid-dependent protein kinase C; PMA, phorbol 12-myristate 13-acetate; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; sphingosine, *trans*-D-*erythro*-2-amino-4-octadecene-1,3-diol (also known as 4-sphinganine); OAG, 1-oleoyl-2-acetylglycerol; PMSF, phenylmethylsulfonyl fluoride.

of total) and soluble fractions (0.18 pmol of ^{32}P per min per μg of protein, 45%; n = 15). Nevertheless, human sperm PKC activity is low and amounts to about 2.5% of the specific activity found in the rat brain enzyme (5). The results might explain the lack of information on human sperm PKC and other reports on the absence of active PKC in ram spermatozoa (12).

Addition of the tumor promoter PMA, a known PKC activator (1), resulted in dose- and time-dependent increases in human sperm motility (Fig. 1), with the maximal effect being observed between 5 and 15 min after addition of the phorbol ester. The effect diminished with time and was not detected after 60 min of incubation. No stimulatory effect was noticed when the inactive phorbol ester analog 4α-phorbol 12,13-didecanoate (4α-PDD, 50 ng/ml) was added to human sperm for 15 min [38% ± 0.6%, 42% ± 2.3%, and 52% ± 0.8% motility for control, 4α-PDD, and 20 ng of PMA per ml (32 nM), respectively].

Addition of the membrane-permeable diacylglycerol analog OAG, a specific PKC activator, to incubated human sperm resulted in increased sperm motility in a dose- and time-dependent fashion (data not shown). The maximal effect was observed at 15 min of incubation following a decrease to basal levels at 60 min, similar to that observed with PMA. The similarity of the PMA and OAG effects suggests that PMA stimulated human sperm motility by activation of PKC.

The stimulatory effect of PMA on human sperm motility was inhibited by known PKC inhibitors such as 1-(5-isoquinoylinylsulfonyl)-2-methylpiperazine (H-7), *trans*-D-*erythro*-2-amino-4-octadecene-1,3-diol (sphingosine; also known as 4-sphinganine), and staurosporine (refs. 13–15; Fig. 2). The IC_{50} values observed for H-7 (6.6 μM), sphingosine (8.8 μM), and staurosporine (0.7 nM) are in excellent agreement with those reported for inhibition of PKC activity (13–15). Whereas H-7 and sphingosine showed no consistent effect on basal motility, staurosporine inhibited basal motility by 30% under the conditions of the present study.

Using a monoclonal antibody (CKmCIβ-a; refs. 5, 9, 16) and a polyclonal antibody (CKpCIβ-a; ref. 10) that are reactive to rat brain α, β, and τ PKC subspecies, we could demonstrate that PKC is localized in the equatorial segment of the sperm cell in a distinct band (Fig. 3 *a* and *c*). Both antibodies also stained the principal piece of the tail to a lesser extent (Fig. 3 *a* and *c*). Occasionally, we noticed staining in the centriole area. The midpiece, nucleus, and acrosome were unstained. The specificity of the immunostaining was demonstrated by pretreatment of both antibodies with purified rat brain PKC (5, 16), which completely blocked the immunostaining (Fig. 3 *b* and *d*). Also, no staining was observed with nonimmune rabbit or mouse serum (data not shown).

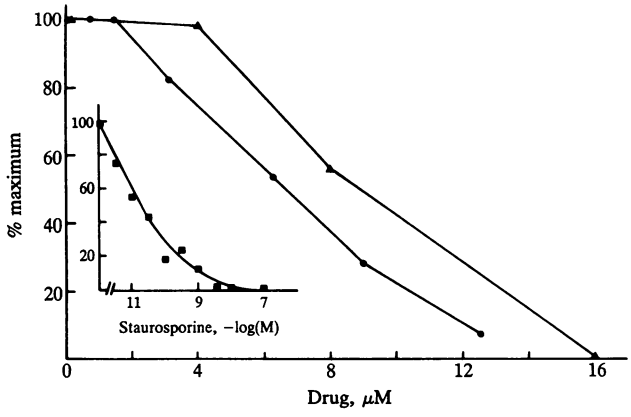

FIG. 2. Effect of PKC inhibitors on PMA-induced sperm motility. Human sperm (3–4 × 10^7 cells per tube) was incubated under similar conditions to those described in the legend to Fig. 1. PKC inhibitors sphingosine (▲, prepared in bovine serum albumin, 1:1 molar ratio) and H-7 (●) were added 10 min before PMA (80 nM) and incubation was continued for another 15 min in the presence of the drugs. (*Inset*) Staurosporine was added for 2 min and cells were washed and incubated with PMA (80 nM) for 15 min in the absence of the drug.

Preliminary studies with type-specific antibodies (1) suggest that type II PKC (β) is present in human sperm. This is based on staining with CKII-90 (rat brain type II-specific monoclonal antibody; ref. 8). As with the previous staining with the polyclonal antibody, the staining was concentrated in the equatorial segment, and the principal piece of the tail was only slightly stained (results not shown). Peptide polyclonal antibodies specific to βI and βII (11, 17, 18) revealed that βI is present in the principal piece of the tail (Fig. 3*e*). The staining of βI was faint, although it was similar to that observed in Fig. 3 *a* and *c*. Thus it indicates a low antigenicity in this region, which may reflect a low concentration of PKC. Antibody to βII stained the equatorial segment only weakly (Fig. 3*f*) compared with Fig. 3 *a* and *c*. It is therefore reasonable to assume that PKC subspecies other than βII (most likely α PKC since it is the most widely expressed subspecies; refs. 1, 10) are also present in this region. These subspecies are recognized by the monoclonal and polyclonal antibodies (CKmCIβ-a, CKpCIβ-a) that are directed against α, β, and τ PKC. No staining was observed with CKI-97 [rat brain type I (τ)]-specific monoclonal antibody, in agreement

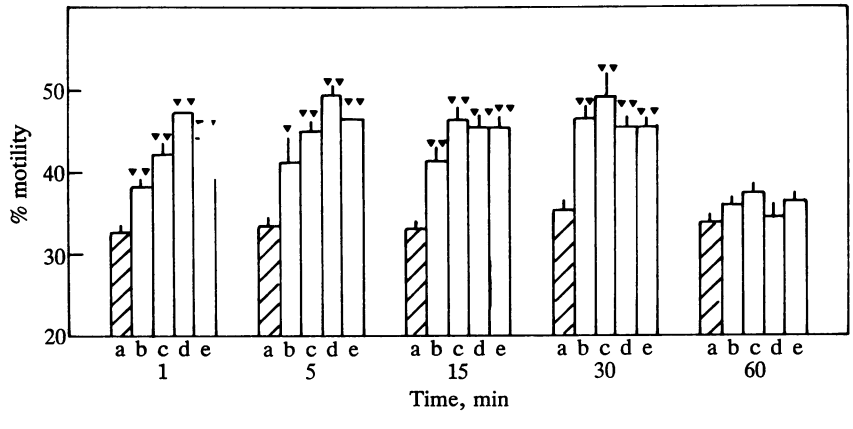

FIG. 1. Effect of PMA on human sperm motility. Frozen human sperm was thawed, washed, and incubated in 0.5 ml of Ham's F-10 medium (3–4 × 10^7 cells per tube) with increasing concentrations of PMA (a = 0, b = 16, c = 32, d = 80, and e = 160 nM) for the times indicated at 33°C. Motility was counted under a microscope using an A. R. Horwell counting chamber (London). Results are expressed as the mean ± SEM from three experiments, each done in triplicate. ▼, $P < 0.05$; ▼▼, $P < 0.01$ vs. basal motility. (Student t test.)

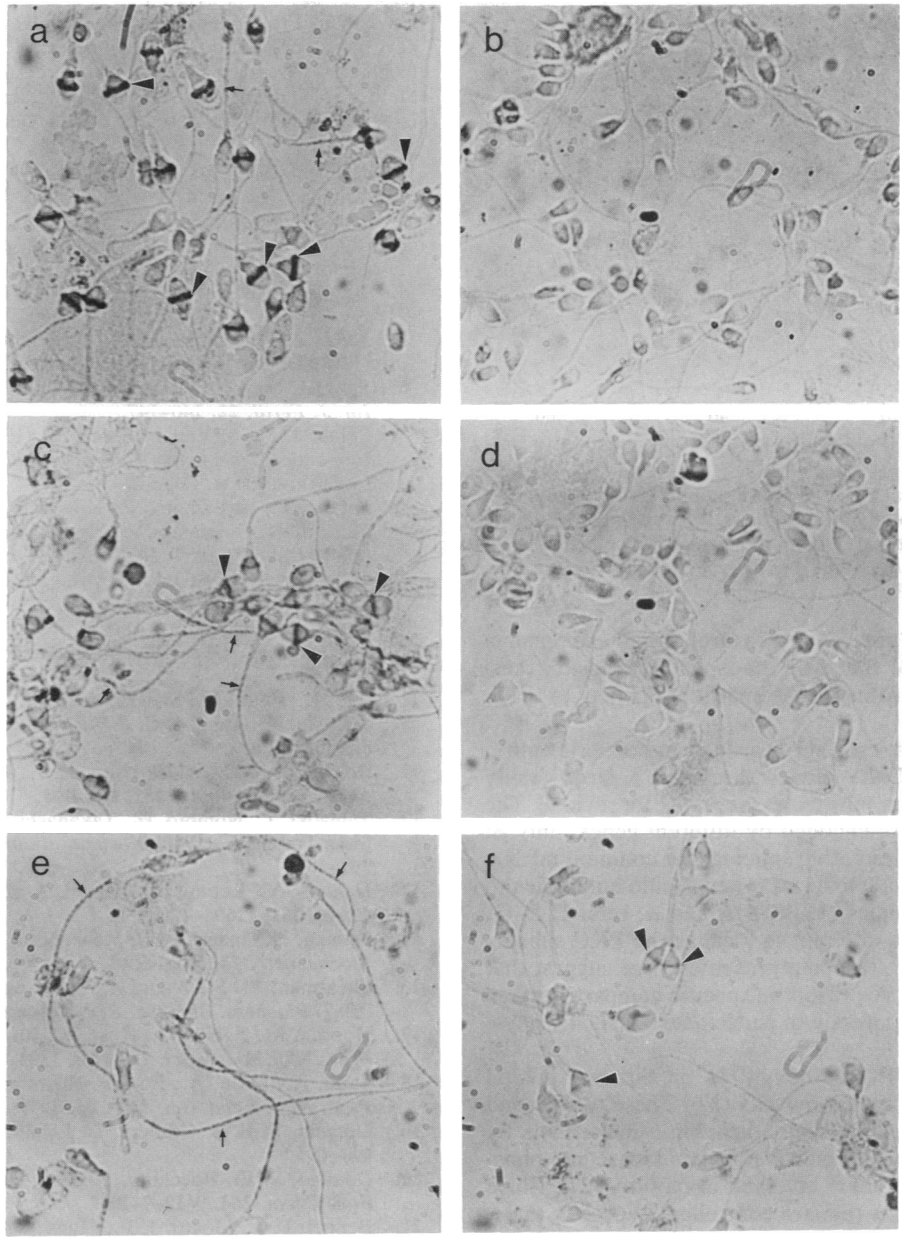

FIG. 3. Localization of PKC in human sperm. Sperm (2×10^6) was collected on glass slides by cytospin and stained for PKC. (*a*) Cells treated with the monoclonal antibody CKmCIβ-a, which is reactive to the rat brain PKC subspecies (α, β, and τ). Note the distinct band in the equatorial segment (arrowheads) and the principal piece of the tail (arrows). (*b*) Cells treated with CKmCIβ-a antibody, which was pretreated with rat brain PKC for 1 hr at 37°C. Note the absence of staining of the equatorial segment and the tail. (*c*) Cells treated with the polyclonal PKC antibody CKpCIβ-a, which is reactive to the rat brain PKC subspecies (α, β, and τ). The results are similar to those in *a*. (*d*) Cells treated with CKpCIβ-a antibody, which was pretreated with rat brain PKC (as in *b*). (*e*) Cells treated with antibody CKpV5βI-a (rat brain βI-specific). Note staining of principal piece of the tail (arrows) but not the equatorial segment. (*f*) Cells treated with antibody CKpV5βII-a (rat brain βII-specific). Note staining of the equatorial segment (arrowheads). The tail is unstained. (×750.)

with the proposal that τ PKC is a "brain-specific" enzyme (1, 10, 16).

To further investigate the possible role of PKC in regulation of human sperm flagellar motility, semen was collected from various donors and motility of the sperm was compared with the percentage of PKC-stained cells. Regression analysis revealed an excellent correlation between the two parameters (Fig. 4) ($r = 0.90$; $P < 0.001$). The results support the notion of a possible role of PKC in flagellar motility.

DISCUSSION

Our results on the presence of PKC in human sperm are in accordance with other reports documenting the presence of signal-transduction units in sperm of other species. The fucose-sulfate glyconjugate of egg jelly was shown to increase inositol phosphates and 1,2-diacylglycerol formation in sea urchin spermatozoa (19, 20). Also, addition of the Ca^{2+} ionophore A23187 to human sperm resulted in accumulation of diacylglycerol and phosphatidic acid in the cells (21). The presence of phosphatidyl inositol-specific phospholipase C in human sperm and phospholipase D in sea urchin spermatozoa was also reported (20, 22), and addition of a diacylglycerol analog or phorbol ester to mouse spermatozoa resulted in changes in the zona pellucida-induced acrosome reaction (23).

Another component of the signal-transduction apparatus—namely, guanine nucleotide-binding protein (G protein)—

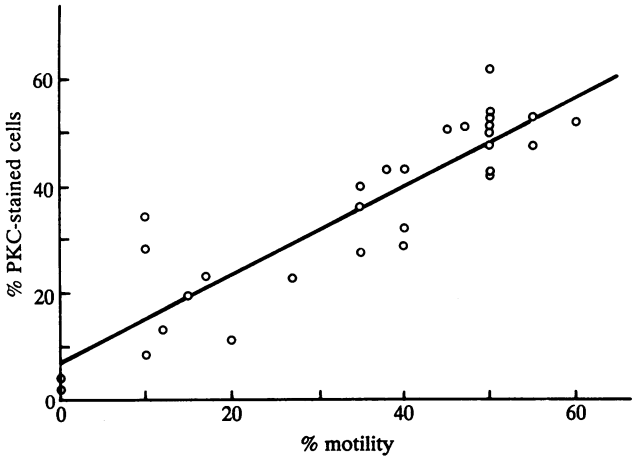

FIG. 4. Regression analysis of sperm motility and percentage of PKC-stained cells among various donors. The sperm was stained with the monoclonal antibody CKmCIβ-a, which is reactive to the rat brain PKC subspecies (α, β, and τ) as described in the legend to Fig. 3*a* ($r = 0.90$; $P < 0.001$).

was reported to be present in sea urchin and mammalian spermatozoa (24–26). Since the phosphatidyl inositol messenger system is operating in the sperm, it is likely to result in PKC activation.

Although PKC was regarded as a single monomeric entity, recent studies of cDNA clones disclosed a large family encoding at least seven subspecies (see ref. 1 for review). The various subspecies are encoded by different genes; only βI and βII result from alternative splicing of a common mRNA transcript (1). The availability of type-specific antibodies to group A PKC subspecies (τ, βI, βII, and α, refs. 1, 8–11, 16–18) prompted us to attempt to localize the PKC subspecies in human sperm. The data presented here suggest that the PKC subspecies are present in specific compartments in the human sperm, apparently to participate in specific physiological roles.

Calcium and cAMP are thought to be the two pivotal regulators of sperm flagellar motility (27). These two second messengers are thought to exert their biological effects by means of activation of a respective protein kinase and phosphorylation of key proteins involved in motility (27). Since most biological systems that are controlled by protein phosphorylation are now recognized to be under complex interaction of Ca^{2+}, cyclic nucleotides, and PKC (1), it is not surprising that PKC might also play a crucial role in regulating sperm motility in concert with cAMP and Ca^{2+} by means of phosphorylation of similar or other endogenous substrate proteins.

Motility initiation in sea urchin sperm is thought to be induced by an increase in intracellular pH, which activates the dynein-ATPase, which, in turn, is responsible for flagellar beating (28). The alkalization is mediated by a Na^+/H^+ exchanger (29). Since PKC is known to activate Na^+/H^+ exchange (30), this might represent one possible mechanism of action. The observation reported here that PKC is highly localized in the equatorial segment suggests a specific compartmentalized role for PKC in human sperm physiology. Our results might be applied to human *in vitro* fertilization programs and veterinary medicine. The results might also have implications for ciliary and flagellar systems in other vertebrates and possibly invertebrates.

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