Localization of cyclooxygenase and production of prostaglandins in bovine spermatozoa

Y. Shalev¹, M. Shemesh^{2,3*}, T. Levinshal¹, S. Marcus² and H. Breitbart¹

¹Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel; ²Department of Hormone Research, Kimron Veterinary Institute, Beit Dagan, 50250, Israel; and ³Koret School of Veterinary Medicine, Hebrew University of Jerusalem, Israel

Ejaculated bovine spermatozoa were examined for their capacity to synthesize prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 , $PGF_{2\alpha}$). It was found that in the absence of exogenous substrate, arachidonic acid, basal $PGF_{2\alpha}$ production was less than that of PGE_2 . However, addition of $61 \mu\text{mol}$ arachidonic acid l^{-1} resulted in at least a twofold increase in PGE_2 and $PGF_{2\alpha}$ above control values (1.3 ng and 0.3 ng per 10^8 spermatozoa, respectively). Addition of calcium and the calcium ionophore A23187 to the incubation medium did not cause a significant increase in the production of either PG. The presence of indomethacin ($100\text{--}200 \mu\text{g ml}^{-1}$) caused a 50–70% inhibition of the production of both PGs. Activity of cyclooxygenase was determined by western blot analysis, using a specific polyclonal antiserum, and by fluorescence immunohistochemistry using a monoclonal antibody. The western blot displayed a clear signal for the presence of cyclooxygenase in ejaculated and epididymal spermatozoa. The immunohistochemical studies showed that the enzyme is localized in the apical region of the head, the post-acrosomal region and the mid-piece of the tail. Since the synthesis of PGs in the absence of exogenous arachidonic acid is low, the effect of melittin, a known phospholipase A_2 activator, on PG production was examined. Incubation of spermatozoa with melittin produced a threefold increase in PGE_2 and a sixfold increase in $PGF_{2\alpha}$. Staurosporine, a protein kinase C inhibitor, inhibited the effect of melittin indicating that activation of phospholipase A_2 by protein kinase C is an obligatory step in PG synthesis by bovine spermatozoa. To determine the physiological role of PG synthesis by bovine spermatozoa the effect of PGE_2 and $PGF_{2\alpha}$ on Ca^{2+} uptake was examined. PGE_2 , but not $PGF_{2\alpha}$, increased the Ca^{2+} uptake linearly during the first 10 min of incubation. These data provide evidence that bovine spermatozoa can synthesize prostaglandins in the presence of arachidonic acid or melittin. Furthermore, it was demonstrated that the spermatozoa contain the key enzyme for prostaglandin synthesis, cyclooxygenase.

Introduction

Prostaglandins (PGs) have been reported to be involved in all of the stages of sperm maturation, from spermatogenesis to the acrosomal reaction (for review see Hayashi *et al.*, 1988). It has also been reported that PGs may inhibit (Cohen *et al.*, 1977) or stimulate (Schlegel *et al.*, 1981) sperm motility. Meizel and Turner (1984) reported that PGE_1 stimulates the acrosome reaction in hamster spermatozoa and Joyce *et al.* (1987) reported that exogenous PGE can overcome inhibition of the acrosome reaction by indomethacin in guinea-pigs. Although nearly all reproductive tissues examined have been shown to produce PGs (semen: Gerozissi and Dray, 1981; uterine fluid: Harrison *et al.*, 1987; Graaffian follicles: LaMaire *et al.*, 1975; Fallopian tubes: Warnes *et al.*, 1978), there has been only one recent report on PG synthesis by human spermatozoa (Roy and

Ratnam, 1992), but Shalev *et al.* (1992) were the first to report this activity in intact bovine spermatozoa. The present studies were conducted (1) to determine whether intact bovine spermatozoa can synthesize PGE_2 or $PGF_{2\alpha}$; (2) to localize cyclooxygenase using an immunofluorescent technique; (3) to confirm the existence of cyclooxygenase in spermatozoa homogenates by western blot analysis; (4) to elucidate the regulatory mechanism of PG synthesis by bovine spermatozoa; and (5) to determine the role of PGs in Ca^{2+} uptake by spermatozoa.

Materials and Methods

Materials

Arachidonic acid, indomethacin, melittin, staurosporine, calcium ionophore, prostaglandins, 3(*N*-morpholino) propane-sulfonic acid (MOPS), tergitol type NP-40, phenylmethylsulfonyl fluoride were all purchased from Sigma (St Louis,

*Correspondence.

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Table 1. Production of prostaglandins (PGs) by bovine spermatozoa in response to increasing doses of arachidonic acid

Arachidonic acid ($\mu\text{mol l}^{-1}$)	Prostaglandin secretion (pg per 10^8 spermatozoa per 3 h)	
	PGF _{2a}	PGE ₂
0	50 $\pm$ 12	437 $\pm$ 31
2.4	65 $\pm$ 6	453 $\pm$ 30
12.6	93 $\pm$ 12	671 $\pm$ 31
61.0	153 $\pm$ 18 ^a	1156 $\pm$ 109 ^b
122.0	500 $\pm$ 31	2515 $\pm$ 124

Results are expressed as means ($\pm$ SEM). Five replicates were carried out for each treatment in each experiment.

Significantly different from control: ^a $P < 0.01$; ^b $P < 0.05$.

MO). GF/C filters were purchased from Tamar Incorporation (Jerusalem). Arachidonic acid and indomethacin were dissolved in ethanol and evaporated to dryness in a vacuum before use. Labelled PGF_{2a} [5,6,8,9,11,12,14,15-³H] (164 Ci mmol⁻¹), PGE₂ [5,6,8,9,11,12,14,15-³H] (191 Ci mmol⁻¹) and ⁴⁵CaCl₂ (10–40 Ci mg⁻¹) were purchased from Amersham International (Amersham, Buckinghamshire). Polyclonal antibody for prostaglandin synthetase (cyclooxygenase) was produced in our laboratory by inoculating rabbits with purified ram seminal vesicle prostaglandin synthetase (Oxford Biochemicals, Oxford, MI) as previously described (Izhar *et al.*, 1992). Horseradish peroxidase conjugated anti-rabbit IgG was purchased from Biomakor (Nes Ziona, Israel). Monoclonal antibodies for cyclooxygenase were purchased from Cayman Chemical (Ann Arbor, MI). The antigen was purified cyclooxygenase from sheep seminal vesicles (crossreactivity: human, rat, bovine and most mammalian species). The second antibody was fluorescein isothiocyanate anti-mouse IgG (Sigma). Since

the specificity of the polyclonal anti-cyclooxygenase was not evaluated for the immunocytochemistry study, a well-characterized commercial monoclonal antibody was used for the localization of cyclooxygenase in the cell and the polyclonal antiserum was used only for the western blot studies.

Sperm preparation

Fresh ejaculated bull semen, collected using an artificial vagina (Artificial Insemination Center, Hafez Haim), was immediately diluted (1:1, v:v) with media containing 110 mmol NaCl l⁻¹, 5 mmol KCl l⁻¹ and 10 mmol MOPS l⁻¹ (pH 7.4) (NKM media). Bovine epididymides were obtained from local abattoirs. Epididymal spermatozoa were collected and washed as described by Babcock *et al.* (1976). The cells were washed with NKM media, centrifuged at 780 g for 10 min (three times) and resuspended in TALP (Lax *et al.*, 1990) to a final concentration of 10⁸ cells ml⁻¹. Only those samples with a minimum of 70% motile spermatozoa were used. The TALP medium contained (in mmol l⁻¹) 94.3 NaCl, 4.0 KCl, 0.7 Na₂HPO₄, 25.9 NaHCO₃, 0.5 MgCl₂ · 6H₂O, 12.8 glucose, 1.3 pyruvate, 7.6 lactate, 20 Hepes (pH 7.4) plus 3.0 mg BSA ml⁻¹ and 10 iu penicillin ml⁻¹.

Cell incubations

Spermatozoa were incubated for 3 h in TALP medium at 37°C in an atmosphere of 5% CO₂:95% air. Samples were then centrifuged at 780 g for 10 min at 4°C and the supernatants were extracted with ethyl acetate acidified with HCl. After evaporation under vacuum, the dried residues were stored at -20°C and redissolved in 0.5 ml steroid buffer (PBS plus 0.1% gelatin) before the radioimmunoassay.

Fig. 1. Time course study of conversion of arachidonic acid to (a) prostaglandin F_{2a} (PGF_{2a}) and (b) PGE₂ by bovine spermatozoa. Spermatozoa were incubated with 61 μmol arachidonic acid l⁻¹. Mean PGF_{2a} and PGE₂ production after 3 h incubation was significantly greater than production at zero time, $P < 0.01$.

Fig. 2. Effect of Ca^{2+} , arachidonic acid and indomethacin on prostaglandins (PGs) $\text{PGF}_{2\alpha}$ and PGE_2 accumulation in the culture media containing bovine spermatozoa. Spermatozoa were prepared and incubated in the following media: A: control media containing 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$; B: addition of 61 μmol arachidonic acid l^{-1} to medium containing calcium; C: addition 61 μmol arachidonic acid l^{-1} in the absence of calcium; D: addition of 10 μg indomethacin ml^{-1} to B; E: addition of 200 μg indomethacin ml^{-1} to B. (□) PGE_2 ; (▨) $\text{PGF}_{2\alpha}$. There was no difference in amount of PGs formed in the control media in the absence or presence of calcium (data not shown). Production of $\text{PGF}_{2\alpha}$ and PGE_2 was significantly ($P < 0.01$) inhibited by 50 and 70%, respectively, by indomethacin.

Treatments

Arachidonic acid was added at the concentration of 61 $\mu\text{mol l}^{-1}$ since this concentration has been shown by Meisel and Turner (1984) to be optimal for the acrosomal reaction in hamster spermatozoa. Indomethacin was added at the concentration of 100 $\mu\text{g ml}^{-1}$ since Joyce *et al.* (1987) showed that this concentration inhibited the acrosomal reaction. Preliminary experiments showed that this concentration caused a 50% inhibition of prostaglandin synthesis by spermatozoa. Melittin was added at 20 $\mu\text{g ml}^{-1}$ since this concentration resulted in a maximal stimulation of PG production over the range of 1.25–20 $\mu\text{g ml}^{-1}$. Staurosporine was used at a concentration of 10^{-9} – 10^{-10} mol l^{-1} ; higher concentrations have been reported to be toxic to rat liver cells (Levine, 1990). Neither indomethacin nor staurosporine treatments had any effect on spermatozoa motility or forward progression.

In the calcium-uptake experiments, PGE_2 was tested at a concentration of 1.3 ng ml^{-1} (the amount produced by spermatozoa cells after 3 h of incubation) and 40 ng ml^{-1} (concentration in seminal fluid). $\text{PGF}_{2\alpha}$ was tested at 350 pg ml^{-1} (the amount produced by spermatozoa cells after 3 h of incubation) and 0.25 $\mu\text{g ml}^{-1}$ (concentration in seminal fluid). The PGs

Fig. 3. Effect of melittin on production of prostaglandins (PGs) by bovine spermatozoa. Arachidonic acid was not added to the incubation media. Melittin was added in the range 0–20 $\mu\text{g ml}^{-1}$ in the presence of (●) 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ for $\text{PGF}_{2\alpha}$ (○) without calcium and (■) for PGE_2 in the presence of calcium.

were incubated with spermatozoa for 10 min before the addition of labelled calcium. In the experiments with indomethacin, the inhibitor was added 5 min before the addition of the PG to be tested.

Radioimmunoassay of prostaglandins

The dried residues of extracted $\text{PGF}_{2\alpha}$ and PGE_2 were dissolved in steroid buffer and assayed directly by radioimmunoassay as described by Shemesh *et al.* (1979).

Preparation of spermatozoa lysates

Spermatozoa ($1 \times 10^8 \text{ ml}^{-1}$) were washed twice with cold PBS. The pellet was suspended in the lysis buffer which contained (mmol l^{-1}): 10 Tris-HCl, pH 7.5; 0.5 MgCl_2 ; 2 EGTA, 1 phenylmethylsulfonyl fluoride plus 1% NP-40 and 0.1% SDS. The lysate was incubated for 15 min in ice, centrifuged (13 000 g, 10 min) and the supernatant stored at -20°C .

SDS-PAGE

The supernatant was diluted 1:1 in sample buffer (0.05 mol Tris-HCl l^{-1} , pH 6.8, 0.5% SDS, 10% mercaptoethanol, 20% glycerol, 0.001% bromophenol blue) and heated for 5 min at

Fig. 4. Effect of staurosporine or indomethacin on melittin-stimulated, or arachidonic acid-stimulated production of prostaglandins (PGs) by bovine spermatozoa. PGF_{2α} and PGE₂ were measured in the media following 3 h of incubation. Treatments: A: TALP + 2 mmol CaCl₂ l⁻¹; B: as A plus 61 μmol arachidonic acid l⁻¹; C: as A plus 20 μg melittin ml⁻¹; D: as C plus 100 μg indomethacin ml⁻¹; E: as C plus 1 × 10⁻¹⁰ mol staurosporine l⁻¹; F: as C plus 1 × 10⁻⁹ mol staurosporine l⁻¹; G: as B plus 1 × 10⁻⁹ mol staurosporine l⁻¹. (□) PGE₂; (■) PGF_{2α}. The stimulatory effect of melittin on PG synthesis was significantly inhibited ($P < 0.01$ for PGE₂; $P < 0.02$ for PGF_{2α}) by staurosporine in a dose-dependent manner.

100°C. The sample was subjected to electrophoresis in a one-dimensional 9% SDS-PAGE slab gel topped with 5% stacking gel (Izhar *et al.*, 1992). Molecular weight markers were used as standards. All electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA).

Western blot analysis

Microsomes from bovine seminal vesicles were separated on SDS-PAGE and electroblotted onto nitrocellulose paper (Amersham) as described by Izhar *et al.* (1992). The paper was then treated with rabbit anti-PGS polyclonal antisera (1:100) followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000) and a colour reaction performed. Protein was determined using γ-globulin as a reference standard and a protein dye-binding method (Bio Rad Lab.).

Immunocytochemistry studies

Bovine spermatozoa were air-dried on slides at room temperature and fixed in absolute methanol for 15 min. After rehydration with PBS, cells were incubated with the monoclonal antibody (1:500) for 30 min. The slides were then

Fig. 5. Western blot analysis of cyclooxygenase. Lane A: 100 μg of spermatozoa homogenate was applied to SDS-PAGE. Lane B: 25 μg protein from bovine seminal vesicle microsomes was used as internal positive marker. Cyclooxygenase (arrow) was readily detectable in the homogenate of bovine ejaculated spermatozoa and in the seminal vesicle microsomes.

washed extensively with PBS and incubated with the fluorescein isothiocyanate antibody (1:5) for 30 min. Slides were again washed with PBS and examined under a fluorescent microscope. Control slides were incubated with normal mouse serum (1:500) instead of the monoclonal antibody or with monoclonal antibody preadsorbed onto purified cyclooxygenase from ram seminal vesicles.

⁴⁵Ca²⁺ uptake measurements

Fresh ejaculated spermatozoa were prepared as described above. ⁴⁵Ca²⁺ uptake was determined using the filtration technique described by Ben-Av *et al.* (1988) with some modifications. The final concentration of washed spermatozoa

Fig. 6. Localization of cyclooxygenase in bovine spermatozoa. (a) Spermatozoa were treated with monoclonal antibody to cyclooxygenase and then with a fluorescein isothiocyanate second antibody. Cyclooxygenase was localized in the apical region of the spermatozoa head, the post-acrosomal region and the mid-piece of the tail. (b) Control spermatozoa treated with normal mouse serum or with preadsorbed antiserum and then with the fluorescein isothiocyanate second antibody.

was 3×10^8 cells in 1 ml of NKM and $0.5 \mu\text{Ci } ^{45}\text{CaCl}_2$ and $2 \text{ mmol CaCl}_2 \text{ l}^{-1}$ were added to each tube. The time of addition was taken as zero time (representing the nonspecific binding) and aliquots were taken at 2, 4, 6, 8, 10, 12 and 15 min intervals and applied to GF/C filters (Ben-Av *et al.*, 1988). The filters were prewashed with the washing buffer

($150 \text{ mmol NaCl l}^{-1}$; $2 \text{ mmol EGTA l}^{-1}$; $10 \text{ mmol Tris l}^{-1}$, pH 7.4). The cells were trapped on the filters and washed three times with ice-cold washing buffer and placed in scintillation vials. Four millilitres of liquiscint was added to each vial and the beta radiation counted in a Packard Scintillation Counter.

Statistical analysis

Values for PGF_{2a} and PGE_2 are expressed as pg PG produced per 10^8 cells ml^{-1} (3 h) $^{-1}$ ($\pm$ SEM). Pooled results from three to five independent experiments using multiple donors are presented. In each experiment there were five replicates for each treatment. Data were analysed using Student's *t* test.

Results

The effect of arachidonic acid concentrations on PGE and PGF_{2a} accumulation

To study the effect of increasing substrate concentration on PGE_2 and PGF_{2a} accumulation by bovine spermatozoa (10^8 ml^{-1}), incubations were performed in the presence of increasing concentrations of arachidonic acid (Table 1). PGE_2 and PGF_{2a} increased linearly concomitant with the increase in the concentrations of arachidonic acid in the incubation medium. At $61 \mu\text{mol}$ arachidonic acid l^{-1} , PGE_2 and PGF_{2a} accumulations had increased about threefold above basal values. However, $122 \mu\text{mol}$ arachidonic acid l^{-1} (maximum dose of substrate tested in this study) increased PGE_2 accumulation by approximately sixfold and PGF_{2a} by tenfold above basal values.

To ensure that cyclooxygenase within the spermatozoa rather than within the semen or adhering to the spermatozoa was responsible for PG production, seminal plasma was incubated with arachidonic acid and there was no increase in PG production with time above basal activity.

Time course study of the conversion of arachidonic acid to PGs

The accumulation of PGE_2 and PGF_{2a} increased linearly for the first 3 h of incubation in the presence of $61 \mu\text{mol}$ arachidonic acid l^{-1} (Fig. 1). Since 50% of the maximum stimulation obtained was seen after 3 h of incubation, this period was used in subsequent experiments.

Effect of Ca^{2+} , calcium ionophore and indomethacin on arachidonic acid conversion to PG

The addition of 0–10 μmol calcium ionophore l^{-1} to the medium alone or in combination with 2 mmol Ca^{2+} l^{-1} did not cause any significant increase in PG metabolites (data not shown). The role of cyclooxygenase in the conversion of arachidonic acid to PGs was determined by incubating spermatozoa with arachidonic acid in the presence of indomethacin, an inhibitor of cyclooxygenase. Indomethacin (100 – $200 \mu\text{g ml}^{-1}$) was added 10 min before the addition of arachidonic acid. The production of PGF_{2a} and PGE_2 was significantly ($P < 0.01$) inhibited by 50% and 70%, respectively, by indomethacin (Fig. 2).

The effect of melittin on PGE and PGF_{2a} accumulation in the incubation media

Melittin has been shown to activate phospholipase A_2 . The effect of increasing concentrations (1.25 – $20 \mu\text{g ml}^{-1}$) of

Fig. 7. Effect of prostaglandin E (PGE_2) or indomethacin, alone and in combination, on calcium uptake by bovine spermatozoa. Spermatozoa (3×10^8 cells ml^{-1}) were preincubated for 10 min with 1.3 or 40 ng $\text{PGE}_2 \text{ ml}^{-1}$. $^{45}\text{Ca}^{2+}$ was added at time 0 and aliquots taken at the indicated times. (●) control media; (□) $20 \mu\text{g}$ indomethacin ml^{-1} ; (○) $100 \mu\text{g}$ indomethacin ml^{-1} ; (▲) $100 \mu\text{g}$ indomethacin ml^{-1} plus 40 ng ml^{-1} PGE_2 ; (■) $100 \mu\text{g}$ indomethacin ml^{-1} plus 1.3 ng $\text{PGE}_2 \text{ ml}^{-1}$; (△) preincubation with $20 \mu\text{g}$ indomethacin ml^{-1} for 5 min followed by 40 ng $\text{PGE}_2 \text{ ml}^{-1}$.

melittin on the accumulation of PGE_2 and PGF_{2a} in the absence of exogenous arachidonic acid was therefore examined. Both PGs increased linearly with increased concentration of melittin in the incubation media. At $20 \mu\text{g}$ melittin ml^{-1} , there was a threefold increase in PGE_2 and a sixfold increase in PGF_{2a} (Fig. 3). The effect of melittin on PGF production was potentiated by the presence of Ca^{2+} in the culture medium.

Factors regulating prostaglandin synthesis by bovine spermatozoa

The effect of 8-bromo-cAMP (0 – 2 mmol l^{-1}) on the conversion of arachidonic acid to PGE_2 and PGF_{2a} was measured to study the pathway of regulation of PG synthesis by bovine spermatozoa. PG synthesis was not increased in the presence of 8-bromo-cAMP (data not shown).

The possible role of protein kinase C in the activation of PG synthesis or release of arachidonic acid was determined by investigating the effect of an inhibitor of protein kinase C, staurosporine (10^{-9} – $10^{-10} \text{ mol l}^{-1}$), on the accumulation of PGE_2 and PGF_{2a} in the presence of arachidonic acid or melittin (Fig. 4). Prostaglandin synthesis in the presence of arachidonic acid was not affected by staurosporine. In contrast, the stimulatory effect of melittin on PG synthesis was significantly inhibited ($P < 0.01$ for PGE_2 ; $P < 0.02$ for PGF_{2a}) by staurosporine in a dose-dependent manner.

Fig. 8. Effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or indomethacin on calcium uptake by bovine spermatozoa. (○) Control media; (●) $100 \mu\text{g indomethacin ml}^{-1}$; (□) $100 \mu\text{g indomethacin ml}^{-1}$ plus $350 \text{ pg } PGF_{2\alpha} \text{ ml}^{-1}$; (■) $100 \mu\text{g indomethacin ml}^{-1}$ plus $0.25 \mu\text{g } PGF_{2\alpha} \text{ ml}^{-1}$.

Western blot analysis of cyclooxygenase

To determine the presence of cyclooxygenase in the bovine ejaculated spermatozoa, $100 \mu\text{g}$ protein from spermatozoa homogenates were applied to SDS-PAGE and $25 \mu\text{g}$ protein from microsomes from bovine seminal vesicles were used as an internal positive marker for enzyme localization. Five homogenates of bovine spermatozoa samples from different bulls were used. Cyclooxygenase was readily detectable in the spermatozoa homogenates (Fig. 5). The enzyme was also localized in homogenates prepared from caput and cauda epididymal spermatozoa.

Localization of cyclooxygenase in bovine spermatozoa

Staining with the monoclonal antibody to cyclooxygenase indicated that the enzyme is localized in the apical region of the spermatozoa head, the post-acrosomal region and the mid-pieces of the tail. No other component in the spermatozoa was stained above background level (Fig. 6).

Role of exogenous prostaglandins in calcium uptake

Uptake of Ca^{2+} increased linearly during the first 10 min of incubation (Fig. 7). In the presence of indomethacin, Ca^{2+} uptake was inhibited, but this effect was abolished by the presence of exogenous PGE_2 . In contrast, when spermatozoa were incubated in the presence of exogenous $PGF_{2\alpha}$ (350 pg ml^{-1} or $0.25 \mu\text{g ml}^{-1}$), Ca^{2+} uptake was significantly inhibited (Fig. 8).

Discussion

In the present study, we report that bovine spermatozoa can produce $PGF_{2\alpha}$ and PGE_2 *in vitro*, although the amounts of

$PGF_{2\alpha}$ produced were lower than those of PGE_2 . We have previously shown (Shalev *et al.*, 1992) that intact bovine spermatozoa can synthesize PG and using a specific monoclonal antibody, that cyclooxygenase is localized in the post-acrosomal region and the mid-piece of the tail of ejaculated spermatozoa. Furthermore, we demonstrated that arachidonic acid can be metabolized to PGE_2 and $PGF_{2\alpha}$. Roy and Ratnam (1992) reported that homogenates of human spermatozoa converted arachidonic acid to PGE_2 and $PGF_{2\alpha}$.

The synthesis of PG by spermatozoa was unaffected by the addition of either Ca^{2+} or the calcium ionophore, indicating that increasing intracellular or extracellular calcium alone does not affect the rate of synthesis. Calcium ionophore has been shown to activate phospholipase A_2 (PLA_2) in human spermatozoa during the acrosomal reaction (Bennet *et al.*, 1987). However, it is not known whether the concentration of calcium affects the synthesis of PGs in humans.

The release of arachidonic acid is considered to be the rate-limiting step for PG production in most tissues (Irvine, 1982). In particular, two major pathways for arachidonic acid release (Smith, 1989) have been reported. One is the activation of PLA_2 , which causes the release of arachidonic acid directly from esterified stores of phospholipids, and the second is the sequential hydrolysis of phosphoinositide by phospholipase C and glycerol lipases. As both $PGF_{2\alpha}$ and PGE_2 increased concomitantly with substrate concentration in the incubation media, it appears that cyclooxygenase activity is not a limiting factor in PG synthesis by bovine spermatozoa. PG synthesis was inhibited by indomethacin indicating that cyclooxygenase is involved in the synthesis rather than a non-specific oxidation of arachidonic acid. Activation of PLA_2 or protein kinase C could be the limiting step in the release of arachidonic acid and subsequent PG synthesis. This concept is supported by the observation that the amount of PG produced in the absence of exogenous arachidonic acid was significantly increased by

adding melittin, which activates PLA₂ via protein kinase C activation. Activation of PLA₂ and phospholipase C is considered to depend upon intracellular Ca²⁺ concentrations (Nozawa *et al.*, 1991; Meldrum *et al.*, 1991). It has been reported that activation of protein kinase C enhances arachidonic acid release in canine kidney cells (Silvka and Insel, 1988), human platelets (Halenda *et al.*, 1989) and mouse peritoneal macrophages (Balsinde *et al.*, 1990).

Since it was demonstrated that arachidonic acid is metabolized by bovine spermatozoa, two enzymes (PLA₂ and PKC) that are responsible for the release of arachidonic acid and for which there is good evidence of their presence in mammalian spermatozoa (Breitbart *et al.*, 1992; Hinkovska *et al.*, 1987). (Protein kinase A is apparently not involved in PG synthesis as cyclic nucleotide did not affect PG synthesis.) Melittin, a known activator of PLA₂ (Shier, 1979; Mollay and Kreill, 1974; Matsumura and Aketa, 1991), significantly increased PG production in a dose-dependent manner. Since this effect was stimulated by the presence of Ca²⁺, it appears that Ca²⁺ is required to activate PLA₂ for the release of arachidonic acid. In addition, staurosporine, an inhibitor of PKC (Sako *et al.*, 1988; King and Rittenhouse, 1989), inhibited the effect of melittin, indicating that PKC activation of PLA₂ is a necessary step in PG synthesis by bovine spermatozoa. The inhibitory effect of staurosporin was not seen in the presence of exogenous arachidonic acid, indicating that the increased PG synthesis was due to release of arachidonic acid stores.

We found that exogenous PGE stimulated calcium uptake by bovine spermatozoa. This agrees with the observations of Aitken *et al.* (1985, 1986) who reported that PGE can enhance the penetrating capacity of human spermatozoa into hamster oocytes and is correlated with the ability of PGs to increase intracellular concentration of free extramitochondrial calcium. Aitken and co-workers showed that intracellular calcium increased in the presence of PGE₁ and PGE₂ but that PGF_{2α} had no effect. This suggests that PGE can act as a Ca²⁺ ionophore. Similarly, Kirtland and Baum (1972) found that PGE, as well as the calcium ionophore A23187, could increase spermatozoa penetration into the zona-free hamster egg.

The data presented here suggest that unknown substances present in seminal fluid (possibly spermine (Shalev *et al.*, 1992)) stimulate protein kinase C activity, which, in turn, activates PLA₂. The resultant release of arachidonic acid increases the synthesis of PG via activation of the cyclooxygenase pathway. The data also indicate that the complete machinery required to produce PGs is present in bovine spermatozoa. The system is active and regulated by the same transduction systems seen in other PG producing tissues. However, the localization of cyclooxygenase in spermatozoa (head and midpiece) suggests that PG synthesis has different functions related to its anatomical localization. Synthesis of prostaglandins in the head could be associated with the acrosomal reaction (Joyce *et al.*, 1987), whereas PG synthesis in the midpiece (a mitochondria-rich area) could be involved in energetic metabolic processes related to motility.

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References

- Aitken RJ and Kelly RW (1985) Analysis of the direct effects of prostaglandins on human sperm function *Journal of Reproduction and Fertility* **73** 139–146
- Aitken RJ, Irvine S and Kelly RW (1986) Significance of intracellular calcium and cyclic adenosine 3'5' monophosphate in the mechanisms by which prostaglandins influence human sperm function *Journal of Reproduction and Fertility* **77** 451–462
- Babcock DF, First NL and Lardy HA (1976) Action of ionophore A23187 at the cellular level. Separation of effects at the plasma and mitochondrial membranes. *Journal of Biological Chemistry* **251** 3881–3886
- Balsinde J, Fernandez B and Diez E (1990) Regulation of arachidonic acid release in mouse peritoneal macrophages *Journal of Immunology* **144** 4298–4304
- Ben-Av P, Rubinstein S and Breitbart H (1988) Induction of acrosomal reaction and calcium uptake in ram spermatozoa by ionophores. *Biochimica et Biophysica Acta* **939** 214–222
- Bennet PJ, Moatti JP, Mansat A, Ribbes H, Cayrac JC, Pontonnier F, Chap H and Dauste-Blazy L (1987) Evidence for activation of phospholipases during acrosome reaction of human sperm elicited by A23187 *Biochimica et Biophysica Acta* **919** 255–265
- Breitbart H, Lax J, Rotem R and Naor Z (1992) Role of protein kinase C in the acrosome reaction of mammalian spermatozoa. *Biochemical Journal* **281** 473–476
- Cohen MS, Colin MJ, Golimbu M and Hotchkiss RS (1977) The effect of prostaglandins on sperm motility *Fertility and Sterility* **28** 78–85
- Gerozissi K and Dray F (1981) Radioimmunoassay of prostaglandins in the semen of fertile men *Journal of Reproduction and Fertility* **61** 487–490
- Halenda SP, Banga HS, Zavoico GB, Lau LF and Feinstein MB (1989) Synergistic release of arachidonic acid from platelets by activators of protein kinase C and Ca²⁺ ionophores: evidence for role of protein phosphorylation in the activation of phospholipase A₂ and independence from the Na⁺/H⁺ exchanger *Biochemistry* **28** 7356–7363
- Harrison FA, Heap RB, Horton EW and Poyser NL (1987) Identification of prostaglandin F_{2α} in uterine fluid from non-pregnant sheep with an autotransplanted ovary *Journal of Endocrinology* **53** 215–222
- Hayashi S, Noda Y and Mori T (1988) Analysis of the role of prostaglandins in the fertilization process *European Journal of Obstetrics and Gynaecology and Reproductive Biology* **29** 287–297
- Hinkovska VTZ, Momchilova AB, Petkova DH and Koumanov KS (1987) Phospholipase A₂ activity in ram spermatozoa plasma membranes *International Journal of Biochemistry* **19** 569–572
- Irvine RF (1982) How is the level of arachidonic acid controlled in mammalian cells? *Biochemical Journal* **204** 3–16
- Izhar M, Pasmanik M, Marcus S and Shemesh M (1992) Dexamethasone inhibition of cyclooxygenase expression in bovine term placenta *Prostaglandins* **43** 239–254
- Joyce C, Nuzzo NA, Wilson L and Zaneveld LJD (1987) Evidence for the role of cyclooxygenase (prostaglandin synthetase) and prostaglandins in the sperm acrosome reaction and fertilization *Journal of Andrology* **8** 74–82
- King WG and Rittenhouse S (1989) Inhibition of protein C by staurosporine promotes elevated accumulation of inositol triphosphates and tetrakisphosphate in human platelets exposed to thrombin *Journal of Biological Chemistry* **264** 6070–6074
- Kirtland SJ and Baum H (1972) Prostaglandin E₁ may act as a calcium ionophore *Nature* **236** 47–49
- LaMaire WJ, Leidner R and Marsh JM (1975) Pre- and post-ovulatory changes in the concentration of prostaglandins in rat Graffian follicles *Prostaglandins* **9** 221–229
- Lax Y, Grossman S, Rubinstein S, Magid N and Breitbart H (1990) Role of lipoxygenase in the mechanism of acrosome reaction in mammalian spermatozoa *Biochimica et Biophysica Acta* **1043** 12–18
- Levine L (1990) Effects of the protein kinase inhibitors, staurosporine and K252a, on PG12 production by rat liver cells (the C-9 cells line) *Prostaglandins* **40** 259–264
- Matsumura K and Aketa K (1991) Does phospholipase A₂ participate in the acrosome reaction of sea urchin sperm? A pharmacological study *Development, Growth and Differentiation* **33** 365–370
- Meizel S and Turner KO (1984) The effects of products and inhibitors of arachidonic acid metabolism on the hamster sperm acrosome reaction *Journal of Experimental Zoology* **231** 283–288
- Meldrum E, Parker PJ and Carozzi A (1991) The PtdIns-PLC super family and signal transduction *Biochimica et Biophysica Acta* **1092** 49–71

- Mollay C and Kreill G (1974) Enhancement of bee venom phospholipase A₂ activity by melittin, direct lytic factor from cobra venom and polymyxin B *FEBS Letters* **46** 141–144
- Nozawa Y, Nakashima S and Negata K (1991) Phospholipid-mediated signaling in receptor activation of human platelets *Biochimica et Biophysica Acta* **1082** 219–238
- Roy AC and Ratnam SS (1992) Biosynthesis of prostaglandins by human spermatozoa *in vitro* and their role in the acrosomal reaction and fertilization *Molecular Reproduction and Development* **33** 303–306
- Sako T, Tauber AI, Jeng AY, Yupsa SH and Blumberg PM (1988) Contrasting actions of staurosporine, a protein kinase C inhibitor, on human neutrophils and primary mouse epidermal cells *Cancer Research* **48** 4646–4650
- Schlegel W, Rotermund S, Faber G and Neischlag E (1981) The influence of prostaglandins on sperm motility *Prostaglandins* **21** 87–99
- Shalev Y, Shemesh M, Marcus S, Rubinstein S and Breitbart H (1992) Localization of cyclooxygenase and production of prostaglandins in bovine sperm *Biology of Reproduction*, Supplement 1 **46** 149 (Abstract)
- Shemesh M, Milaguir F, Ayalon N and Hansel W (1979) Steroidogenesis and prostaglandin synthesis by cultured bovine blastocysts *Journal of Reproduction and Fertility* **56** 181–185
- Shier WT (1979) Activation of high levels of endogenous phospholipase A₂ in cultured cells. *Proceedings National Academy of Sciences, USA* **76** 195–199
- Silvka SR and Insel PA (1988) Phorbol ester and neomycin dissociate bradykinin receptor-mediated arachidonic acid release and polyphosphoinositide hydrolysis in Madin Darby canine kidney cells *Journal of Biological Chemistry* **263** 14 640–14 647
- Smith WL (1989) The eicosanoids and their biochemical mechanism of action *Biochemical Journal* **259** 315–324
- Warnes GM, Amato F and Seamark RF (1978) Prostaglandins in the Fallopian tube secretion of the ewe *Australian Journal of Biological Science* **31** 275–282