Stimulation of platelet-activating factor synthesis by progesterone and A23187 in

Stimulation of platelet-activating factor synthesis by progesterone and A23187 in human spermatozoa

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The presence of platelet-activating factor (PAF) has been demonstrated recently in mammalian spermatozoa, together with evidence for a role of this phospholipid in enhancing sperm motility and fertilizing ability. To investigate whether PAF synthesis and release occurs in human spermatozoa following incubation with stimuli that induce acrosome reaction, spermatozoa were incubated with progesterone and A23187, two known inducers of the exocytotic event. PAF synthesis (remodelling pathway) was assessed by [^{3}H]acetate incorporation into PAF. Treatment of spermatozoa with progesterone and A23187 resulted in an increase of [^{3}H]acetate incorporation into PAF. Most of the newly synthesized [^{3}H]PAF formed in response to acrosome reaction was found in the supernatant, suggesting a release of the phospholipid from spermatozoa. PAF-like material extracted from human spermatozoa was able to induce aggregation of rabbit platelets and showed identical retention time and the same ion *m/e* values as authentic PAF when analysed with g.c.–m.s. Lyso-PAF:acetyl-CoA acetyltransferase (EC 2.3.1.67) activity in human spermatozoa was also studied and showed similar kinetic parameters to those described for other cell systems. Stimulation of spermatozoa with progesterone and A23187 induced an increase of [^{3}H]arachidonic acid release, suggesting an activation of phospholipase A. In conclusion, our results demonstrated increased production and release of PAF in human sperm following stimulation with progesterone and A23187 and suggest a role for this phospholipid in the activation of spermatozoa.

INTRODUCTION

Platelet-activating factor (PAF) is a potent pro-inflammatory, lipid-derived mediator having several biological effects, including aggregation and degranulation of platelets and neutrophils and increased vascular permeability (for review see Snyder, 1990). PAF has been detected in rabbit and human washed spermatozoa (Kumar et al., 1988; Kuzan et al., 1990; Parks et al., 1990; Minhas et al., 1991), indicating that these cells are able to synthesize this phospholipid. Recently, addition of PAF to sperm incubation medium has been reported to enhance motility of human sperm (Ricker et al., 1989; Hellstrom et al., 1991) and *in vitro* fertilization of rabbit and mouse oocytes (Roudebush et al., 1990; Kuzan et al., 1990). Conversely, treatment of rabbit spermatozoa with PAF-receptor antagonists inhibits fertilization ability (Kumar et al., 1988; Harper et al., 1989). At present, it is not known whether PAF production by spermatozoa can be enhanced by stimulation, in particular by stimuli that induce the acrosome reaction. This exocytotic event occurs in spermatozoa *in vitro* following treatments that stimulate an increase in the concentration of intracellular free calcium (Yanagimachi, 1988), such as calcium ionophores (Aitken et al., 1984) and steroids present in the follicular fluid, in particular progesterone and 17α-hydroxyprogesterone (Osman et al., 1989; Thomas and Meizel, 1989; Blackmore et al., 1990). Besides an increase of intracellular calcium, ionophores and progesterone activate sperm phospholipase C (Roldan and Harrison, 1989; Thomas and Meizel, 1989), and ionophores increase sperm phospholipase A2 activity (Takkar et al., 1983; Bennet et al., 1987). The activation of these phospholipases might affect cellular PAF production. In particular, phospholipase A2 activation might mobilize a fatty acid from the *sn*-2 position of l-alkyl-linked phosphatidylcholine (PC), a major phospholipid subclass in spermatozoa (Nikolopoulou et al., 1985; Aveldano et al., 1992). This release leads to the generation of lyso-PAF which may be acetylated at the *sn*-2 position by lyso-PAF:acetyl-CoA acetyltransferase (EC 2.3.1.67) to form PAF. The major aim of the present study was to evaluate whether incubation with A23187 and progesterone stimulates PAF production (remodelling pathway) and release from human spermatozoa. In addition, some kinetic parameters of lyso-PAF:acetyl-CoA acetyltransferase activity of human sperm were measured.

MATERIALS AND METHODS

Materials

A23187, PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) and lyso-PAF (1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine) were obtained from Calbiochem (La Jolla, CA, U.S.A.). The sodium salts of [^{3}H]acetic acid (1.9 Ci/mmol) and [^{3}H]acetyl-CoA (4 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]Arachidonic acid (207 Ci/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). T.l.c. phospholipid standards [PC, lysophosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM) and arachidonic acid], fatty-acid-free BSA, progesterone, acetyl-CoA, *N*-formylmethionyl-leucylphenylalanine (fMLP), horseradish peroxidase (Type IV) and phospholipase C (from *Bacillus cereus*) were from Sigma (St. Louis, MO, U.S.A.). tert-Butyldimethylchlorosilyl/imidazole (TBDMSI) reagent was obtained from Alltech Applied Science (Deerfield, IL, U.S.A.).

Abbreviations used: PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; fMLP, formylmethionyl-leucylphenylalanine; DMSO, dimethyl sulphoxide; TBDMSI, tert-butyldimethylchlorosilyl/imidazole; TNS, 2-*p*-toluidinylnaphthylene-6-sulphonate; m.i.d., multiple ion detection; s.i.m., selected ion monitoring; BWW, Biggers-Whitten–Whittingham medium.

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H.p.t.l.c. and t.l.c. silica gel 60 plates were from E. Merck (Darmstadt, Germany). Organic solvents were from Carlo Erba (Milan, Italy). L659,989 was a generous gift from Merck Sharp and Dohme (Rahway, NJ, U.S.A.). A23187 (10 mM) and progesterone (10 mg/ml) were dissolved in dimethyl sulphoxide (DMSO).

Preparation of spermatozoa

Human semen was collected, according to the WHO recommended procedure (1987) by masturbation after 3–4 days of abstinence from 29 normozoospermic men undergoing semen analysis for couple infertility. Samples with a linear progressive motility of less than 50% at 60 min and with leucocytes and/or immature germ-cell concentrations greater than 10^6/ml were not included in the study. Occasionally, two to three semen samples were pooled in order to achieve a sufficient number of spermatozoa. Semen samples were processed as previously described (Baldi et al., 1991). Briefly, after complete semen liquefaction, spermatozoa were routinely separated on 40% and 80% Percoll gradients, combined and washed once in Biggers-Whitten-Whittingham (BWW) medium (Biggers et al., 1971) containing 3 mg/ml BSA, and finally resuspended in the same medium at the concentration of 5×10^6 cells/ml. The presence of leucocytes was checked after Percoll separation by careful counting under an optical microscope, and by measuring formation of reactive oxygen species by luminol-loaded cells after stimulation with fMLP using a chemiluminescent method as described by Krausz et al. (1992). An increase of reactive-oxygen-species formation following fMLP stimulation was considered to be an indication of the presence of activated leucocytes in the sample (Krausz et al., 1992), and samples showing an increase of chemiluminescence after fMLP treatment were discarded. Samples were always pre-incubated for 2 h at 37 °C in BWW medium in an atmosphere of 5% CO_2 in humidified air before the experiments were performed. We have previously shown that such a pre-incubation time was sufficient to obtain a maximal response to progesterone (Baldi et al., 1991). Sperm motility was assessed at 60 min and after pre-incubation in BWW medium using the HTM-2000 motility analyser (Hamilton–Thorn Research, Danvers, MA, U.S.A.).

Cell labelling with [^{3}H]acetate and [^{3}H]PAF production

[^{3}H]PAF formation was measured by quantitative incorporation of [^{3}H]acetate into PAF as described previously (Baldi et al., 1990; Villani et al., 1991). Briefly, spermatozoa were centrifuged at 600 ***g*** for 10 min, resuspended in BWW medium supplemented with 3 mg/ml fatty-acid-free BSA at a concentration of 5×10^6 cells/ml and incubated for 30 min at 37 °C in the presence of [^{3}H]acetate (20 μCi/ml). Stimuli or vehicle (0.1% DMSO) were then added at the indicated concentrations for the indicated times. The reaction was stopped by adding 3 ml of ice-cold chloroform/methanol/acetic acid (1:2:0.04, by vol.), and lipids were extracted twice in chloroform/methanol (1:1, v/v) according to the Bligh and Dyer (1959) extraction procedure. The combined chloroform extracts were washed once with methanol/water (10:9, v/v), dried under nitrogen and reconstituted in chloroform/methanol (9:1, v/v). Samples were then applied on heat-activated h.p.t.l.c. silica gel 60 plates and developed in chloroform/methanol/water (65:35:6, by vol.). The labelled phospholipid products were identified by co-chromatography with known standards. The R_F value of PAF was 0.2. The lipid fractions were visualized under a u.v. lamp after 2-*p*-toluidinylnaphthylene-6-sulphonate (TNS) exposure, and areas corresponding to PAF carefully scraped and the radioactivity counted by liquid scintillation. For [^{3}H]PAF-release experiments, spermatozoa, after incubation with stimuli, were centrifuged for 10 min (600 ***g***, 4 °C) and lipids were separately extracted from supernatant and cell pellet.

Assay of lyso-PAF:acetyl-CoA acetyltransferase activity

PAF acetyltransferase activity was determined in lysates of spermatozoa by the incorporation of [^{3}H]acetyl from radiolabelled acetyl-CoA into [^{3}H]PAF, using lyso-PAF as substrate, as described previously (Villani et al., 1991). Spermatozoa, after incubation with stimuli for the indicated times, were centrifuged and washed in a buffer containing 250 mM sucrose, 0.5 mM EGTA, 1 mM dithiothreitol and 10 mM Hepes (pH 7), and finally resuspended in the same buffer containing 1 mM $CaCl_2$. Cells were then sonicated (3×15 s bursts) and efficiency of sonication was checked each time under an optical microscope. The homogenate was centrifuged at 600 ***g*** for 5 min at 4 °C and aliquots of supernatant were incubated for 5 min at 37 °C, in a shaking waterbath, in the presence of 30 μM lyso-PAF or otherwise indicated. [^{3}H]Acetyl-CoA (5 μCi, 100 μM, or otherwise indicated) was then added and incubation proceeded for the indicated times. The reaction was stopped by addition of chloroform/methanol/acetic acid (1:2:0.04, by vol.); lipids were extracted and PAF separated by t.l.c. as described above.

Identification of [^{3}H]PAF-like material as authentic PAF

We tested sperm-derived PAF-like material, isolated as described above, for its ability to aggregate washed rabbit platelets. The assay was performed as described previously (Wang et al., 1988; Baldi et al., 1990). Briefly, 5×10^7 platelets were stirred at 37 °C in an aggregometer in Tris-buffered Tyrode's solution supplemented with 0.25% gelatin, in the presence of 10 μM indomethacin. A calibration curve with PAF standard was performed and PAF-like material present in the sample calculated accordingly. In some experiments, aliquots of the sample were pretreated with 30 mM sodium hydroxide in methanol at 22 °C for 5 min to inactivate PAF by deacylation. Also, the ability of L659,989 (10 μM), a third-generation PAF-receptor antagonist (Ponpipom et al., 1988), to block platelet aggregation induced by the sample was tested.

We also performed g.c.–m.s. analysis of an A23187-stimulated sperm sample by using a Perkin Elmer 8420 gas chromatography–ion-trap mass spectrometer. The sample, extracted as described above, was re-extracted from t.l.c. powder and exposed to phospholipase C (1 unit/ml in 0.5 M Tris, pH 7.6) hydrolysis. The obtained diglyceride was then derivatized with TBDMSI according to Triolo et al. (1991) and applied to the gas chromatograph–ion-trap mass spectrometer. Chromatographic separation was obtained using a DB-1 (100% methylsilicone) fused-silica capillary column. Injection was performed using a splitless programmed temperature vaporizer (250 °C). Acquisitions were made both in multiple ion detection (m.i.d.) and selected ion monitoring (s.i.m.) by comparison with a PAF $C_{16:0}$ standard. When analysed by m.i.d., the ions selected with PAF $C_{16:0}$ were *m/e* 117, 131, which are formed by rearrangement of the acetyl group and dimethylsilanol ($CH_3CO + 74$), and 415 ($M - 57$), which is produced by cleavage of the tert-butyl radical from the parent ion. When analysis was performed with s.i.m., the ion *m/e* 415 ($M - 57$) was selected. These three ions are

representative of 1-*O*-alkyl-*sn*-2-acetylglycerophosphocholine (PAF $C_{16:0}$).

[^{3}H]Arachidonic acid release from prelabelled spermatozoa

In this set of experiments, spermatozoa (5×10^6 cells/ml), after separation on a Percoll gradient, were pre-incubated in BWW medium supplemented with fatty-acid-free BSA. [^{3}H]Arachidonic acid (1 μCi/ml) was added to the incubation medium during the last 90 min and incubation proceeded in a shaking waterbath. As shown by Bennet et al. (1987), arachidonate incorporation into sperm lipids was dependent on the total concentration of arachidonic acid, with maximal incorporation into phospholipids occurring at a concentration of 8–10 μM. Accordingly, we incubated spermatozoa with a final [^{3}H]arachidonic acid concentration at 8 μM. At the end of incubation, spermatozoa were centrifuged, washed once in fresh BWW medium, and incubated in the presence of stimuli for 120 min. Total radioactivity incorporated into spermatozoa was determined by counting aliquots of the final cell suspension. The reaction was stopped by placing tubes on ice and centrifuging at 600 *g* for 10 min at 4 °C. Supernatants were collected and lipids extracted as described above. The chloroform phase was dried under nitrogen, reconstituted in chloroform/methanol (9:1, v/v), and applied to heat-activated silica gel 60 t.l.c. plates. The solvent system used was hexan/ethyl ether/acetic acid (70:30:1, by vol.). [^{3}H]Arachidonic acid was identified by co-chromatography with a known standard and visualized under a u.v. lamp after exposure to fluorescein. Areas corresponding to arachidonic acid were scraped and counted by liquid scintillation. In some experiments, cell pellets (corresponding to 1.5×10^7 spermatozoa) were reconstituted in buffer, an aliquot was counted to determine residual radioactivity, and the remaining sample was extracted according to the procedure of Bligh and Dyer (1959). The chloroform phase was dried under nitrogen, reconstituted in chloroform/methanol (9:1, v/v) and applied to heat-activated silica gel 60 t.l.c. plates. The solvent system used was chloroform/ethanol/triethylamine/water (30:34:35:8, by vol.), which allows separation of phospholipids, free fatty acids and neutral lipids (Touchstone et al., 1980). Different spots were identified by parallel chromatography with known standards visualized under a u.v. lamp, after exposure to TNS. Areas corresponding to different phospholipids (PC, PE and PS) and free fatty acids were carefully scraped and counted.

Statistical analysis

Results are expressed as means ± S.E.M. Statistical comparisons were performed using paired Student's *t*-test. For time-course experiments, results were analysed using ANOVA. The progesterone dose–response curve was analysed with the ALLFIT program (De Lean et al., 1978).

RESULTS

Effect of progesterone and A23187 on [^{3}H]PAF production by spermatozoa

PAF production ([^{3}H]acetate incorporation into PAF) by A23187 (10 μM)-stimulated human spermatozoa was time-dependent (Figure 1), with a maximal response at 60 min followed by a plateau. When spermatozoa from different men were treated for 2 h with progesterone (10 μg/ml) and A23187 (10 μM), an increase of [^{3}H]PAF formation [1.76 ± 0.26 ($n = 9$) and 2.79 ± 0.39 ($n = 11$) fold increase over basal respectively] was observed (Figure 2). A similar effect of progesterone (10 μg/ml) was also observed after 60 min incubation (1.7 ± 0.17-fold in-

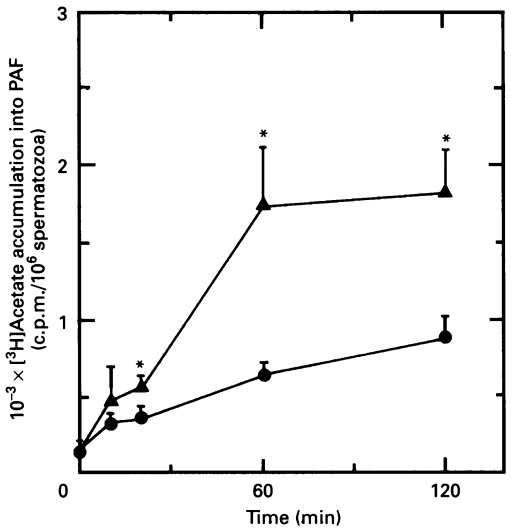

Figure 1 Time course of [^{3}H]acetate incorporation into PAF in control and A23187-stimulated human sperm

Spermatozoa were incubated at 37 °C for the indicated times with (▲) or without (●) A23187 (10 μM) in the presence of [^{3}H]acetate. After lipid extraction and t.l.c. separation, areas corresponding to PAF were scraped and radioactivity counted. n = four experiments. *$P < 0.005$ versus control spermatozoa; raw c.p.m. were five times higher.

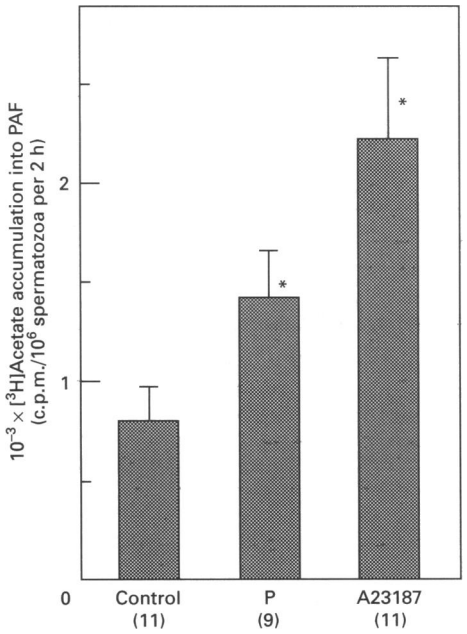

Figure 2 Effect of progesterone and A23187 on [^{3}H]acetate incorporation into PAF in human spermatozoa

Spermatozoa were incubated at 37 °C for 2 h with progesterone (P) (10 μg/ml) and A23187 (10 μM) in the presence of [^{3}H]acetic acid. After lipid extraction and t.l.c. separation, areas corresponding to PAF were scraped and counted. The number of experiments is given in parentheses. Control = non-stimulated spermatozoa; raw c.p.m. data were five times higher. Statistical significance: *$P < 0.005$ versus control.

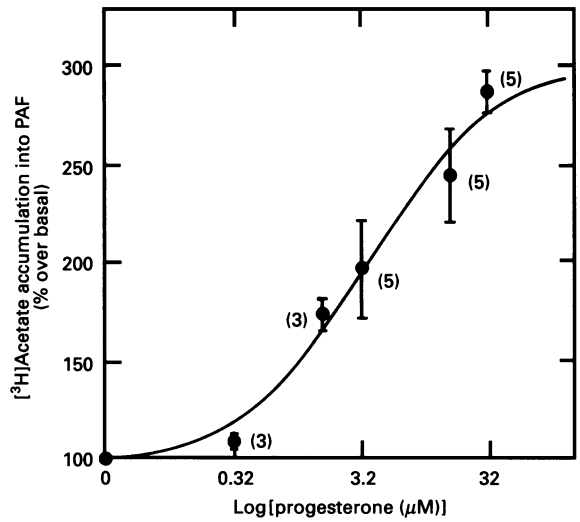

Figure 3 Dose–response curve of progesterone-stimulated [^{3}H]acetate incorporation into PAF in spermatozoa

Spermatozoa were incubated at 37 °C for 2 h with increasing concentrations of progesterone in the presence of [^{3}H]acetate. After lipid extraction and t.l.c. separation, areas corresponding to PAF were scraped and radioactivity counted. C.p.m. in the control samples were $2306 \pm 333/5 \times 10^6$ spermatozoa. The number of experiments is given in parentheses.

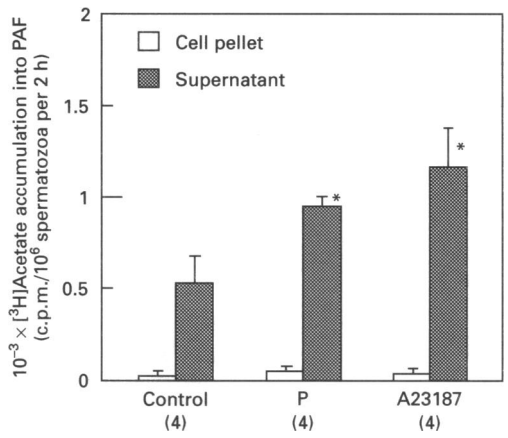

Figure 4 [^{3}H]PAF release in the medium by human spermatozoa

Spermatozoa were incubated at 37 °C for 2 h with progesterone (10 μg/ml) and A23187 (10 μM) in the presence of [^{3}H]acetic acid. At the end of incubation, samples were centrifuged and lipids extracted from supernatants and cell pellets. After t.l.c., areas corresponding to PAF were scraped and radioactivity was counted. The number of experiments is given in parentheses. Control = non-stimulated spermatozoa; raw c.p.m. data were five times higher. Statistical significance: $^*P < 0.05$ versus control.

crease over basal, $n = 3$). The effect of progesterone was dose-dependent (Figure 3), reaching a plateau at the concentration of 5 μg/ml (16 μM). The EC_{50} of the curve (defined as the concentration with an expected half-maximal response) was 1.12 μg/ml (3.56 μM).

When cell pellets and supernatants were analysed separately, most of newly synthesized [^{3}H]PAF was found in the supernatants of both unstimulated and stimulated spermatozoa (Figure 4). The treatment with progesterone (10 μg/ml) and A23187 (10 μM) stimulated an increase of [^{3}H]PAF release in the supernatant of 1.78 ± 0.59- and 2.2 ± 0.54-fold respectively. We suggest that [^{3}H]PAF release occurs from the acrosome as a consequence of the acrosome reaction. Indeed, although no PAF activity has been found in seminal plasma from both rabbit (Kumar et al., 1988) and human spermatozoa (Minhas et al., 1991), release of PAF occurs in mouse spermatozoa after incubation for 3 h (Kuzan et al., 1990). As mouse spermatozoa undergo spontaneous acrosome reaction during *in vitro* incubation, PAF release might be due to this exocytotic event. Moreover, Davis et al. (1980) reported an increase of phospholipids in the incubation medium of rat spermatozoa when incubated for prolonged periods of time, that they suggest is due to spontaneous exocytosis. Spontaneous acrosome reaction, to a certain extent, may occur in the absence of stimulation in human sperm (Takahashi et al., 1992; Falsetti et al., 1993). However, whether PAF is released into the medium as a consequence of acrosome reaction remains to be established.

Lyso-PAF:acetyl-CoA acetyltransferase activity in human spermatozoa

The results of the experiments designed to characterize and optimize the enzyme assay are reported in Figure 5. Figure 5(a) shows the time course of the reaction, indicating that, in our conditions, PAF synthesis was linear up to 90 min, followed by a plateau. Increasing the number of spermatozoa in the sonicate up to 1.5×10^7 spermatozoa/tube, PAF synthesis increased linearly up to a level of 5×10^6 spermatozoa/tube (Figure 5b). Accordingly, experiments were performed using 5×10^6 spermatozoa. The reaction was linearly dependent on lyso-PAF concentration up to 50 μM (Figure 5c); however, as shown in the figure, significant incorporation of [^{3}H]acetate from [^{3}H]acetyl-CoA was also present in the absence of exogenously added lyso-PAF, suggesting that high concentrations of lyso-PAF are present in unstimulated cells. The dependence of acetyltransferase activity on the concentration of acetyl-CoA is shown in Figure 5(d). The observed K_m value for acetyl-CoA, obtained from a double-reciprocal plot (Figure 5d, inset) was 46.6 μM. These results indicate that kinetic parameters of lyso-PAF:acetyl-CoA acetyltransferase of human spermatozoa are similar to those described for different cell preparations (Wykle et al., 1980; Snyder et al., 1987). The treatment with progesterone (10 μg/ml) and A23187 (10 μM) enhanced lyso-PAF:acetyl-CoA acetyltransferase activity at early times (Table 1).

Identification of sperm-derived PAF-like material as authentic PAF

PAF-like material co-migrating with authentic PAF isolated from A23187-stimulated human spermatozoa was able to induce aggregation of rabbit platelets, which was inhibited by the PAF-receptor antagonist L659,989 (10 μM) or by alkaline methanolic hydrolysis (results not shown). Based on the PAF dose–response curve, we estimated that 1×10^8 A23187-stimulated spermatozoa produce 0.37 pmol of bioactive PAF/2 h.

G.c.–m.s. analysis of phospholipase C-treated and TBDMSI-derivatized PAF-like material isolated from human spermatozoa, showed the presence of a chromatographic peak with identical retention time of standard PAF $C_{16:0}$. Ions *m/e* 117, 131 and 415, with identical ratios to those of PAF standard ions, were present in the sample when analysed by m.i.d. [Figures 6(a) and 6(b)]. When monitored by s.i.m., the ion *m/e* 415 (M − 57) showed the same retention time as the PAF standard (results not shown). These results confirm the presence of 1-*O*-alkyl-*sn*-2-acetyl-glycerophosphocholine, the biologically active PAF, because the acyl-PAF analogue (1-*O*-acyl-*sn*-2-acetylglycerophospho-

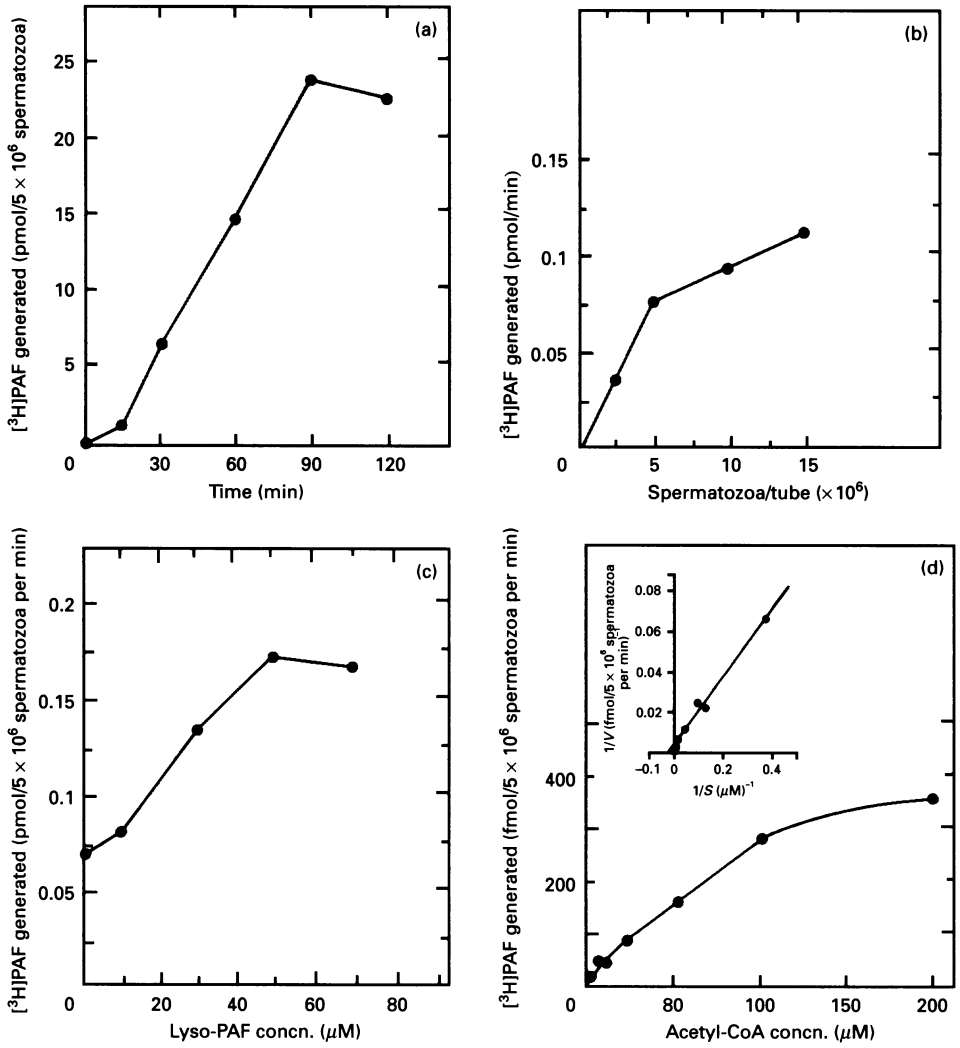

Figure 5 Lyso-PAF:acetyl-CoA acetyltransferase activity in human spermatozoa

Acetyltransferase activity was assessed in sonicated spermatozoa by measuring [^{3}H]acetyl-CoA incorporation into PAF as a function of incubation time (**a**), cell number (**b**), lyso-PAF concentration (**c**) and acetyl-CoA concentration (**d**). The inset in (**d**) shows the double-reciprocal plot of substrate dependence of acetyltransferase activity towards acetyl-CoA. The results are representative of at least two different experiments done in duplicate.

Table 1 Time-course of lyso-PAF:acetyl-CoA acetyltransferase activation (pmol/5 × 10^6 spermatozoa per min) in human spermatozoa after incubation with progesterone (10 μg/ml) and A23187 (10 μM)

Spermatozoa were incubated with progesterone and A23187 for the indicated times and acetyltransferase activity measured in sperm sonicates as described in the text. Values are the mean ± S.E.M. of two experiments.

	Acetyltransferase activity (pmol/5 × 10^6 spermatozoa per min)					
Time (min)...	2	5	10	20	60	120
Control	0.05 ± 0.004	0.07 ± 0.007	0.07 ± 0.014	0.09 ± 0.009	0.10 ± 0.000	0.10 ± 0.014
Progesterone	0.08 ± 0.014	0.11 ± 0.015	0.14 ± 0.000	0.11 ± 0.004	0.12 ± 0.009	0.11 ± 0.021
A23187	0.09 ± 0.019	0.12 ± 0.007	0.13 ± 0.007	0.12 ± 0.000	0.11 ± 0.014	0.11 ± 0.009

choline) has different electron impact mass spectra and a different gas-chromatographic retention time in our system.

Effect of progesterone on arachidonic acid metabolism of human spermatozoa

Under steady-state conditions, 310 ± 41.6 c.p.m./10^6 spermatozoa were recovered in the material co-migrating with authentic arachidonic acid of the supernatant of unstimulated, prelabelled spermatozoa after 2 h. Free [^{3}H]arachidonic acid represented 21.2 ± 1.1 % of total radioactivity. Exposure of [^{3}H]arachidonic-acid-prelabelled spermatozoa to progesterone (10 μg/ml) and A23187 (10 μM) for 2 h, stimulated a significant increase in the release of [^{3}H]arachidonic acid (Figure 7) and an increase of radioactivity in the free-fatty-acid fraction of the cell pellet (Table 2). In four different experiments, the effect of progesterone was tested at concentrations of 1, 5, and 10 μg/ml, which elicited

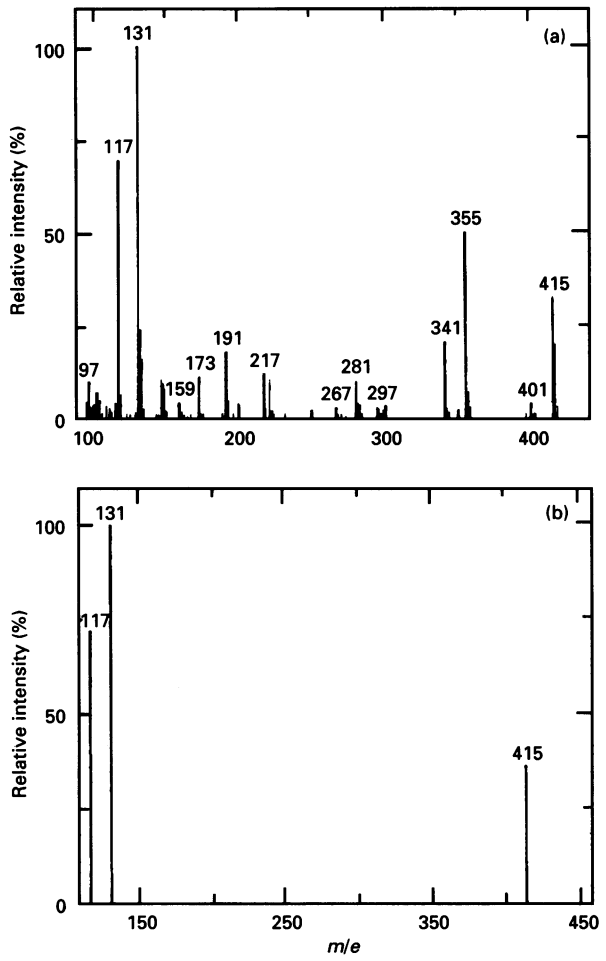

Figure 6 G.c.–m.s. analysis of sperm-derived PAF-like material

PAF-like material extracted from human spermatozoa, after exposure to phospholipase C hydrolysis and derivatization with TBDMSI, was injected into a Perkin Elmer 8420 gas chromatograph–ion-trap mass spectrometer, and acquisition performed with m.i.d. mode. Identification was made by comparison with PAF $C_{16:0}$ standard. Full-scan acquisition of PAF $C_{16:0}$ standard is represented in (**a**). Masses 117, 131 and 415, fragment ions characteristic of PAF $C_{16:0}$, were present in the sample at the same retention time as the standard (**b**).

a stimulation of 1.06±0.23-, 1.09±0.3- and 1.19±0.35-fold increase over basal respectively.

Among the cellular phospholipids examined, a decrease of radioactivity was observed in PC after stimulation with A23187 (Table 2), whereas the changes in PE and PS were not significant. Progesterone determined a slight decrease in PC radioactivity (4.9%), which was approaching statistical significance ($P = 0.07$) (Table 2).

DISCUSSION

The results presented in this paper demonstrate that incubation of human spermatozoa, either with physiological (progesterone) and non-physiological (A23187) stimuli of acrosome reaction, produces a significant increase of PAF production, as assessed by [^{3}H]acetate incorporation into PAF. Moreover, we report the presence of lyso-PAF:acetyl-CoA acetyltransferase activity in human spermatozoa. Such activity was dependent on time and substrate (lyso-PAF and acetyl-CoA) concentrations, with similar kinetic parameters to those found in other cells. The occurrence of PAF in mammalian spermatozoa has been reported in several studies (Kumar et al., 1988; Kuzan et al., 1990; Parks et al., 1990; Minhas et al., 1991); however, these studies do not

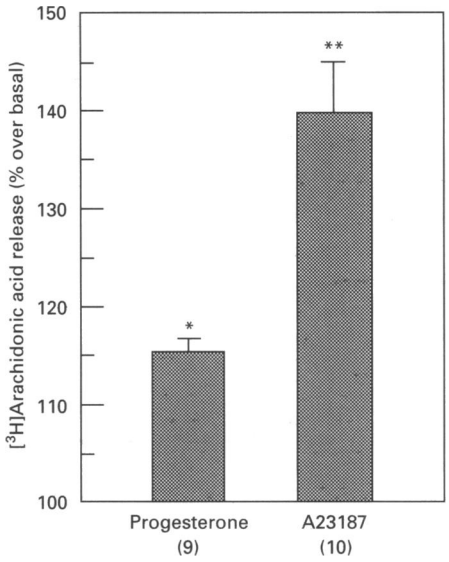

Figure 7 Effect of progesterone and A23187 on [^{3}H]arachidonic acid release from prelabelled human spermatozoa

Spermatozoa were incubated with [^{3}H]arachidonic acid for 90 min, washed and stimulated or not with A23187 (10 μM) and progesterone (10 μg/ml) for 2 h. After centrifugation, supernatant was collected and lipids extracted and subjected to t.l.c. Areas corresponding to standard arachidonic acid were scraped and counted. C.p.m. in the control samples were $1550 \pm 205/5 \times 10^6$ spermatozoa per 2 h. The number of experiments is given in parentheses. Statistical significance: $^*P < 0.05$; $^{**}P < 0.005$.

provide information concerning the enzymical routes involved in PAF synthesis in these cells. Indeed, PAF can be synthesized by at least two different pathways, namely the remodelling and the *de novo* anabolic pathways (Snyder, 1990). The evaluation of [^{3}H]acetate incorporation into PAF is considered a reliable method to study the anabolic, remodelling pathway of PAF synthesis. Our results indicate that PAF synthesis in human spermatozoa may occur at least through the remodelling pathway, and that this route can be increased by stimuli that induce Ca^{2+} entry and the acrosome reaction.

Perturbations of membrane phospholipid metabolism after induction of the acrosome reaction of spermatozoa have been reported. In particular, progesterone and follicular fluid are able to stimulate phospholipase C, with increased production of inositol phosphates and, presumably, of diacylglycerol (Thomas

Table 2 Changes in radioactivity of [^{3}H]arachidonic acid-labelled lipids in progesterone- (10 μg/ml) and A23187- (10 μM) stimulated human spermatozoa

[^{3}H]Arachidonic acid-labelled spermatozoa were stimulated with progesterone and A23187 at 37 °C for 2 h. Results are expressed as a percentage of total cellular radioactivity (5837±500.3 c.p.m./10^6 spermatozoa) and are means±S.E.M. of five experiments done in triplicate. Statistical significance: $^{**}P < 0.005$, $^*P < 0.05$, versus control.

	Change in radioactivity (%)		
Lipid	Control	Progesterone	A23187
Free fatty acids	6.6±1.1	8.15±1.4*	10.2±1.3**
PC	40.3±3.3	35.4±3.2	21.3±4.5*
PE	17.5±1.8	16.7±2.2	13.9±1.4
PS	7.04±2.1	5.1±1.5	5.4±1.7

and Meizel, 1989). Similarly, stimulation of acrosome reaction of mammalian spermatozoa with A23187 induces an increase of phospholipase C (Roldan and Harrison, 1989), phospholipase A (Takkar et al., 1983; Bennet et al., 1987) and lipoxygenase (Lax et al., 1990) activities. Futhermore, the induction of acrosome reaction of sea-urchin sperm with fucose sulphate activates both a phospholipase C (Domino and Garbers, 1988) and a phospholipase D which stimulates phosphatidic acid and diacylglycerol accumulations (Domino et al., 1989). All these activities are entirely dependent upon calcium influx in the cells, indicating that an increase of intracellular calcium is the primary necessary event for stimulation of phospholipases. Our results demonstrate that progesterone and A23187 stimulated an increase of [^{3}H]arachidonic acid release from prelabelled spermatozoa and [^{3}H]acetate accumulation into PAF, and enhanced lyso-PAF:acetyl-CoA acetyltransferase activity, further confirming the occurrence of increased phospholipid metabolism after incubation with stimuli that induce the acrosome reaction. The high [^{3}H]acetate incorporation into PAF found in unstimulated spermatozoa suggests that both phospholipase A2 and acyl(acetyl)transferase are active even in unstimulated cells. Indeed, in our hands, [^{3}H]arachidonic acid release in unstimulated sperm was very high (representing about 20% of total cell radioactivity), suggesting the presence of high phospholipase A2 activity in these cells. Moreover, the high level of [^{3}H]acetate incorporation into PAF in sperm sonicate in the absence of exogenously added lyso-PAF indicates that lyso-PAF content (or production) of spermatozoa is quite high. High levels of lyso-PC were found in epididymal rat spermatozoa (Aveldano et al., 1992) and alkyl-acyl-PC is a major phospholipid in spermatozoa (Nikolopoulou et al., 1985; Aveldano et al., 1992), suggesting that a certain percentage of lyso-PC belongs to the alkyl species. Furthermore, Jones and Plymate (1989) reported high levels of incorporation of palmitic acid into PC in human sperm in the absence of exogenous lyso-PC, suggesting the presence of high levels of the lysophospholipid.

We also showed that newly synthesized and biologically active PAF in control and stimulated spermatozoa is almost entirely released into the medium, suggesting a potentially important physiological role for PAF during sperm–egg interaction. Indeed, it has been shown recently that the addition of physiological concentrations of PAF to sperm incubation medium enhances both motion parameters (Ricker et al., 1989; Hellstrom et al., 1991) and *in vitro* fertilization rate (Roudebush et al., 1990), indicating an active role for the phospholipid in fertilization. Although the mechanism(s) of PAF action on spermatozoa is still unclear, PAF released by these cells might act both by increasing sperm motility parameters and by facilitating sperm–egg interactions during the fertilizing process.

The mechanism of progesterone action on spermatozoa is quite peculiar and appears to be shared only by germinal cells. Indeed, only spermatozoa (Thomas and Meizel, 1989; Blackmore et al., 1990) and oocytes (Chien et al., 1986, 1991; Stith et al., 1991) respond to progesterone with an immediate increase of intracellular free calcium and alterations of membrane phospholipid metabolism. Furthermore, such unusual progesterone-mediated signal transduction pathways appear to be mediated, at least in spermatozoa, by membrane receptors (Meizel and Turner, 1991; Blackmore et al., 1991), which seem to be different from classic intracellular receptors present in somatic cells (Baldi et al., 1991; Blackmore et al., 1991). Although we have no evidence for the involvement of such receptors in progesterone-mediated [^{3}H]PAF production or [^{3}H]arachidonic acid release, the rapidity of action of progesterone seems to exclude the stimulation of intracellular progesterone receptors.

In summary, progesterone and A23187 elicit an increase of PAF production and arachidonic acid release in human spermatozoa, which may play a physiological role in the process of fertilization.

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