Significance of intracellular calcium and cyclic adenosine 3',5'-monophosphate in the mechanisms by which prostaglandins influence human sperm function

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Summary. The selective ability of PGE-1 and PGE-2 to enhance the capacity of human spermatozoa for hamster oocyte penetration was dependent upon high concentrations (1.7 mM) of extracellular Ca^{2+} and a prolonged (4 h) duration of exposure, and insensitive to the Ca^{2+} channel antagonist, verapamil. Studies with the intracellular calcium indicator Quin-2 indicated that exposure to PGE-1 and PGE-2, but not PGF-2 α , induced a significant rise in the levels of cytoplasmic Ca^{2+} , suggesting that an ionophore-like action might be responsible for the ability of the E-series prostaglandins to influence sperm function.

Stimulation of oocyte penetration with PGE-1 and PGE-2 was significantly enhanced when these compounds were presented to the spermatozoa in medium of high osmolarity (410 mosmol). A combination of PGE-1 in hyperosmotic medium did not significantly influence sperm function in cases of oligozoospermia, although it was effective with patients exhibiting idiopathic infertility.

Exposure to high doses of PGE-1 and PGE-2, but not 19-hydroxy PGE or PGF-2 α , also induced a significant rise in the cyclic AMP content of human spermatozoa. This effect did not appear to be involved in the enhancement of fertilization rates because (1) it did not exhibit the same absolute dependence on high levels of extracellular Ca^{2+} as did the fertilization responses and (2) the enhancement of oocyte penetration and the elevation of cAMP were independent of each other within the patient population.

Introduction

The finding that prostaglandins, especially those of the E series, can exert direct effects upon the functional competence of human spermatozoa (Aitken & Kelly, 1985) has important clinical implications for the treatment of male infertility and may also be of biological importance in suggesting mechanisms by which sperm function may be regulated *in vivo*. In the light of these considerations we have undertaken the following study to investigate the cellular mechanisms by which prostaglandins influence the fertilizing capacity of human spermatozoa. Particular attention has been paid to changes in the intracellular levels of free, extramitochondrial calcium and cyclic 3',5'-adenosine monophosphate (cAMP), both of which are influenced by prostaglandins in other cell types (Kirtland & Baum, 1972; O'Donnell, 1974) and both of which have been implicated in the regulation of human sperm function (Yanagimachi, 1981; Aitken, Best, Richardson, Schats & Simm, 1983a).

Materials and Methods

Donors. Unless otherwise stated these studies were carried out on semen samples provided by a panel of 25 donors, all of whom were normospermic according to previously established criteria

(Aitken, Best, Richardson, Djahanbakhch & Lees, 1982a) in that the concentration of spermatozoa exceeded $20 \times 10^6/\text{ml}$ and the proportion of cells that were motile or exhibited a normal morphology exceeded 40%. Samples were also collected from a group of 17 infertile patients presenting at the infertility clinic with at least 2 years infertility associated with oligozoospermia (sperm counts of $<20 \times 10^6/\text{ml}$ on at least 2 occasions) or idiopathic infertility (male normospermic as defined above and female partners exhibiting normal history and examination, normal menstruation cycles of 28 ± 4 days, normal elevated progesterone concentrations during the luteal phase and a normal diagnostic laparoscopy, including confirmation of tubal patency; Aitken *et al.*, 1982b).

In-vitro fertilization. The penetrating ability of the spermatozoa was assessed using the zona-free hamster ovum penetration test (Yanagimachi, Yanagimachi & Rogers, 1976) using Medium BWB, a modified Krebs–Ringer bicarbonate buffer described by Biggers, Whitten & Whittingham (1971). The protocol was exactly as described by Aitken, Wang, Lui, Best & Richardson (1983c) and Aitken & Kelly (1985) involving a capacitation period of 3 h in hyperosmotic medium (410 mosmol) followed by a 3 h incubation with the zona-free hamster oocytes under normosmotic conditions (305 mosmol.). The oocytes were subsequently removed from the sperm suspensions, washed, and scored for the presence of spermatozoa with decondensing nuclei by phase-contrast microscopy.

The result of the test was assessed on at least 20 oocytes in terms of the percentage of oocytes penetrated and the mean number of penetrated spermatozoa per oocyte. The assay was performed using a total sperm concentration of 10×10^6 spermatozoa/ml and the results were subsequently adjusted for a standard concentration of 5×10^6 motile spermatozoa/ml. This correction was based upon the formula described by Aitken & Elton (1984) where $P_2 = 1 - \exp(-rm_1)$ in which P_2 = the corrected penetration rate for a motile sperm concentration of $5 \times 10^6/\text{ml}$; r = the ratio of the theoretical concentration of motile spermatozoa (i.e. 5) to the actual concentration recorded in the experiment and m_1 = the observed outcome of the penetration test in terms of the mean number of spermatozoa penetrating each oocyte.

Intracellular calcium measurements. The influence of prostaglandins on the intracellular concentration of free, extramitochondrial Ca^{2+} was assessed by monitoring the fluorescence signal emitted by the Ca^{2+} chelating agent, Quin-2, as described by T sien, Pozzan & Rink (1982). The spermatozoa were washed 3 times with Medium BWB, and finally resuspended at a total sperm concentration of $20 \times 10^6/\text{ml}$. Quin-2-tetracetoxymethyl ester was added from a stock solution in dimethylsulphoxide to achieve a final concentration of $50 \mu\text{M}$, and the cell suspension was incubated at 37°C in 5% CO_2 in air for 20 min. The cell suspension was then diluted 1:9 with fresh medium BWB and the incubation continued for a further 100 min. The cells were subsequently washed twice to remove extracellular Quin-2, by centrifugation at 500 *g* and finally resuspended in fresh medium BWB to a concentration of $10 \times 10^6/\text{ml}$. Fluorescence measurements were performed in an Aminco-Bowman spectrofluorometer, with the excitation wavelength set to 339 nm and the emission wavelength set to 492 nm. The suspension of spermatozoa was maintained at 37°C in quartz cuvettes for the duration of each series of measurements and before the start of each experiment the entry of Quin-2 into the cells was checked by observing the shift in the fluorescence maxima for Quin-2 from 430 to 492 nm.

To measure the effect of prostaglandins on intracellular Ca^{2+} , a 1-ml sample of Quin-loaded spermatozoa was transferred to the spectrofluorometer and the fluorescence signal was recorded. An equal volume of Medium BWB was then added containing either no additive (control) or 60 μg prostaglandin/ml. The changed fluorescence signal was recorded immediately and then at 10-min intervals for 40 min.

Cyclic AMP determinations. The cAMP content of the spermatozoa was assessed by radioimmunoassay, according to the method of Steiner, Parker & Kipnis (1972) as modified by Harper & Brooker (1975). For these determinations the spermatozoa were washed 3 times with Medium

BWW and resuspended at a total concentration of $10 \times 10^6/\text{ml}$. Extraction of the cAMP for the dose-response studies was performed by treating 400 μl of the sperm suspension with 100 μl 2.5 M-perchloric acid. The suspensions were then vigorously vortexed and incubated at 4°C for 30 min. After this time 250 μl 0.714 M- K_3PO_4 were added and the solutions were again vortexed and incubated for a further 20 min at 4°C. After centrifugation at 2000 g for 10 min the supernatants were removed and stored at -20°C. Incorporation of phosphodiesterase inhibitors such as caffeine into the sperm suspensions, as in the time-course studies, was found to interfere with the measurement of cAMP when the above extraction procedure was used. Therefore, for the time-course studies, 500 μl samples of the sperm suspensions were centrifuged at 600 g for 5 min before extraction of the pellet with 500 μl ice-cold ethanol. After extensive vortexing and an incubation period of 20 min at 4°C, these extracts were centrifuged at 2000 g for 10 min and the supernatants removed. The latter were then dried down under a stream of nitrogen and resuspended in 500 μl 0.01 M-phosphate-buffered saline (PBS), pH 7.4. In the absence of phosphodiesterase inhibitors the results obtained with these two extraction procedures were highly correlated ($r = 0.846$).

Intracellular cAMP levels were monitored in 100 μl duplicate samples each of which was treated with 5 μl of freshly prepared acetylating reagents (2.7 parts triethylamine:1 part acetic anhydride) mixed and incubated for 1 h. Subsequently, 100 μl antiserum (diluted 1:70 000) raised against a conjugate of human serum albumin and succinyl cyclic AMP (Steiner *et al.*, 1972) and 100 μl (30 000 c.p.m.) ^{125}I -2'-O-succinyl cAMP-tyrosine methyl ester tracer (Sigma Chemical Co, St Louis, MO, U.S.A.) were added to each tube and incubated for 16 h at 4°C; cAMP standards (0–2500 fmol) were treated in precisely the same way. Separation of free from bound labelled tracer was achieved by centrifugation at 2000 g for 30 min at 4°C after addition of 17% (w/v) polyethylene glycol. The radioactivity associated with the pellets was measured with a Nuclear Enterprises 1600 multihead gamma counter and the results were expressed as fmol cAMP/ 10^6 spermatozoa. The antibody used in these assays exhibited the following cross-reactivities: cAMP, 100%; cGMP, 0.0035%; AMP, 0.0005%; ADP, 0.00045%; ATP, 0.00069%; GTP 0.0005%; theophylline 0.0004%; isobutyl methyl xanthine 0.00001%. The intra- and inter-assay coefficients of variation were 6.3% and 14.5% respectively.

Materials. Prostaglandins E-1, E-2 and F-2 α were purchased from Sigma Chemical Co, St Louis, MO (U.S.A.) while verapamil was a gift from Abbott Laboratories Ltd, Queensborough, U.K.

A 19-hydroxy PGE-1 + 2 extract was prepared from a pool of human seminal plasma which had been stored in acetone at -20°C. The extraction of 19-hydroxy PGE was performed exactly as described by Aitken & Kelly (1985) and the final product was >90% pure, containing <2% PGE and consisting of equal amounts of 19-hydroxy PGE-1 and 19-hydroxy PGE-2.

All PG preparations were taken up in ethanol, transferred to clean flat-bottomed tubes and dried down at 37°C under a stream of nitrogen, before addition of Medium BWW.

Statistics. All statistical comparisons were made using the non-parametric Mann-Whitney U and Wilcoxon matched pairs signed rank tests.

Results

In-vitro penetration

The purpose of the first series of experiments was to determine (1) the importance of extracellular Ca^{2+} in mediating the effects of the E-series prostaglandins on human sperm function and (2) the time course over which these prostaglandins exerted their action.

Human spermatozoa were capacitated in hyperosmotic Medium BWW (410 mosmol) with or without the addition of 1.7 mM- Ca^{2+} (measurements with a calibrated Orion Ca^{2+} electrode,

Fig. 1. Influence of extracellular calcium and duration of exposure on the responses of human spermatozoa to (a) PGE-1 and (b) PGE-2 in the zona-free hamster oocyte penetration test. * $P < 0.05$; ** $P < 0.01$.

Table 1. Response of human spermatozoa to 60 μg PGE-1/ml in the presence and absence of 10 μM -verapamil

Donor	Penetration rate (%)		
	Control	PGE-1	PGE-1 + vera-pamil
1	51.7	88.9	95.5
2	8.5	84.5	26.1
3	0.0	44.5	14.0
4	4.2	21.3	20.8
Mean $\pm$ s.e.m.	16.1 $\pm$ 11.9	59.8 $\pm$ 16.2	39.1 $\pm$ 18.9

model 93-20, indicated that, in the absence of added Ca^{2+} , the Ca^{2+} concentration of Medium BWB is about 10 μM). During this 4 h capacitation period the spermatozoa were exposed to 60 μg PGE-1 or PGE-2/ml for one of 3 different periods: (1) the first hour, (2) the last hour or (3) the entire 4 h capacitation period. Once this 4 h incubation period had been completed the spermatozoa were washed in normo-osmotic Ca^{2+} -supplemented Medium BWB (305 mosmol) and incubated for a further 3 h with zona-free hamster oocytes. The results of this study are summarized in Figs 1a and b. In the presence of added Ca^{2+} a significant ($P < 0.05$) increase in oocyte penetration rates was observed in the presence of PGE-1 and PGE-2, but only after being exposed to the latter for the entire 4 h capacitation period. The absence of Ca^{2+} during the capacitation period made no significant difference to the rates of penetration observed in the control incubations but, under these conditions, no stimulation was observed in the presence of PGE-1 or PGE-2, even after exposure for 4 h.

To determine whether the interaction between Ca^{2+} and prostaglandins of the E-series involved voltage-sensitive calcium channels, the stimulatory effects of a 4 h exposure to 60 μg PGE-1/ml were examined in the presence and absence of 10 μM verapamil. The results of 4 separate experiments are presented in Table 1; overall, the presence of PGE-1 increased the mean ($\pm$ s.e.m.)

Table 2. Response of human spermatozoa to PGE in the presence of normo-osmotic (305 mosmol) or hyperosmotic (410 mosmol) Medium BWB

Prostaglandin	Treatment	n	Penetration rate (%)	
			Normo-osmotic	Hyperosmotic
PGE-1	Control	6	14.5 ± 6.3	16.8 ± 7.1
	60 µg/ml	6	50.2 ± 10.4**	60.8 ± 13.4**
	Control	4	17.1 ± 8.8	6.5 ± 2.6
	270 µg/ml	4	27.3 ± 12.6	28.6 ± 5.3**
PGE-2	Control	4	14.4 ± 9.2	11.1 ± 6.4
	60 µg/ml	4	33.8 ± 17.3	53.4 ± 13.4*
	Control	4	17.1 ± 8.8	6.5 ± 2.6
	270 µg/ml	4	37.1 ± 8.1	56.6 ± 10.1**

* $P < 0.05$; ** $P < 0.01$.

penetration rates from a control value of $16.1 \pm 11.9\%$ to $59.8 \pm 16.2\%$. The presence of verapamil caused a slight, though statistically insignificant, diminution in the response to PGE-1, to a mean value of $37.9 \pm 18.9\%$ (Table 1).

The contribution of the hyperosmotic conditions used during capacitation to the enhanced fertilization rates observed with PGE-1 and PGE-2 was examined at 2 doses of prostaglandin, 60 and 270 µg/ml (Table 2). The spermatozoa were capacitated for 3 h in the presence of prostaglandin dissolved in Medium BWB with an osmolarity of 305 or 410 mosmol (Aitken *et al.*, 1983c). At the end of this incubation period the spermatozoa were pelleted and resuspended in normo-osmotic Medium BWB (305 mosmol) before addition of the zona-free hamster oocytes and a further 3 h incubation to effect penetration. PGE-1 and PGE-2 both increased significantly the percentage of oocytes penetrated in hyperosmotic medium, at all doses tested (Table 2). Incorporation of the prostaglandins into normo-osmotic medium also resulted in a consistent increase in the proportion of oocytes penetrated, but this effect was only statistically significant in the incubations containing 60 µg PGE-1/ml.

Patient responses

To assess the therapeutic potential of hyperosmotic medium supplemented with PGE-1 for the artificial stimulation of human sperm function, analyses were carried out with samples from 18 patients attending the infertility clinic (Tables 3 & 4). The samples from 12 oligozoospermic patients were treated with 20 or 60 µg PGE-1/ml and the level of penetration observed was compared with that obtained in the presence of the calcium ionophore, A23187 (Aitken, Ross, Hargreave, Richardson, & Best, 1984). Overall, 5 of these oligozoospermic patients showed an increase in penetration rates after exposure to PGE-1 and in 2 cases the responses were superior to those observed in the presence of ionophore. Statistically, however, the percentage of oocytes penetrated in the presence of PGE-1 was not significantly different from that for the hyperosmotic controls in the oligozoospermic group as a whole. In contrast, the presence of A23187 increased the penetration rates observed in 7 of these oligozoospermic patients ($P < 0.05$).

The patients exhibiting idiopathic infertility were much more sensitive to the stimulatory effects of PGE-1 (Table 4). At a dose of 60 µg PGE-1/ml, 5 of the patients showed an increase in penetration rates, raising the overall level of oocyte penetration from a control value ($\pm$ s.e.m.) of $9.2 \pm 3.8\%$ to $23.1 \pm 8.4\%$ ($P < 0.05$). In the same patients, A23187 raised the penetration rates to a mean ($\pm$ s.e.m.) of $27.1 \pm 14.0\%$, but this figure was not significantly different from the controls.

Table 3. Influence of PGE-1 on the penetrating capacity and intracellular cAMP content of spermatozoa from oligozoospermic patients

Patient	PG dose ($\mu\text{g/ml}$)	Penetration rate (%)			cAMP (fmol/ 10^6 spermatozoa)*	
		Control	PGE-1	A23187†	Control	PGE-1
A	20	49.7	30.9	99.9	187	298
B	20	0	0	12.3	201	513
C	20	38.9	38.9	75.7	104	92
D	20	0	0	0	—	—
E	20	15.6	31.9	32.6	—	—
F	20	6.6	50.0	20.0	—	—
G	60	44.5	59.8	99.1	340	184
H	60	0	0	0	237	743
I	60	11.3	5.2	0	278	413
J	60	7.0	14.7	30.9	121	176
K	60	0	7.0	0	329	742
L	60	0	0	0	—	—

*1 min exposure to PG in presence of 2 mM-caffeine.

†Aqueous suspension of Ca/Mg salt at 100 μM .**Table 4.** Influence of PGE-1 on the penetrating ability and intracellular cAMP content of spermatozoa from patients with idiopathic infertility

Patient	Penetration rate (%)				cAMP (fmol/ 10^6 spermatozoa)*		
	Control	PGE-1		A23187†	Control	PGE-1	
		20 $\mu\text{g/ml}$	60 $\mu\text{g/ml}$			20 $\mu\text{g/ml}$	60 $\mu\text{g/ml}$
M	10	—	36.8	31.8	148	—	102
N	24.5	—	55.2	91.3	87	—	98
O	0	—	0	0	346	—	490
P	13.8	17.1	17.1	20.3	99	140	124
Q	0	14.1	7.3	19.3	259	138	152
R	6.8	5.6	22.1	0	302	1583	1456

*1 min exposure to PG in presence of 2 mM-caffeine.

†Aqueous suspension of Ca/Mg salt at 100 μM .

Intracellular calcium

The fluorescence signal emitted by human spermatozoa loaded with Quin-2 was used to carry out a time-dependent study of the way in which PGE-1, PGE-2 and PGF-2 α influenced the levels of free, intracellular calcium. PGE-1 and PGE-2 significantly elevated intracellular calcium levels but PGF-2 α was ineffective (Fig. 2). When the formulae described by Tsien *et al.* (1982) were applied to these changes in fluorescence, to calculate the actual levels of intracellular calcium ($[\text{Ca}^{2+}]_i$), the mean, resting level of $[\text{Ca}^{2+}]_i$ in human spermatozoa was found to be 140 nM, rising to 280 nM and 274 nM after the addition of PGE-1 and PGE-2 respectively. Despite their statistical significance, these PGE-induced changes are not as dramatic as those observed in the presence of A23187 which, over the same time course, elevates $[\text{Ca}^{2+}]_i$ to a mean value of around 600 nM (data not shown).

cAMP

To investigate whether prostaglandins might enhance the penetrating potential of human spermatozoa by elevating the intracellular concentrations of cAMP (Aitken *et al.*, 1983a) the latter

Fig. 2. Influence of prostaglandins on the fluorescence signal emitted by the intracellular calcium indicator Quin-2. * $P < 0.05$; ** $P < 0.01$ compared with control values.

Fig. 3. Dose-dependent study of the influence of prostaglandins on the cyclic AMP content of human spermatozoa: (a) PGE-2, (b) PGE-1, (c) 19-hydroxy PG and (d) PGF-2α. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 4. Time-dependent study of the influence of prostaglandin on the cyclic AMP content of human spermatozoa in (a) the presence and (b) the absence of 2 mM-caffeine. * $P < 0.05$; ** $P < 0.01$.

was measured in cells which had been incubated for 3 h in hyperosmotic medium supplemented with PGE-1, PGE-2, PGF-2 α or 19-hydroxy PGE, over a dose range from 4.2 to 270.0 $\mu\text{g/ml}$ (Aitken & Kelly, 1985). This study revealed significant, dose-dependent, effects on the cAMP content of human spermatozoa for PGE-1 and PGE-2, but not for PGF-2 α or 19-hydroxy PGE (Fig. 3).

Time-dependent studies were then carried out for each prostaglandin, at a fixed dose of 60 $\mu\text{g/ml}$ (Fig. 4). Significant increases in intracellular cAMP were again observed for both PGE-1 and PGE-2, but not for PGF-2 α or 19-hydroxy PGE, regardless of whether or not caffeine was present as a phosphodiesterase inhibitor. The results obtained in these time-course studies revealed some variation between samples in the duration and extent of the changes in cAMP levels, although in general the response to PGE-1 and PGE-2 appeared to be optimal within the first 5 min, particularly in the absence of caffeine (Fig. 4).

The choice of caffeine as a phosphodiesterase inhibitor did not influence the outcome of these experiments, since a direct comparison of the changes in cAMP induced by 60 μg PGE-1/ml in the presence of 2 mM-caffeine or 0.2 mM-isobutyl methyl xanthine on samples from 3 separate donors, at 1, 5, 30 and 60 min, revealed a rise in intracellular cAMP which was maximal after 5 min and of similar magnitude for both compounds, equivalent to a rise ($\pm$ s.e.m.) over control values of $125 \pm 26\%$ and $148 \pm 25\%$ for caffeine and isobutyl methyl xanthine respectively.

Unlike the in-vitro penetration response, the rise in cAMP observed after exposure to PGE-1 was still observed in the absence of added calcium. In 5 separate samples extracted 1 min after exposure to 60 µg PGE-1/ml in hyperosmotic Medium BWB lacking added calcium, intracellular cAMP was elevated from a control value ($\pm$ s.e.m.) of 112.6 ± 19.2 to 135.2 ± 17.0 fmol/ 10^6 spermatozoa ($P < 0.05$).

The changes in cAMP induced by PGE also differed from the oocyte penetration responses in that they were influenced by prior capacitation of the spermatozoa. In an experiment performed with 3 separate donors the cAMP levels observed 1 min after exposure to PGE-1 rose to a mean ($\pm$ s.e.m.) of 173.0 ± 51.4 fmol/ 10^6 spermatozoa before capacitation, but after incubation for 3 h in hyperosmotic medium the same degree of stimulation resulted in a cAMP level of only 87.3 ± 14.3 fmol/ 10^6 spermatozoa ($P < 0.05$). Over the same period the control levels of cAMP had not changed significantly, giving values of 92.6 ± 18.4 and 70.6 ± 29.2 fmol/ 10^6 spermatozoa before and after capacitation respectively.

In 14 of the infertile patients examined, sufficient spermatozoa were available to evaluate the changes in cAMP induced by PGE-1 in addition to the capacity for oocyte penetration (Tables 3 & 4). In 10 (71%) of these patients (6 oligozoospermic and 4 with idiopathic infertility) exposure to PGE-1 resulted in a rise in intracellular cAMP levels within 1 min. However, this response to PGE-1 did not appear to relate directly to the ability of the prostaglandin to influence the fertilizing potential of the spermatozoa. Hence in 4 samples (A, B, H and O) PGE-1 induced a rise in cAMP but no change in penetration rate, while in a further 3 samples (G, M and Q) the cAMP values fell but the penetrating capacity of the spermatozoa was nevertheless enhanced.

Discussion

The ability of prostaglandins of the E-series to enhance the penetrating capacity of human spermatozoa appears to be associated with their ability to elevate the intracellular concentration of free extramitochondrial calcium. Studies with the fluorescent indicator Quin-2 indicated that exposure to PGE-1 and PGE-2 caused a rapid and sustained rise in intracellular calcium from a resting level of 140 nM to around 280 nM within 5 min. Furthermore, this effect was specific in that exposure to a similar concentration of PGF-2 α did not influence $[Ca^{2+}]_i$, in keeping with the inability of this compound to influence the penetrating capacity of human spermatozoa *in vitro* (Aitken & Kelly, 1985). The results obtained in this study also suggest that the source of this calcium is extracellular, since neither PGE-1 nor PGE-2 could enhance the performance of human spermatozoa in the hamster egg assay in the absence of high concentrations of calcium in the external medium. In contrast, the ability of PGE-1 and PGE-2 to elevate the intracellular concentrations of cAMP did not depend upon the presence of added calcium, indicating that the cAMP response is probably not a critical component of the mechanisms by which PGE influences the penetrating potential of human spermatozoa. This conclusion was reinforced by the observations made on the infertile patients, some of whom exhibited a clear rise in intracellular cAMP in response to PGE-1 but no corresponding increase in the penetrating capacity of the spermatozoa.

These findings strongly suggest that the mechanisms by which PGE-1 and PGE-2 influence the level of oocyte penetration involves their ability to act as ionophores, facilitating the transport of calcium from the external medium to the interior of the cell. Ionophore-like properties have previously been described for prostaglandins of the E-series (Kirtland & Baum, 1972) and recognized calcium ionophores such as A23187 are known to influence profoundly the performance of human spermatozoa in the zona-free hamster egg penetration test (Aitken *et al.*, 1984). Voltage-sensitive calcium channels do not appear to be involved since verapamil did not significantly depress the response to PGE-1. Independent studies of the influence of calcium channel blockers such as verapamil and diltiazem have, indeed, failed to provide any evidence for the presence of such channels in the plasma membrane of the human spermatozoon (S. Irvine & R. J. Aitken, unpublished observation).

Direct comparison of the levels of intracellular calcium achieved after exposure to PGE and A23187 indicated that the latter is a far more potent ionophore, increasing $[Ca^{2+}]_i$ to around 600 nM in contrast to the values 270–280 nM achieved with PGE-1 and PGE-2. This difference in relative efficiency may explain why maximal enhancement of fertilization rates is achieved after incubation with A23187 for 30 min (Aitken *et al.*, 1984) but requires at least 4 h of exposure to the PGEs.

The time-course studies also indicated that the prostaglandins did not appear to be able to initiate capacitation since exposure to PGE for the first hour of the 4 h capacitation period did not profoundly influence the penetration rates observed. This possibility was examined because human spermatozoa are known to begin their capacitation sequence as they pass through cervical mucus (Overstreet, Gould, Katz & Hanson, 1980) and the latter is richly endowed with prostaglandins (Charbonnel, Kremer, Gerozissis & Dray, 1982). Furthermore, the PGEs did not significantly influence penetration rates when incorporated into the medium for the last hour of the capacitation period, suggesting that they do not readily precipitate the acrosome reaction in capacitating spermatozoa. Longer periods of capacitation may have revealed an increased sensitivity to the stimulatory effects of PGE, as observed with the response of hamster spermatozoa to arachidonic acid for example (Meizel & Turner, 1983). However the hyperosmotic conditions used in this study are known to effect capacitation rapidly, producing penetration rates after 3 h equivalent to 7 h incubation in normo-osmotic medium (Aitken *et al.*, 1983c).

The hyperosmotic conditions employed during the capacitation period clearly enhanced the responses of human spermatozoa to the stimulatory effects of both PGE-1 and PGE-2. Hence, under normo-osmotic conditions only the 60 μ g dose of PGE-1 was able to induce a statistically significant elevation of penetration rates (Table 2). In the presence of hyperosmotic Medium BWB, however, every dose of PGE investigated produced a significant increase in the proportion of oocytes penetrated. In such circumstances the high osmolarity of the medium may enhance the ionophore-like action of the prostaglandins by facilitating the removal of decapacitation factors from the sperm surface, which may limit the permeability of the sperm plasma membrane to calcium.

In a therapeutic context these results clearly suggest that PGE-1 should be incorporated into hyperosmotic medium to achieve the maximal stimulation of sperm function. A group of infertile patients was therefore examined to assess the potential of such treatment for artificially stimulating the penetrating capacity of human spermatozoa. In general the results suggest that PGE-1 is an effective stimulatory agent particularly in cases of idiopathic infertility, in which the lesions in sperm function may be less severe (Aitken *et al.*, 1983b). The fact that just over 40% of the oligozoospermic patients responded with an increase in oocyte penetration rates after exposure to PGE-1 is also encouraging. If ionophores are to find clinical application in the treatment of defective sperm function, PGE-1 would certainly be the reagent of choice, for while it may be a less potent ionophore than A23187, it is a natural compound which does not exhibit the same degree of toxicity as the latter.

The selective ability of PGE to exert a direct effect on human sperm function suggests that this action cannot be due to non-specific perturbation of the sperm plasma membrane. The specific action of the E-series prostaglandins is noteworthy because the doses of PGE used in this study to increase penetration rates (60 μ g/ml or 170 μ M) or elevate cAMP (16.8 μ g/ml or 47 μ M) are several-fold higher than the 10 μ M dose of PGE normally used in biological experiments to achieve, for example, maximal suppression of macrophage function through the elevation of intracellular cAMP (O'Donnell, 1974; Schultz, Pavlidis, Stylos & Chirigos, 1978; Taffet, Pace & Russell, 1981). Nevertheless, the doses of prostaglandin employed in the present study are physiological in the sense that seminal plasma from normal fertile men may contain 2–272 μ g PGE/ml and 53–1094 μ g 19-hydroxy PGE/ml (Templeton, Cooper & Kelly, 1978) respectively.

However, whether the enhanced penetration rates observed in the presence of PGE are of biological importance, in reflecting the physiological mechanisms by which sperm function is

controlled, is a separate question. The high doses of PGE found in human semen are clearly irrelevant in this respect because human spermatozoa cannot begin to capacitate until they have lost their association with seminal plasma. The latter is an unsuitable vehicle for PGs to influence sperm capacitation because it contains low levels of free ionic calcium (a mean $\pm$ s.e.m. of 0.123 ± 0.006 mM, $n=40$; S. Irvine & R. J. Aitken, unpublished observations) and, as yet, uncharacterized decapacitation factors (Kanwar, Yanagimachi & Lopata, 1979). It is doubtful whether microgram quantities of PGE would be encountered by human spermatozoa during their ascent of the female reproductive tract, except possibly, in the local environment of the cumulus mass, where the acrosome reaction is thought to be initiated. An alternative possibility is that prostaglandins are actually synthesized by the spermatozoa themselves to facilitate the calcium influx associated with the terminal stages of the capacitation sequence (Triana, Babcock, Lorton, First & Lardy, 1980). Activation of phospholipase A_2 and arachidonic acid release are thought to be important components of sperm capacitation in the hamster and guinea-pig (Llanos, Lui & Meizel, 1982; Ono, Yanagimachi & Huang, 1982; Fleming & Yanagimachi, 1984; Meizel & Turner, 1984), while evidence has been obtained for a calmodulin-regulated phospholipase A_2 in the plasma membrane of human spermatozoa (Thakkar, East & Franson, 1984).

The biological significance of the high doses of PGE present in human seminal plasma may reside in their ability to stimulate sperm motility (Aitken & Kelly, 1985), through an effect on the intracellular concentration of cyclic AMP. Both PGE-1 and -2 were able to elevate the intracellular concentrations of cAMP in human spermatozoa, at doses equivalent to the concentrations normally observed in human seminal fluid. Since this increase in cAMP values was observed in the presence in phosphodiesterase inhibitors, the mechanism through which the PGEs achieve their effect might involve the receptor-mediated activation of adenylate cyclase. Such a mechanism would be consistent with the well-documented influence of PGE on adenylate cyclase activity in other cell types although evidence for prostaglandin receptors on the surface of human spermatozoa is currently controversial (Schlegel, Rotermund, Farber & Nieschlag, 1981). Such a mechanism would also not be consistent with the apparent absence of an N regulatory subunit in the adenylate cyclase complex of human spermatozoa (Cheng & Boettcher, 1982) or the inability of prostaglandins to activate the adenylate cyclase of primate spermatozoa (Casillas & Hoskins, 1971). However, most studies with human or primate spermatozoa are carried out on ejaculated cells which have already been exposed to the high concentration of prostaglandins present in seminal plasma. Therefore, desensitization of the adenylate cyclase complex may have occurred, leading to a loss and a reduction in the affinity (Kenimer, 1982) of prostaglandin receptors as well as an inactivation or loss of the regulatory subunit (Garrity, Andreassen, Storm & Robertson, 1983). Desensitization of the adenylate cyclase complex by seminal prostaglandins might explain why such high doses of PGE were required to increase cyclic AMP levels in this study and why such difficulty has been experienced in demonstrating the existence of prostaglandin receptors or a cAMP response in the past.

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