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Hyaluronic acid enhances induction of the acrosome reaction of human sperm through interaction with the PH-20 protein

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Summary

When capacitated human sperm were treated with hyaluronic acid (HA) for 30 min prior to the addition of progesterone or solubilised human zonae pellucidae, there was a significant increase in the percentage of acrosome reactions. Progesterone treatment alone increased acrosome reactions from 10.5% to 21.8% and pretreatment with 100 µg / ml HA resulted in 33.0% acrosome reactions. With zonae pellucidae treatment alone the increase was from 9.0% to 23.5% and with HA pretreatment it was 48.8%. HA treatment alone had no direct effect on acrosome reactions, and the enhancing effect of HA was not removed when sperm were washed prior to the addition of either acrosome reaction agonist. Experiments with sperm 5 min after HA treatment demonstrated that enhancement of acrosome reactions was apparent as early as 1 min after addition of zonae and within 5 min after addition of progesterone. When sperm were pretreated with Fab fragments of anti-PH-20 IgG, then with HA and then with progesterone or zonae pellucidae, there was no enhancement of the acrosome reaction. Fab treatment did not induce acrosome reactions and did not interfere with the action of either agonist in the absence of HA. Sperm that were treated with HA had significantly higher intracellular calcium levels, and pretreatment with Fab reduced this increase to 42.7%. Addition of progesterone to HA-treated sperm was followed by another large increase in intracellular calcium, which was lower when sperm were pretreated with Fab. These results suggest that HA interacts with the PH-20 protein to increase basal levels of intracellular calcium and thereby potentiates the acrosome reaction. The data support the hypothesis that HA in the cumulus matrix may act to prime the fertilising sperm for induction of the acrosome reaction by constituents of the cumulus and / or zona pellucida.

Keywords: Acrosome reaction, hyaluronic acid, Progesterone, Sperm, Zona pellucida

Introduction

Prior to ovulation, the cumulus cell-oocyte complex synthesises and organises an extensive extracellular matrix that is enriched in hyaluronic acid (HA). Hyaluronic acid is a glycosaminoglycan (GAG) and consists of a large polymer of alternating *N*-acetyl glucosamine and glucuronic acid residues. In addition to being a major constituent of the cumulus matrix, HA is found in the follicular fluid as well as in the zona pellucida and perivitelline space of the oocyte (Eppig, 1979; Grimek, 1984; Laurent *et al.*, 1992). In other cell types, HA has been shown to function in the regulation of cell–cell interactions as well as in the stimulation of cell motility, which may involve interactions between HA and the cell surface (Turley, 1991). Addition of HA to human sperm suspensions *in vitro* appears to improve retention of sperm motility and velocity (Huszar *et al.*, 1990; Mortimer *et al.*, 1990). HA at concentrations as low as 10 µg / ml was shown to induce the acrosome reaction of hamster sperm (Meizel & Turner, 1986). Handrow *et al.* (1982) also showed that GAGs with various degrees of sulphation induce the

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acrosome reaction of bovine sperm. Recent studies in this laboratory have shown that HA enhances the zona-induced acrosome reaction in cynomolgus macaque sperm (VandeVoort *et al.*, 1997).

The HA binding proteins constitute a family of macromolecules that serve as structural components of extracellular matrices or as receptors that bind to the surface of the cells. They have been grouped together to form a large family known as hyaladherins (Toole, 1990). Antibodies against purified HA binding proteins have been used to identify surface components in human sperm (Kornosky *et al.*, 1994; Ranganathan *et al.*, 1994), as well as in sperm of rats, mice and bovines (Ranganathan *et al.*, 1994). Immunofluorescent techniques have been used to demonstrate the presence of these putative HA receptors on the sperm head, midpiece and tail, and it has been suggested that these receptors are involved in the complex mechanisms that control sperm motility (Kornosky *et al.*, 1994; Ranganathan *et al.*, 1994).

The PH-20 protein is another component of the sperm cell that may act as an HA binding protein. PH-20 is a glycosylphosphatidylinositol (GPI) anchored membrane protein (Phelps *et al.*, 1988; Gmachl *et al.*, 1993) that has been localised to the sperm head in several species, including guinea pigs (Primakoff *et al.*, 1985), macaques (Overstreet *et al.*, 1995) and humans (Sabeur *et al.*, 1997). Ultrastructural studies of human sperm (Sabeur *et al.*, 1997) and macaque sperm (Overstreet *et al.*, 1995) have shown that PH-20 is present on the plasma membrane and on the inner acrosomal membrane of the sperm head. PH-20 has been shown to have hyaluronidase activity (Gmachl *et al.*, 1993; Lin *et al.*, 1994; Thaler & Cardullo, 1995) and it appears to be the only hyaluronidase in macque sperm (Cherr *et al.*, 1996; Li *et al.*, 1997*a*), human sperm (Sabeur *et al.*, 1997) and guinea pig sperm (Hunnicutt *et al.*, 1996). Plasma membrane PH-20 may facilitate passage of sperm through the cumulus by localised depolymerisation of the extracellular matrix (Cherr *et al.*, 1996; Li *et al.*, 1997*a*, Meyers *et al.*, 1997) and also may function non-enzymatically as a zona-binding molecule (Li *et al.*, 1997*b*).

The widely accepted model for mammalian sperm–oocyte interaction proposes that the fertilising sperm binds to the zona pellucida with an intact acrosome and that this interaction results in a cascade of events that culminate in the acrosome reaction, as reviewed by Yanagimachi (1994). The possibility has been discussed that other constituents of the oocyte–cumulus complex also participate in the signalling which leads to induction of the acrosome reaction as reviewed by Meizel (1985). These molecules include the components of follicular fluid such as progesterone (Osman *et al.*, 1989; Meizel *et al.*, 1990). Recent observations in this laboratory suggest that HA can dramatically enhance the zona-induced acrosome reaction of macaque sperm and that this enhancement is not due to effects on capacitation (VandeVoort *et al.*, 1997). Human sperm, like macaque sperm, appear to undergo the acrosome reaction as a consequence of binding to the zona pellucida (Cross *et al.*, 1988). HA has not been shown to have any direct effect on the human sperm acrosome reaction, but it is not known whether HA has any synergistic or priming action in concert with other acrosome reaction agonists. In the experiments that we report in this communication, we investigated the synergistic effect of HA on the human sperm acrosome reaction as induced by progesterone or by solubilised human zonae pellucidae. In addition, we investigated the possible function of PH-20 in mediating these effects.

Materials and methods

All chemicals used were reagent grade. Bovine serum albumin (BSA), human serum albumin (HSA), polyvinyl alcohol (PVA) and Percoll were purchased from Sigma (St Louis, MO). HA was purchased from Calbiochem (La Jolla, CA). This HA preparation was obtained from human umbilical cord and was provided as the potassium salt. The preparation contained < 3.0% chondroitin sulphate and 0.69% protein. The relative viscosity was > 40. Fab fragments were prepared from the IgC fraction of antisera raised in rabbits against recombinant macaque PH-20 (Lin *et al.*, 1994) and were purified according to a method described by Cherr *et al.* (1996).

Sperm preparation and treatments

Sperm washing and capacitation

Human semen was obtained by masturbation from five healthy donors. Liquefied semen samples were centrifuged at 300 *g* for 20 min on a two-step Percoll gradient (80%/40%). This procedure yields a population of > 95% motile sperm. The medium used for the Percoll gradient and for the subsequent washing step was a modified Biggers Whitten & Whittingham (BWW) medium (Overstreet *et al.*, 1980) containing 2 mM glucose, 0.25 mM pyruvate, 19 mM lactate, 3 mg/ml BSA, 750 units/ml streptomycin sulphate and 1670 units/ml penicillin G. The sperm pellets from the Percoll gradient were resuspended and diluted to a concentration of 6×10^6 sperm/ml in the medium described above except with 35 mg/ml HSA. Resuspended sperm were capacitated by incubating 500 µl aliquots in 15 ml polystyrene conical centrifuge tubes (Fisher Scientific, Fair Lawn, NJ) at 39 °C for 6 h in a humid atmosphere of 5% CO_2/95% air (pH 7.4–7.6).

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Induction of acrosome reactions with progesterone
After 6 h of capacitation, sperm were washed by centrifugation and resuspension in BWW with 3 mg/ml HSA to a final concentration of 8×10^6/ml. Acrosome reactions were induced by addition of progesterone (final concentration 3.18 µM, 0.1% DMSO for progesterone and solvent control). After 5 min incubation at 39 °C, the sperm were fixed in 4% formaldehyde.

Induction of acrosome reactions with solubilised zonae pellucidae
Human ovarian oocytes were isolated and stored as described by Cross *et al.* (1988). Zonae pellucidae were isolated mechanically by passing the oocyte through a small-bore pipette and then the zonae were disaggregated by treatment with 5 mM NaH_2PO_4, pH 2.5 (Bleil & Wassarman, 1980; Cross *et al.*, 1988). Zonae were first rinsed free of medium by passage through two droplets of 5 mM NaH_2PO_4, pH 2.5, and then placed under oil in 1 µl of the same solution. After 30 min, 1 µl of double-strength medium (medium containing all components at twice the concentration of HSA-BWW, pH 7.4) was added to each droplet followed 5 min later by 1 µl of sperm suspension (50×10^6/ml). Sperm were capacitated as above at 39 °C. The final concentration of zonae in the sperm suspension was 2 zonae/µl. After 1 h of incubation at 37 °C, the sperm were fixed by transferring the droplet into 4% formaldehyde.

Assessment of sperm motility and acrosome reactions
The percentage of acrosome reactions was determined by the method of Cross *et al.* (1986) as modified by Overstreet *et al.* (1995). Sperm were fixed in 4% formaldehyde and in ethanol and the percentage of acrosome-reacted sperm was determined using the filter method as described by Morales *et al.* (1989) and using fluorescein-isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA; Vector Laboratories, Burlingame, CA) (100 µg/ml). Two hundred sperm were counted in each sample to determine the percentage of sperm that were acrosome-reacted according to the criteria of Cross *et al.* (1986). A small aliquot of the sperm suspension (5 µl) was used for determination of the percentage of motile sperm (Thomas & Meizel, 1988).

Treatment of sperm with HA
To test for an effect of HA on the acrosome reaction, capacitated sperm were washed in BWW, resuspended in BWW with 3 mg/ml HSA and treated for 30 min with HA at concentrations of 1, 10 and 100 µg/ml prior to the addition of progesterone. A similar experiment was carried out utilising a 30 min preincubation of capacitated sperm in 100 µg/ml HA prior to incubation with solubilised zonae pellucidae. To test for the reversibility of this effect, sperm that had been incubated for 30 min in 100 µg/ml HA were washed by centrifugation and dilution with BWW before the addition of progesterone or solubilised zonae pellucidae. To determine the time course of the acrosome reaction after treatment with acrosome reaction agonists in the presence of HA, capacitated sperm were preincubated with HA (100 µg/ml) for 5 min before the addition of progesterone or solubilised zonae pellucidae. At 1, 5, 15 and 60 min after the addition of the agonists the sperm were evaluated for motility and fixed for evaluation of acrosome reactions.

Treatment of sperm with anti-PH-20 fragments
To discover whether PH-20 is involved in the effect of HA on the acrosome reaction, capacitated sperm were treated with 100 µg/ml of anti-PH-20 Fab fragments for 10 min and then for 5 min with HA at concentrations of 1, 10 and 100 µg/ml prior to the addition of progesterone. A similar experiment was carried out utilising a 10 min preincubation of capacitated sperm in 100 µg/ml of anti-PH-20 Fab fragments and then 5 min incubation in 100 µg/ml HA prior to incubation with solubilised zonae pellucidae. Sperm were assessed for motility and fixed for evaluation of acrosome reactions at 5 min and 60 min following treatment with progesterone and solubilised zonae, respectively.

Measurements of intracellular calcium concentrations
Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was assayed according to the procedures of Thomas & Meizel (1988) as modified by Meizel & Turner (1993). Briefly, sperm were capacitated in 500 µl aliquots of 6×10^6/ml for 6 h at 39 °C in a humidified 95% air/5% CO_2 atmosphere in BWW medium containing 35 mg/ml HSA. Aliquots of capacitated sperm were pooled (2 ml/tube). Fura-2/Am was then added to the capacitated sperm suspension at a concentration of 1 µM and the sperm incubated for an additional 30 min at 37 °C. Each 2 ml sample was then centrifuged through 40% Percoll at 300 *g* for 20 min, then washed and resuspended in a Hepes-buffered medium containing 10 mM HCO_3^-, and equilibrated in a fluorometric cuvette at 37 °C. Before Ca^{2+} measurements were made, the percentage of motile sperm was determined and the quality of motility rated using subjective estimates (Thomas & Meizel, 1988).

Fluorescence caused by the binding of Ca^{2+} to fura-2 was monitored using a Hitachi F-2000 spectrofluorometer with excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm (Meizel & Turner, 1993). Excitation and emission bandpass widths were each 10 nm. The data were corrected for autofluorescence (Blackmore *et al.*, 1990). $[Ca^{2+}]_i$ was computed as previously described (Meizel & Turner, 1993) using a K_d of 285 nM for Fura-2 at 37 °C.

Immediately following $[Ca^{2+}]_i$ assay, aliquots

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(200 µl) of the remaining fura-2 loaded sperm were used for studies of the acrosome reaction. Anti-PH-20 Fab (100 µg / ml, final concentration) was added to the suspension for 10 min followed by addition of HA (100 µg / ml, final concentration) for 5 min at 37 °C prior to the addition of progesterone as described above. Control suspensions with Fab alone were incubated for 15 min and with HA alone for 5 min prior to addition of progesterone. Cells were fixed and assessed for acrosome reactions as described above.

Statistical analysis

Data were analysed using ANOVA and differences among means were tested by Tukey's test (Snedecor & Cochran, 1969). Values of $p < 0.05$ were considered significant. Results are expressed as mean ± standard error of the mean (SEM).

Results

Enhancing effect of HA on the progesterone-induced acrosome reaction

There was a dose-dependent increase in the percentage of acrosome reactions when capacitated sperm were treated with HA for 30 min prior to the addition of progesterone (Fig. 1). Progesterone treatment alone increased the percentage of acrosome reactions from 10.5% (solvent control) to 21.8%, and pretreatment of sperm with 100 µg / ml HA resulted in 33.0% acrosome reactions. However, treatment with HA alone had no effect on acrosome reactions (Fig. 1). There were no significant differences between the percentages of motile sperm in any of the treatment groups (Fig. 1). The enhancing effect of HA on the induction of acrosome reactions was not removed when sperm were washed following incubation with 100 µg / ml HA and prior to addition of progesterone (35.66 ± 1.20% acrosome reactions in washed sperm versus 36.00 ± 3.51% in unwashed sperm, $n = 3$).

Involvement of PH-20 in the enhancing effect of HA on the progesterone-induced acrosome reaction

In preliminary studies with immunofluorescence microscopy, Fab fragments of anti-PH-20 IgG were shown to bind to the human sperm head, including the acrosomal region (data not shown). When sperm were pretreated with Fab fragments of anti-PH-20 IgG, then with HA and then with progesterone, Fab prevented the HA enhancement of the progesterone-induced acrosome reaction (Table 1). This effect of Fab was shown at all concentrations of HA that were tested (Table 1). Treatment with Fab alone had no significant

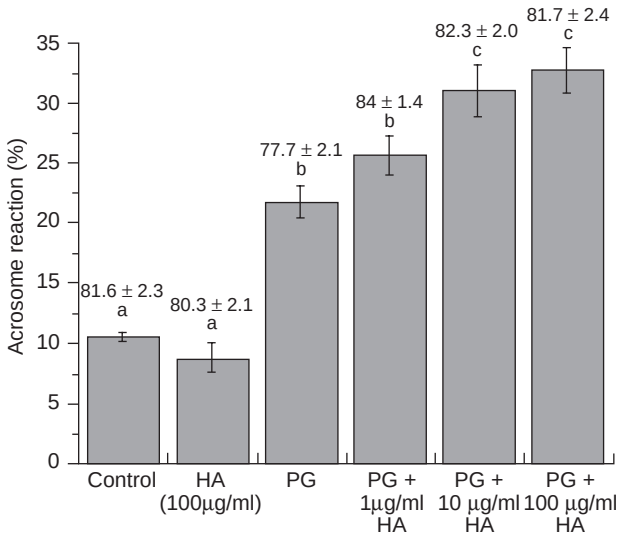

Figure 1 Effect of different concentrations on hyaluronic acid (HA) on the progesterone-induced acrosome reaction ($n = 4$). Capacitated sperm were treated with HA for 30 min before the addition of progesterone (PG) or solvent control and were fixed 5 min later for the FITC-PSA acrosome reaction assay. Data are presented as mean values ± SEM ($n = 4$). Bars with different letters are significantly different from one another ($p < 0.05$). The percentage of motile sperm in each treatment group (mean ± SEM, $n = 3$) is given above each bar. There was no significant difference in percentage motility between treatments in any one experiment.

effect on the acrosome reaction of capacitated sperm and Fab treatment did not interfere with the progesterone-induced acrosome reaction in the absence of HA (Table 1). There were no significant differences between the percentages of motile sperm in any of the treatment groups (Table 1).

Enhancing effect of HA on the zona pellucida-induced acrosome reaction

In experiments of similar design, sperm were pretreated with 100 µg / ml HA before induction of acrosome reactions with solubilised human zonae pellucidae. Because there was a limited supply of human zonae, HA was tested at the concentration that resulted in the greatest enhancement of progesterone-induced acrosome reactions. There was a significant increase in acrosome reactions when sperm were preincubated with HA and then treated with solubilised zonae in comparison with treatment with zonae alone (Table 2). As in the progesterone experiments, sperm washing after treatment with HA did not diminish the enhancing effect of HA on the zona-induced acrosome reaction (43% acrosome reactions in washed sperm versus 47% in unwashed sperm, $n = 2$). When sperm were pretreated with Fab fragments of anti-PH-20 IgG, then with HA and then with solubilised zonae pellucidae,

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Table 1 Inhibitory effects of anti-PH-20 IgG Fab fragments (FAB) on the hyaluronic acid (HA) enhancement of progesterone-induced acrosome reaction in capacitated human sperm

Treatment				
Progesterone: +/- (3.18 µM)	HA: conc./- (µg/ml)	FAB: +/- (100 µg/ml)	% acrosome reaction	% motility
–	–	–	10.50 ± 0.50[a]*	81.67 ± 1.67[a]
–	–	+	13.00 ± 0.57[a]	76.67 ± 1.67[a]
+	–	–	22.50 ± 1.25[b]	77.67 ± 1.45[a]
+	–	+	22.50 ± 1.32[b,c]	80.00 ± 2.89[a]
+	1.0	–	25.75 ± 1.60[c]	80.33 ± 1.45[a]
+	10.0	–	31.25 ± 2.25[d]	84.00 ± 1.00[a]
+	100.0	–	35.00 ± 3.50[d]	82.33 ± 1.45[a]
+	1.0	+	20.00 ± 0.57[b]	76.67 ± 1.67[a]
+	10.0	+	21.75 ± 1.43[b,c]	81.33 ± 1.33[a]
+	100.0	+	22.00 ± 0.82[b,c]	82.67 ± 1.45[a]

Capacitated sperm were treated with FAB (100 µg/ml) for 10 min then HA for 5 min before the addition of progesterone or solvent control and fixed 5 min later for the FITC-PSA acrosome reaction assay.
Data are presented as mean values ± SEM (n = 4 for acrosome reactions and n = 3 for motility).
* Values for the untreated solvent control are the same as reported in Fig. 1, because the experiments reported in Fig. 1 and Table 1 were performed using the same preparations of capacitated sperm.
Values in columns with different superscripts are significantly different from one another ($p < 0.05$).

Table 2 Effects of hyaluronic acid (HA) and anti-PH-20 Fab fragments (FAB) on the zona pellucida (ZP)-induced acrosome reaction in capacitated human sperm

Treatment				
Zonae pellucidae: +/– (2 ZP/µl)	HA: +/– (100 µg/ml)	FAB: +/– (100 µg/ml)	% acrosome reaction	% motility
–	–	–	9.00 ± 1.08[a]	73.75 ± 1.25[a]
–	–	+	12.50 ± 0.86[a]	68.75 ± 2.39[a]
–	+	–	10.87 ± 0.65[a]	76.25 ± 1.25[a]
+	–	–	23.50 ± 1.26[b]	74.50 ± 1.66[a]
+	+	–	48.75 ± 1.88[c]	73.75 ± 1.75[a]
+	+	+	27.00 ± 0.70[d]	71.25 ± 1.25[a]
+	–	+	19.00 ± 0.41[e]	70.00 ± 2.04[a]

Capacitated sperm were treated with FAB (100 µg/ml) for 10 min then HA for 5 min before the addition of ZP or solvent control and fixed 1 h later for the FITC-PSA acrosome reaction assay.
Data are presented as mean values ± SEM (n = 4 for acrosome reactions and for motility).
Values in columns with different superscripts are significantly different from one another ($p < 0.05$).

Fab also prevented the HA enhancement of the zona-induced acrosome reaction (Table 2). As in the progesterone experiments, treatment with Fab alone did not induce acrosome reactions.

Time course of the acrosome reaction after treatment with HA and progesterone or solubilised zonae pellucidae

When sperm were preincubated with HA for 5 min before the addition of solubilised zonae, enhancement of the acrosome reaction by HA was apparent as early as 1 min after the addition of zonae (23.6% for HA/ZP versus 16.6% for ZP alone; Fig. 2), and this enhancement was apparent at all time points of the experiment. In sperm suspensions treated with HA and then with zonae there was a progressive increase in the percentage of acrosome-reacted sperm over the 60 min time course of the experiment. In contrast, when sperm were treated with zonae alone, there was no additional increase in the percentage of sperm that were acrosome-reacted after the 5 min time point (Fig. 2).

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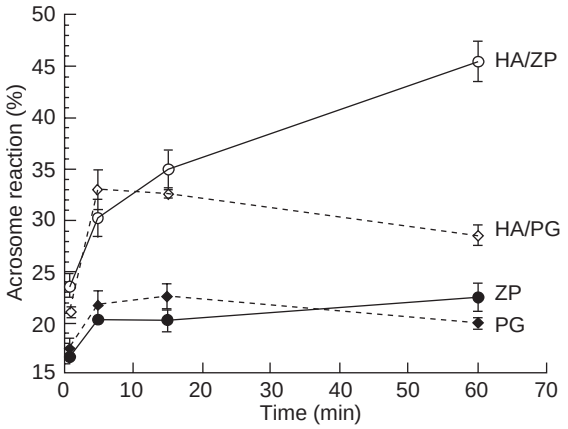

Figure 2 Effect of different times of incubation with hyaluronic acid (HA), progesterone (PG) or solubilised zonae pellucida (ZP) on the human sperm acrosome reaction (n = 3). Capacitated sperm were treated with HA for 5 min before the addition of progesterone, ZP or solvent control and were fixed 1 min, 5 min, 15 min or 60 min later for the FITC-PSA acrosome reaction assay. Data are presented as mean values ± SEM (n = 3). The differences between HA / ZP and HA treatments and between HA / PG and PG treatments are significant at all time points ($p < 0.05$). For sperm treated with HA / ZP there was a significant increase in acrosome reactions between 1 min and 5 min and between 5 min and 60 min ($p < 0.05$). For sperm treated with ZP, PG and HA / PG there was a significant increase in acrosome reactions between 1 min and 5 min ($p < 0.05$).

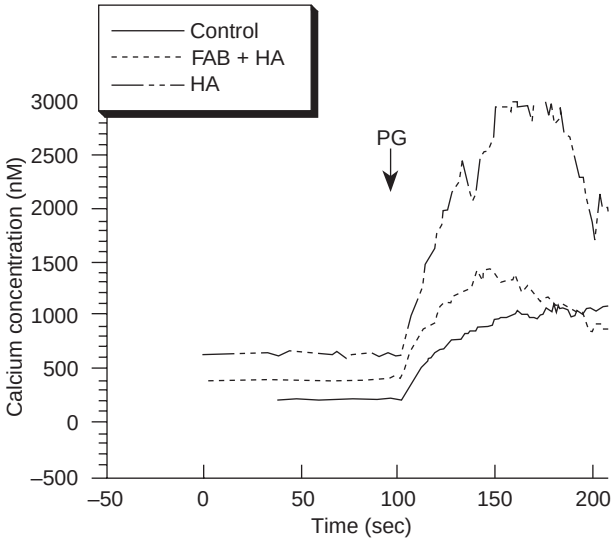

Figure 3 Effect of HA and the Fab fragment of anti-PH-20 IgG (FAB) on progesterone (PG)-mediated Ca^{2+} influx. Fura-2-loaded capacitated human sperm were preincubated for 10 min with FAB then 5 min with HA before the addition of PG. Traces are from one experiment representative of three similar separate experiments. Sperm motility, determined at the end of the experiments was 65–80% and was not different between treatments and control in any single experiment. Sperm concentration was 10×10^6 sperm / ml. Arrow indicates time of PG addition.

When sperm were pretreated with HA before the addition of progesterone, there was a significant increase in the percentage of acrosome reactions by 5 min after addition of progesterone in comparison with sperm that received no HA treatment (Fig. 2). The percentage of acrosome reactions did not further increase after 5 min following treatment with progesterone alone. The combination of HA and progesterone also resulted in no further increase in acrosome reactions after the 5 min time point. There were no differences in the percentage of motile sperm or in sperm progression at the 1 min to 15 min time points (range 74–82%), but at 60 min the percentage of motile sperm was significantly lower than at the earlier time points (range 57–60%).

Involvement of changes in intracellular calcium in the enhancing effect of HA on induction of the acrosome reaction

Capacitated sperm that were preincubated for 5 min with HA alone had significantly higher intracellular calcium levels than did untreated control sperm (599.4 ± 11.5 nM vs 257.3 ± 34.9 nM, $p < 0.05$, n = 3). When sperm were preincubated with Fab fragments of anti-PH-20 IgG for 10 min before the addition of HA, intracellular calcium levels (343.5 ± 59.7 nM) were reduced by 42.7% in comparison with HA-treated sperm but not to the control levels. The addition of progesterone to HA-treated sperm was followed by a large increase in intracellular calcium, which was lower when sperm were pretreated with Fab fragments (Fig. 3). The percentages of induced acrosome reactions in these experiments were 23.6% ± 0.6% for the control, 32.3 ± 0.8% for HA-treated sperm, and 22 ± 0.6% for sperm treated with Fab and HA.

Discussion

The results of these experiments demonstrate that incubation with HA increased the percentage of capacitated human sperm that could be induced to undergo the acrosome reaction by challenge with either progesterone or solubilised human zonae pellucidae. This appears to be a priming or enhancing effect rather than an additive effect, because HA alone was not capable of inducing acrosome reactions in capacitated human sperm.

In these experiments, HA had a similar enhancing effect on the acrosome reaction, regardless of whether progesterone or zona pellucida was used to trigger the event. This observation is particularly important, because these two agonists may act on sperm cells through different mechanisms. The zona-induced

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acrosome reaction is believed to be mediated by specific sperm proteins that are sometimes referred to as sperm receptors for zona and which may be involved in primary sperm binding to specific components of the zona pellucida. In mouse sperm, these proteins include the 56 kDa plasma membrane protein (Bleil & Wassarman, 1990), the 95 kDa protein with intrinsic tyrosine kinase activity termed ZRK (Leyton & Saling, 1989) and a receptor that may be related to the Gi protein class (Ward *et al.*, 1992). On the other hand, progesterone has been shown to interact with other components of human sperm, including a γ-aminobutyric acid type A (GABA)-like receptor (Wistrom & Meizel, 1993), a 50–52 kDa plasma membrane protein (Sabeur *et al.*, 1996) and a receptor with tyrosine kinase activity (Tesarik *et al.*, 1993*a*). The different sensitivities of the two agonists to pertussis toxin suggests that a G protein could be involved in the acrosome reaction induction by zona, but not by progesterone (Tesarik *et al.*, 1993*b*; Murase & Roldan, 1996). In mouse sperm, the generation of diacylglycerol (DAG) and subsequent acrosomal exocytosis that are stimulated by zona pellucida involve activation of both G proteins and protein tyrosine kinase, whereas progesterone stimulation of DAG and exocytosis involves activation of tyrosine kinase but not G protein (Murase & Roldan, 1996).

The present study demonstrated that HA treatment of human sperm resulted in a threefold increase in basal calcium levels as compared with the control. The higher levels of calcium in the presence of HA are consistent with the results of other studies with human sperm, which showed an increase in calcium influx after treatment with HA (Slotte *et al.*, 1993). There is also evidence from experiments with other types of cell that HA induces calcium mobilisation (Bourguignon *et al.*, 1993; Galluzo *et al.*, 1995), and this cellular response is consistent with the requirement for cytosolic calcium in the mechanisms that mediate cell adhesion (Bourguignon *et al.*, 1993). It is known that an extracellular calcium-dependent increase in intracellular calcium is required for the initiation of the acrosome reaction by physiological agonists including progesterone and zona pellucida (Thomas & Meizel, 1988, 1989; Florman *et al.*, 1989). The enhancing effect of HA on the agonist-induced acrosome reaction may involve the increase in basal calcium levels as well as this initial influx of calcium at the initiation of the acrosome reaction.

In the present study, we used the Fab fragment of anti-PH-20 IgG to partially block the enhancing effect of HA on both the progesterone-induced and the zona-induced acrosome reaction, suggesting that interactions between HA and PH-20 are involved in a common mechanism of action. This interpretation is consistent with the observation that these Fab fragments also inhibited the HA-induced calcium increase in sperm. The fact that Fab treatment did not fully inhibit the calcium increase can probably be explained by the interaction of HA with other binding proteins that regulate calcium, particularly those in the midpiece and flagellum that may influence motility (Kornosky *et al.*, 1994; Ranganathan *et al.*, 1994). Pretreatment with HA also caused a threefold increase in sperm calcium during the progesterone-initiated acrosome reaction in comparison with sperm that were treated with progesterone alone (Fig. 3). This increase was inhibited by 50% when HA-treated sperm were first exposed to anti-PH-20 Fab fragments.

PH-20 is a GPI-anchored sperm plasma membrane protein (Phelps *et al.*, 1988; Gmachl *et al.*, 1993) that has been characterised in sperm of several species including humans (Sabeur *et al.*, 1977). We hypothesise that when plasma membrane PH-20 interacts with HA, rapid aggregation of PH-20 protein occurs on the sperm surface, resulting in signal transduction and increased intracellular calcium. Such a function of PH-20 would presumably be due to HA binding activity rather than hydrolytic activity; and PH-20 is known to possess a putative HA binding domain (Gaseca *et al.*, 1994). Such a mechanism of action is consistent with reports that high molecular weight HA can crosslink cell surface receptors and induce signal transduction in other cell types (Tamoto *et al.*, 1993), and that aggregation and crosslinking of GPI-anchored proteins in other cell types can result in increased intracellular calcium and tyrosine phosphorylation (Morgan *et al.*, 1993; reviewed by Brown, 1993). The possibility that HA-induced aggregation of PH-20 is involved in potentiating human sperm acrosome reactions is further supported by our studies of macaque sperm. In the monkey model, HA also enhances the zona-pellucida-induced acrosome reaction (VandeVoort *et al.*, 1997). Immunoaggregation of macaque sperm PH-20 by anti-PH-20 IgG results in intracellular calcium increases and acrosomal swelling (Yudin *et al.*, 1998). This calcium increase also can be blocked by pretreatment with Fab fragments of anti-PH-20 IgG (Yudin *et al.*, 1998). In the present study, we used the same Fab fragments to block the interaction of HA with human sperm PH-20. Although these Fab fragments were obtained from an antibody raised against recombinant macaque PH-20, the antibody cross-reacts with the plasma membrane PH-20 of human spermatozoa (Sabeur *et al.*, 1997).

In conclusion, the present study has demonstrated a possible function for HA in potentiating the induction of the human sperm acrosome reaction. Our results suggest that HA interacts with the PH-20 protein to induce this change in sperm function. The enhancing effect of HA on the acrosome reaction involves an increase in basal levels of intracellular calcium and may also involve an increase in the rate of calcium mobilisation at the initiation of the acrosome reaction.

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It appears that HA potentiates the acrosome reaction by the same mechanism regardless of whether the acrosome reaction agonist is progesterone or zona pellucida. These findings support the hypothesis that under physiological conditions, HA in the cumulus matrix may act to prime the fertilising sperm for induction of the acrosome reaction by constituents of the cumulus and / or the zona pellucida.

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