G-Protein Regulation of the Solubilized Human Zona Pellucida-Mediated Acrosome Reaction and Zona Pellucida Binding

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Purpose: *The study aimed to evaluate the (i) regulatory role of G_i-like protein during the acrosome reaction (AR) of normal sperm donors and (ii) the role of intact acrosomes during sperm–zona binding.*
Methods: *The acrosomal exocytosis of spermatozoa incubated with solubilized zona pellucida (ZP) at a final concentration of 1 ZP/μl was compared with 10 μM calcium ionophore A23187 and 30% (v/v) pooled human follicular fluid (HFF). Spermatozoa were incubated with 1, 10, and 100 ng/ml pertussis toxin (PT) during capacitation to functionally inactivate the G_i-like protein. The sperm–zona binding potential of 100 ng/ml PT-treated spermatozoa followed, by exposure to 1 ZP/μl, revealed significantly higher zona-bound spermatozoa compared to controls treated with 1 ZP/μl only.*
Results: *PT treatment of spermatozoa did not affect sperm motility, however, inhibited the percentage AR induced by solubilized ZP. In contrast, the A23187- and HFF-induced ARs were not sensitive to PT treatment. PT inhibition of the ZP-induced AR occurred in a concentration-dependent manner, with maximal effects observed at 100 ng/ml PT.*
Conclusions: *In conclusion, it seems that PT-sensitive G_i-like protein in human spermatozoa plays an important regulatory role in the AR induced by the human ZP, and this underlines the importance of intact acrosomes during sperm–zona binding.*

KEY WORDS: acrosome reaction; G proteins; zona pellucida.

INTRODUCTION

The astounding success rates achieved by intracellular sperm injection (ICSI) (1) emphasized the need to refine sperm functional evaluation. This is particularly true in cases of profound male-factor infertility, and therefore, contemporary andrology laboratories should be able to select the most appropriate form of treatment for each couple, especially those diagnosed with male-factor infertility (2).

Precise timing of acrosomal response was the rationale for the development of the acrosome reaction ionophore challenge test (ARIC test) (3,4). The ARIC test as well as the concept of acrosomal inducibility (5) is a reliable predictive tool of sperm fertilizing ability compared with tests that simply measure the frequency of the spontaneous acrosome reaction (AR). The inducibility of the AR, i.e., the difference between spontaneous and percentage acrosome-reacted spermatozoa after induction, correlates significantly with fertilization rates (5). The acrosome inducing activity of the zona pellucida (ZP) in both the intact and the solubilized state, has been illustrated to be powerful (6–8).

The ZP, and specifically glycoprotein 3 (ZP3), is thought to be the primary zona protein involved in the initial sperm–egg recognition and mediation of the AR. A sperm-associated G protein of the G_i type mediates the ZP3-induced AR in mouse spermatozoa (9) and the ZP-induced AR in bovine spermatozoa (10). This particular class of G proteins consists of substrates for pertussin toxin (PT)-catalyzed ADP-ribosylation and which are functionally inactivated by such a covalent modification. G proteins play important intermediary roles as signal transducing elements in coupling many ligand–receptor interactions to intracellular secondary messenger cascades/ionic changes (11), and all mammalian spermatozoa studied thus far, including the human, contain G_i-like proteins (12).

Little is known about ZP-mediated sperm signal transduction in the human, due to, for the most part, an inability to obtain sufficient quantities of human ZP for experimental purposes. The human ZP has been

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shown to bind human spermatozoa and to induce the AR of spermatozoa (13, 14). The present study aimed (i) to determine the optimal exposure time of varying PTs, (ii) to compare PT-exposed spermatozoa's AR results, after induction with A23187, human follicular fluid (HFF), and solubilized human ZP in parallel experiments, and (iii) to evaluate the zona binding capacity of PT-treated sperm samples that were exposed to 1 ZP/μl solubilized ZP.

MATERIALS AND METHODS

Preparation of Sperm Samples

Semen samples were obtained by masturbation after 2–3 days of sexual abstinence from normozoospermic fertile donors. Semen samples were analyzed according to the World Health Organization criteria (15) together with strict sperm morphology assessment (16). Motile sperm fractions were collected from samples using a slightly modified double-wash swim-up technique. Retrieved sperm samples were resuspended in human tubal fluid medium (HTF) (17) supplemented with 3% bovine serum albumin (BSA; Seravac, Cape Town, South Africa) to a sperm concentration of 10 $\times$ 10^6 cells/ml. Before the onset of AR studies, sperm samples were allowed to capacitate at 37°C in 5% CO_2 for 3 hr in HTF-BSA. Prepared sperm samples were exposed to varying concentrations (1, 10, and 100 ng/ml) of PT (P-9452; Sigma Chemical Co, St. Louis, MO) for 15, 30, and 60 min.

Preparation of Solubilized ZP

Oocytes were retrieved from postmortem-derived ovarian material. Great care was taken to ensure that all legal, ethical, and moral guidelines were adhered to at all times during oocyte collection. Oocytes were stored in a dimethylsulfoxide/sucrose solution at −196°C in liquid nitrogen (18). Twenty-four hours prior to each test, oocytes were removed from storage and thawed at 37°C. Retrieved oocytes were placed in 0.25 *M* sucrose and 3% BSA in HTF. On the day of the experiment, 50 oocytes were placed in a plastic Eppendorf tube containing 3% BSA in HTF and centrifuged for 15 min at 1800*g*, after which the HTF was removed under microscopic vision (Olympus SZ40; Wirsam Scientific, Cape Town, South Africa), leaving only the 50 oocytes at the bottom of the tube. A total volume of 5 μl of 10 m*M* HCl was then added to the oocytes in the tube; solubilization of the ZP was microscopically observed and controlled. Ooplasma of all oocytes were left at the bottom of the Eppendorf tube; test samples included ZP-free oocytes. Following solubilization, 5 μl of 10 m*M* NaOH was added to the solubilized ZP, to render a final zona volume of 10 μl, containing 5 ZP/μl. The final ZP concentration, after the addition of spermatozoa, was 1 ZP/μl.

Acrosome Reaction Studies

The procedure to determine the AR has been published in detail elsewhere (13, 14). AR status were determined for PT-treated spermatozoa incubated in 5% CO_2 at 37°C for 15, 30, and 60 min, respectively, with the following: (i) 1 ZP/μl (test), (ii) spontaneous (control), (iii) 10 μ*M* A23187 (C-7522; Sigma Chemical Co., control), and (iv) pooled HFF (30%, v/v) (control). Blood-free follicular fluid was aspirated from mature follicles of females attending the assisted reproductive programme. Spermatozoa from the different experiments described above were fixed and air-dried, after which the acrosomal status was determined using fluorescein-labeled *Pisum Sativum* agglutinin (FITC-PSA; 125 μg/ml; L-0770; Sigma Chemical Co.). A minimum of 200 spermatozoa was scored for each determination at the different time points.

Zona Pellucida Binding

Parallel with the acrosomal studies, spermatozoa (10 $\times$ 10^6 cells/ml; test) were pretreated with 100 ng/ml PT (60 min) before exposure to 1 ZP/μl for 60 min. Control spermatozoa were simultaneously incubated in synthetic HTF prior to ZP exposure. Both test and control sperm droplets (50 μl) were incubated under mineral oil for 30 minutes. Hemizonae were then added in a match-controlled fashion. Hemizona assays (HZA) were performed 10-fold and coincubation lasted for 4 hr. Following the coincubation period, hemizonae were removed and washed (5×) to strip the loosely attached spermatozoa from the hemizonae. Hemizonae were then evaluated, while the number of spermatozoa tightly bound to the ZP was recorded for each test and matching control hemizona.

Statistical Analyses

Sperm–zona binding results were expressed as the mean number of sperm bound to matching hemizonae which were used as tests and controls during the experiments. Hemizona assay results were compared using

a Student's paired *t* test. The percentage of acrosome-reacted sperm was comapred using Student's paired *t* test for control and test samples.

RESULTS

Pertussis Toxin and the AR

Acrosome reactions induced either spontaneously (observed in the absence of ZP) or nonspecifically (in the presence of A23187 and HFF), in contrast, were completely insensitive to PT treatment of the spermatozoa. Following exposure to 100 ng/ml PT for 15, 30, and 60 min, the mean percentage of acrosome-reacted spermatozoa remain unchanged, namely, 47, 51, and 50%, respectively. Similar results were observed in the presence of 30% (v:v) HFF, which mediated the AR among 30, 29, and 27% of the spermatozoa following PT pretreatment periods of 15, 30, and 60 min, respectively. The inhibitory effect of PT on the ZP-induced AR was dependent on the concentration and exposure time of PT. The maximum AR inhibition occurred after 60-min PT treatment, when only 14% spermatozoa were reported to be acrosome-reacted (Table I).

Sperm–Zona Binding

Since the acrosome plays an important role during the binding and penetration of the ZP, the zona-binding capacity of PT-treated sperm populations was recorded. PT-treated sperm populations exposed to 1 ZP/μl for 60 min bound significantly more spermatozoa (mean ± SD) to the ZP compared with the control spermatozoa that were incubated in a solution containing 1 ZP/μl only, namely, 134.1 ± 15 compared to 84.3 ± 19 ($P < 0.001$) (Table II). The PT-treated sperm population therefore bound significantly more spermatozoa compared with the sperm population (control) that were exposed to 1 ZP/μl only.

DISCUSSION

Sperm-associated G protein has been shown to be involved during induced acrosomal exocytosis of different species (19). The present results illustrate the possible regulatory effect of PT on the ZP-induced AR. Functional inactivation of G_i by PT inhibits downstream events leading to acrosomal exocytosis. Stimulation of spermatozoa with ZP depolarizes sperm membrane potential. The ZP and specific ZP3 stimula-

Table I. Influence of Pertussis Toxin Exposure on the Acrosome Reaction (Mean ± SD) Mediated by Zona Pellucida (ZP), Follicular Fluid (FF), and Calcium Ionophore

AR inducer	Percent acrosome-reacted spermatozoa in triplicate experiments (exposure time)			*P* value
	15 min	30 min	60 min	
Culture medium				g vs b; NS
	18 ± 2	19 ± 3	18[a] ± 3	g vs d; NS
				g vs f; NS
1 ZP/μl	18[b] ± 3	24 ± 5	30[i] ± 3	h vs i; 0.001
				g vs i; 0.001
		1 ng/ml pertussis toxin		
A23187	49 ± 6*	50 ± 4*	47 ± 4*	NS*
FF	31 ± 7	30 ± 7	28 ± 9	NS
ZP	30[a] ± 4	20 ± 7	18[b] ± 3	b vs a; 0.001
				b vs d; NS
				b vs f; NS
		10 ng/ml pertussis toxin		
A23187	51 ± 7*	48 ± 8*	49 ± 5*	NS*
FF	29 ± 7	32 ± 9	30 ± 4	NS
ZP	30[c] ± 4	19 ± 2	17[d] ± 1	c vs d; 0.001
				d vs f; NS
		100 ng/ml pertussis toxin		
A23187	47 ± 3*	51 ± 7*	50 ± 8*	NS*
FF	30 ± 4	29 ± 3	27 ± 7	NS
ZP	19[e] ± 3	17 ± 5	15[f] ± 3	e vs f; NS

Table II. Sperm–Zona Binding and Acrosome Reaction Results After Pertussis (PT) Toxin Treatment Followed by Exposure to Solubilized Zona Pellucida (ZP)*

Tests		Control	
Spermatozoa exposed to PT followed by treatment of 1 ZP/μl solubilized zona		Spermatozoa treated with 1 ZP/μL solubilized zona only	
% acrosome-reacted sperm (n = 3)	Mean (±SD) No. of zona-bound sperm (n = 10)	% Acrosome-reacted sperm (n = 3)	Mean (±SD) No. of zona-bound sperm (n = 10)
$15^a \pm 3$	$134.1^c \pm 15$	$30^b \pm 3$	$84.3^d \pm 19$

* a vs b, $P = 0.0001$; c vs d, $P = 0.001$.

tion therefore activates a depolarization mechanism with the characteristics of a poorly selective cation channel. Pretreatment of spermatozoa with PT prevents activation of the Ca^{2+}-selective channel by ZP3/ZP (20). The results can be interpreted to support the idea that the ZP-induced AR is the physiologically relevant exocytotic event since it is the ZP-induced AR, and not the spontaneous or A23187- and HFF-induced AR, which appears to be mediated through a G protein-mediated signal transduction process.

Human spermatozoa were capacitated in the presence of PT to determine whether functional inactivation of sperm G_i affected the ability of the treated cells to undergo the AR. PT treatment of spermatozoa inhibited the ability of the cells to undergo acrosomal exocytosis in the presence of solubilized ZP. Fertile donor spermatozoa that were first exposed to 1, 10, and 100 ng/ml PT concentrations for 15, 30, and 60 min, followed by a second incubation in 1 ZP/μl for 60 min, showed increasing inhibition of the AR. Sperm–zona binding studies with PT-treated, acrosome-intact spermatozoa, however, revealed significantly higher numbers of spermatozoa firmly bound to the zona under controlled HZA conditions. It is interesting to note that a difference of 15% in the acrosome-reacted sperm population following treatment with solubilized ZP caused a significant decrease in the zona-binding potential of the spermatozoa (Table II). Despite the presence of 70% (350,000 sperm/hemizona) acrosome-intact sperm in the sample, significantly fewer sperm were reported bound to the zona under hemizona assay conditions. The inhibition of sperm–ZP binding by previous incubation with solubilized ZP may also be due partly to the occupation of ZP-binding sites on still acrosome intact spermatozoa. PT may moderate this effect not only by inhibiting the AR, but also by delaying sperm capacitation, leading to a reduced availability of sperm plasma membrane binding sites for solubilized ZP. This possibility was tested by comparing ZP binding ability of PT-treated and spermatozoa with exposure to solubilize ZP during pilot studies. The mean number of PT-treated sperm versus untreated sperm that were zona bound after coincubation was 118 ± 12 and 124 ± 17, respectively.

The results highlight the importance of intact acrosomes during tight zona binding and underline the possible regulatory effect of PT on the ZP-induced AR. Although it is generally accepted that the spermatozoa must be acrosome-reacted to complete penetration of the zona (21), the exact site of the AR has not been defined and appears to differ between species. PT treatment of human spermatozoa does not affect the ability of spermatozoa to bind to structural intact human ZP. The results indicate the importance of intact acrosomes on the spermatozoa to ensure tight binding to the ZP, i.e., those sperm populations with a decreased AR; namely, PT-treated spermatozoa bound significantly higher numbers of sperm during HZA conditions (8).

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