Journal of Andrology, Vol. 17, No. 5, September/October 1996

Voltage-Dependent Calcium Channels and G_i Regulatory Protein Mediate the Human Sperm Acrosomal Exocytosis Induced by *N*-Acetylglucosaminyl/Mannosyl Neoglycoproteins

ADRIANO BRANDELLI,* PATRICIA V. MIRANDA,† AND JORGE G. TEZÓN†

*From the *Departamento de Bioquimica, Instituto de Biociencias, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; and the †Institute de Biologia y Medicina Experimental (IBYME)–CONICET, Buenos Aires, Argentina.*

ABSTRACT: Mammalian spermatozoa must undergo an exocytotic event during fertilization, the acrosome reaction (AR). In most species studied this process is induced by specific glycoproteins of the oocyte extracellular matrix, the zona pellucida (ZP), and it involves guanine nucleotide-binding regulatory proteins (G-proteins), resulting in an uptake of extracellular calcium by the sperm. In the bull, this event has been reported to be mediated by voltage-dependent calcium channels (VDCC). Previous observations showed that neoglycoproteins (NGPs) with *N*-acetylglucosamine or mannose (GlcNAc-BSA or Man-BSA) residues induce the AR in capacitated human spermatozoa. We report here that the pretreatment of spermatozoa with 125 ng/ml pertussis toxin (PTx) inhibited GlcNAc-BSA- or Man-BSA-induced AR, whereas 1 μg/ml cholera toxin had no effect. These data indicate that the transduction mechanism for GlcNAc-BSA- and Man-BSA-induced AR involves G-proteins of the inhibitory type (G_i). An increase in the AR rate was observed when capacitated spermatozoa were incubated with increasing concentrations of potassium ions (K^+) in Biggers–Whitten–Whittingham (BWW) modified medium (2.6 ± 0.3-fold at 80 mM K^+). This induction was observed only when the pH was raised to 8.5, and it was inhibited by verapamil, nitrendipine, ω-conotoxin, nickel ions (Ni^{2+}), lanthanum ions (La^{3+}), or cadmium ions (Cd^{2+}) in a concentration-dependent manner, indicating the participation of VDCC activated by membrane depolarization. The GlcNAc-BSA- or Man-BSA-induced AR was completely inhibited by preincubation of spermatozoa with VDCC blockers and calcium antagonists, indicating a link between the binding of sugar residues of the NGPs and channel activation. The AR induced by membrane depolarization with high K^+ medium was not inhibited by PTx, suggesting that Ca^{2+} entry is downstream to G_i-protein activation. These data show that the induction of the AR in human spermatozoa by GlcNAc- or Man-NGPs involves VDCC and G_i-like regulatory proteins similar to the induction described for ZP in other mammalian species.

Key words: Carbohydrate, G-protein, acrosome reaction, VDCC, neoglycoproteins.

J Androl 1996;17:522–529

In order to assess the fertilizing ability of human spermatozoa, several approaches were aimed at functional studies, such as the exocytosis of sperm acrosomal content, or acrosome reaction (AR). The identification of factors with the ability to trigger this process under physiological conditions and the description of the mechanisms involved, are crucial for the characterization of possible agonists for the AR and the development of new functional tests for spermatozoa. All of the known inducers of AR, such as calcium ionophore (Yanagimachi and Usui, 1974; Hyne and Garbers, 1979; Jamil and White, 1981), progesterone (Prg) (Blackmore et al, 1990; Aurell Wistrom and Meizel, 1993; Turner et al, 1994), follicular fluid proteins (Siiteri et al, 1988; Saragüeta et al, 1994), or zone pellucida (ZP) (Cross et al, 1988; Florman et al, 1992; Bailey and Storey, 1994), directly or indirectly increase the concentration of intracellular calcium in spermatozoa. Of the inducers present in the female reproductive tract, the signal transduction mechanisms are being studied more intensely for ZP and Prg. Specific carbohydrate residues in the glycoproteins of the ZP (Florman and Wassarman, 1985; Bleil and Wassarman, 1988; Leyton and Saling, 1989) are recognized by receptors on the sperm surface that cluster after binding (Bleil and Wassarman, 1983; Leyton and Saling, 1989; Macek et al, 1991). As demonstrated for bull spermatozoa (Florman et al, 1992), this is followed by an increase in intracellular

Supported by Coordenaçao de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) of Brazil; The Population Council, New York, New York; Centro Argentino–Brasileiro de Biotecnologia (CABBIO Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET, project no. 251-0518\91) of Argentina.

Correspondence to: Dr. Jorge G. Tezón, Instituto de Biología y Medicina Experimental, Obligado 2490, Buenos Aires 1428, Argentina.

Received for publication October 19, 1995; accepted for publication May 15, 1996.

calcium that can be blocked by nitrendipine, prenylamine, or verapamil, known inhibitors of voltage-dependent calcium channels (VDCC). Similar results are obtained when VDCC are directly activated by membrane depolarization with high concentrations of potassium ions (K^+) (Babcock and Pfeiffer, 1987; Cox and Peterson, 1989; Florman et al, 1992). In contrast to ZP, Prg induction of AR is linked to a chloride channel complex (Aurell Wistrom and Meizel, 1993), and the elevation of intracellular calcium was not affected by VDCC inhibitors (Foresta et al, 1993), suggesting the existence of two different mechanisms for signal transduction in the acrosomal exocytosis.

A regulatory link between ligand binding, either ZP or Prg, and the triggering of AR is being intensively studied. Inhibitory G-proteins (G_i-proteins), which are inhibited when ADP-ribosylated by pertussis toxin (PTx) (Bentley et al, 1986; Kopf et al, 1986), appear to play an important role in the ZP-induced acrosomal exocytosis (Endo et al, 1987; Kopf and Wilde, 1990). The treatment with PTx inhibits the AR induced by ZP in mouse (Endo et al, 1988), cow (Florman et al, 1989, 1992), and human (Lee et al, 1992; Tesarik et al, 1993) spermatozoa, whereas no effect is seen in spontaneous, Prg-stimulated or calcium ionophore-induced AR in human spermatozoa (Tesarik et al, 1993). These results indicate that a G_i-protein would connect the occupancy of ZP receptors with the increase in intracellular calcium and pH necessary for the AR (Endo et al, 1987; Florman et al, 1989). Activation of VDCC, reported as necessary for the signal transduction of ZP in mouse spermatozoa, has been shown to follow (or occur downstream from) the activation of a G-protein sensitive to PTx (Florman et al, 1992).

Among the tests developed to assess sperm functions, the binding (Burkman et al, 1988) and the penetration of ZP (Liu and Baker, 1994a) exhibited the highest correlation with *in vitro* fertilizing capacity (Liu and Baker, 1992). Moreover, the occurrence of the AR at the surface of the ZP helped to identify a new cause of male infertility (Liu and Baker, 1994b). The scarcity of human zonae, however, has prevented the use of these tests on a routine basis. Because the biological activity of ZP resides on its carbohydrate moieties, the presence of functional receptors for specific sugars on the sperm surface should be indicative of its function related to ZP recognition.

Binding sites for neoglycoproteins (NGPs) bearing mannose residues (Man-BSA) in human spermatozoa were related to ZP binding capacity (Benoff et al, 1993a; Chen et al, 1995), and our laboratory has reported that NGPs with *N*-acetylglucosamine (GlcNAc-BSA) or mannose residues specifically induce the acrosome reaction in capacitated human spermatozoa (Brandelli et al, 1994). Moreover, the degree of induction for GlcNAc-BSA correlates with the *in vitro* fertilization rate (Brandelli et al, 1995). These findings suggest that GlcNAc and mannose residues may interact with the putative receptor for ZP in human spermatozoa. To explore this hypothesis, we have investigated the signal transduction mechanism activated by GlcNAc and mannose NGPs. In the present work we studied the effect of modulators of VDCC, G_i, and stimulatory (G_s) regulatory proteins in the AR induced by GlcNAc- and mannose-BSA in order to compare their effects to those reported for the physiological induction by ZP.

Materials and Methods

Reagents

Calcium ionophore A23187 was from Calbiochem-Novabiochem (La Jolla, California); cholera toxin was from Schwarz/Mann (Orangeburg, New York); nitrendipine was from Research Biochemical Corporation; inorganic salts were from Fisher Scientific (Fair Lawn, New Jersey); and pertussis toxin, verapamil, ω-conotoxin GVIA, albumin, bovine-*p*-aminophenyl-*N*-acetyl-α-D-glucosaminide (GldNAc-BSA), and albumin, bovine-*p*-aminophenyl-α-D-mannopyranoside (Man-BSA) were from Sigma Chemical Co. (St. Louis, Missouri).

Semen Samples and Sperm Capacitation

Semen was obtained from fertile donors. The normal samples used were selected according to World Health Organization (WHO, 1992) standards. After complete liquefaction, spermatozoa were separated from seminal plasma by layering 500 μl of each sample onto 700 μl of a 50% isotonic Percoll (Pharmacia Inc., Piscataway, New Jersey) and centrifuging for 10 minutes at 900 × *g*. The sperm pellet was resuspended and washed twice in Biggers–Whitten–Whittingham (BWW) medium (Biggers et al, 1971), containing 35 mg/ml of bovine serum albumin (BSA) (BWW35), by centrifugation at 900 × *g*. The final pellet was resuspended in BWW35 (2×10^6 cells/ml) and incubated 6 hours at 37°C and 5% CO_2 in air for sperm capacitation (DeJonge et al, 1989). To study the effect of exotoxins from *Bordetella pertussis* or *Vibrio cholerae*, spermatozoa were incubated in the presence of these toxins during or after capacitation. The percentage of motile spermatozoa was examined with phase-contrast optics. In all of the cases studied, it was found that at least 80% of the cells were motile at the end of the incubation.

Acrosome Reaction (AR) Assays

Capacitated spermatozoa were treated with 1 μg/ml GlcNAc-BSA or Man-BSA neoglycoproteins (NGPs), 10 μM A23187 calcium ionophore, 1 μg/ml Prg, or medium as control, as previously described (Brandelli et al, 1994, 1995). Briefly, incubations were carried out in BWW35 at 37°C in 5% CO_2 in air for 30 minutes. Sperm cells were then washed three times in phosphate-buffered saline (PBS), followed by centrifugation at 500 × *g*, and the final pellet was resuspended in 30 μl of PBS. An aliquot of this suspension was air dried onto immunofluorescence slides coated with polylysine, submerged for 15 seconds in methanol at 4°C for cell permeabilization (Mendoza et al,

1992), and air dried again immediately. The AR was evaluated by staining with 50 μg/ml fluorescein isothiocyanate (FITC)–*Pisum sativum* agglutinin (Cross et al, 1986) in duplicate assays. Stained cells (200 cells per treatment) were scored using a Nikon Labrophot epifluorescence microscope.

To induce the AR by membrane depolarization, noncapacitated spermatozoa, purified as described above, were transferred to BSA-free modified BWW (pH 8.5) with elevated concentrations of K^+ (prepared by replacement of NaCl with KCl), and incubated for 30 minutes at 37°C. In experiments where the effect of calcium channel modulators was studied, spermatozoa were preincubated for 10 minutes at pH 6.5 with the indicated concentrations of these agents to facilitate binding. The pH was then raised to 8.5 by the addition of 1 M Tris to controls (no K^+) and treated cells (80 mM K^+). The VDCC blockers, verapamil, nitrendipine, and ω-conotoxin, were used in the 1 nM to 0.1 μM concentration range, and inactive antagonists, such as ultraviolet (UV)-irradiated nitrendipine or adenosine monophosphate (AMP), were used at 1 μM or 1 mM, respectively. The calcium antagonists, Ni^{2+}, La^{3+}, and Cd^{2+}, were used in the micromolar to millimolar range. In these experiments, the percentage of acrosome-reacted spermatozoa (AR) was normalized (NAR) as follows:

$$NAR = \frac{ARt - ARc}{ARK^+ - ARc} \times 100,$$

where ARK^+ (80 mM K^+) minus ARc (no K^+) represents maximal stimulation for a given sample. The stimulation in the presence of different blockers and antagonists is represented by ARt (80 mM K^+ plus inhibitors) minus ARc. The ratio indicates the fraction of maximal response after treatment with antagonists.

When studying the effect of VDCC antagonists on the NGP-induced AR, the cells were also preincubated for 10 minutes at pH 6.5 with or without these compounds, and the pH was then adjusted to 7.4 for incubations with either NGP or control medium.

Statistical Analysis

Data processing was done on a personal computer using the GraphPad InStat program (GraphPad Software, San Diego, California). The results, expressed as the percentages of acrosome-reacted spermatozoa, were normalized, and the Dunnett multiple comparison test was applied.

Results

Effect of Pertussis and Cholera Toxins on the Induction of the Acrosome Reaction by N-Acetylglucosamine and Mannose Neoglycoproteins

In a previous work we demonstrated that the NGPs GlcNAc-BSA and Man-BSA were capable of inducing the AR in human spermatozoa (Brandelli et al, 1994). Following a typical concentration-dependent effect, GlcNAc-BSA stimulates the AR with an ED_{50} (effective dose for 50% stimulation) of 0.3 μg/ml, whereas Man-BSA was slightly less effective (ED_{50}: 0.6 μg/ml) (Fig. 1). Both NGPs reached a similar maximal effect at 1 μg/ml. This effect is not observed with NGPs bearing *N*-acetylgalactosamine, lactose, or glucose residues (Brandelli et al, 1994), or with typical glycoproteins such as lactalbumin (results not shown). In order to analyze the relevance of this effect, we tried to determine whether the transduction mechanisms involved in the NGP-induced AR are similar to those described for ZP.

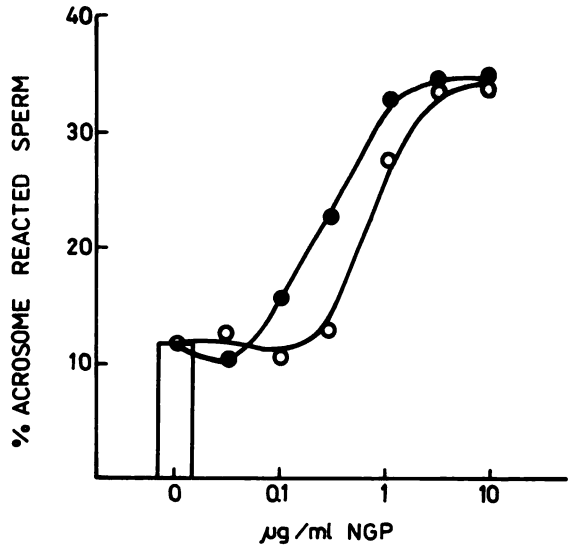

FIG. 1. Induction of the acrosome reaction (AR) by neoglycoproteins: concentration-dependent effect. Capacitated spermatozoa were incubated with medium as control (bar), GlcNAc-BSA (●), or Man-BSA (○), and the AR was evaluated as described in the Materials and Methods section. Results, expressed as the percentage of acrosome-reacted cells, are representative of three similar experiments. Each point represents the mean of duplicate assays.

Pertussis toxin (PTx) has been reported to inhibit the ZP-induced AR in human spermatozoa (Lee et al, 1992), whereas no effect was observed on the Prg-induced AR (Tesarik et al, 1993). The exposure of capacitated spermatozoa to 1 μg/ml GlcNAc-BSA or Man-BSA induced a 2.5-fold increase in the AR. When PTx was added during capacitation, a marked inhibition of the NGP-induced AR was observed at toxin concentrations of 125 ng/ml (Fig. 2). The rates of spontaneous AR or induction after exposure to calcium ionophore or Prg were not affected by PTx (Fig. 3).

The involvement of stimulatory G-proteins (G_s) in the ZP-induced AR in human spermatozoa had been ruled out based on the lack of effect of cholera toxin (CTx) (Lee et al, 1992). The effect of CTx on the AR induced by GlcNAc-BSA or Man-BSA was also investigated. The treatment of spermatozoa with CTx caused no effect on the spontaneous AR or that induced by NGPs, A23187, or Prg (Fig. 3).

The incubation of humán spermatozoa with PTx does not alter the parameters considered so far to be indicative of capacitation, such as spontaneous AR, motility patterns, or ZP binding (Lee et al, 1992). In order to establish

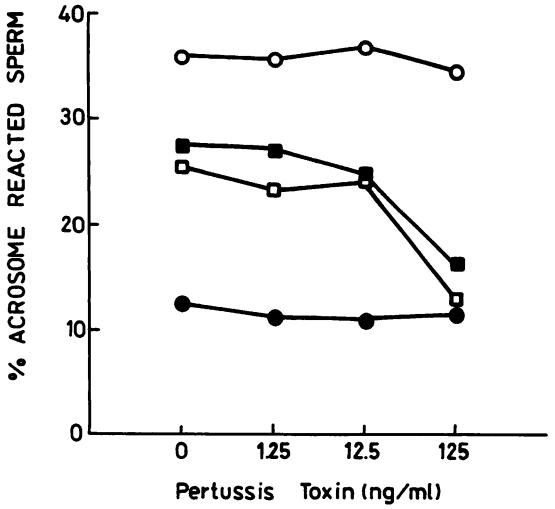

FIG. 2. Induction of the acrosome reaction by neoglycoproteins: effect of capacitation in the presence of pertussis toxin (PTx). Capacitated spermatozoa were incubated in medium as control (●), with GlcNAc-BSA (■), Man-BSA (□), or 10 μM calcium ionophore (○), with increasing concentrations of PTx, as described in the Materials and Methods section. Results, expressed as the percentage of acrosome-reacted cells, are representative of three similar experiments. Each point represents the mean of duplicate assays.

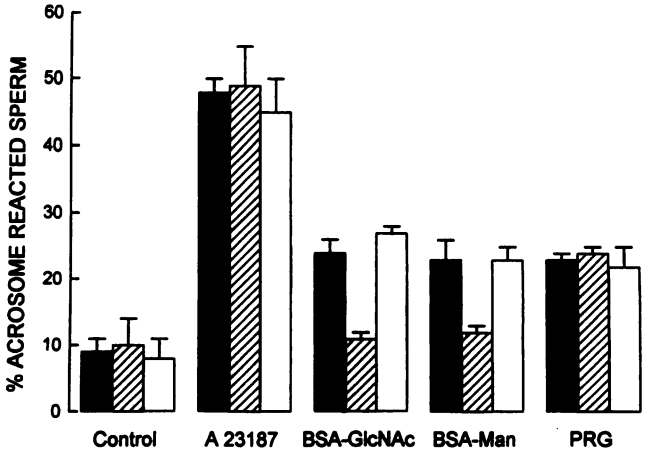

FIG. 3. Differential effects of G-protein modulators on the acrosome reaction (AR). Spermatozoa were capacitated in medium alone (■), with 0.5 μg/ml pertussis toxin (▨), or with 1 μg/ml cholera toxin (□), before the addition of different AR-inducers: 10 μM calcium ionophore (A23187), 1 μg/ml GlcNAc-BSA, 1 μg/ml Man-BSA, and 1 μg/ml Progesterone (PRG). Bars show the mean ± standard error of the mean (SEM) of triplicate experiments.

whether the effect of PTx observed for GlcNAc-BSA or Man-BSA was specific for the AR, the spermatozoa were incubated in capacitation medium for 5 hours. PTx was then added at a concentration of 125 ng/ml, and, 1 hour later, the cell suspensions were treated with 1 μg/ml of either GlcNAc-BSA or Man-BSA for one additional hour. The results, depicted in Figure 4, show that the AR induced by both NGPs was inhibited by PTx when added shortly before the stimulus, supporting the idea that the toxin would affect the AR rather than the capacitation process.

Effect of VDCC Antagonists on the Acrosome Reaction-induced N-acetylglucosamine or Mannose Neoglycoproteins and by Membrane Depolarization

The binding of ZP to its putative receptor on bull spermatozoa results in the activation of VDCC, as evidenced by the inhibition of the AR by specific antagonists (Florman et al, 1992). We then investigated the participation of these channels in the AR induced by GlcNAc or mannose NGPs.

The induction of the human sperm AR by membrane depolarization was characterized. When spermatozoa were incubated with different K^+ concentrations in control medium (pH 7.4), no change in the percentage of acrosome-reacted spermatozoa was observed (Fig. 5, insert). However, at pH 8.5 an increase in the AR was observed, correlating with the increment of K^+ concentration. The maximum effect (38% of acrosome-reacted spermatozoa, 2.6 ± 0.3-fold stimulation) was observed at a concentration of 80 mM K^+.

In order to analyze the participation of VDCC in the depolarization-induced AR, the effect of channel blockers and Ca^{2+} antagonists was investigated; the results are shown in Figure 5. Nitrendipine and verapamil, which specifically block L-type VDCC, were more effective than ω-conotoxin (N-type specific) at 1 μM. These antagonists reached their IC_{50} (effective dose for 50% inhibition) at the nM range (60, 100, and 400 nM, respectively). Nickel, cadmium, and lanthanum ions, known to compete

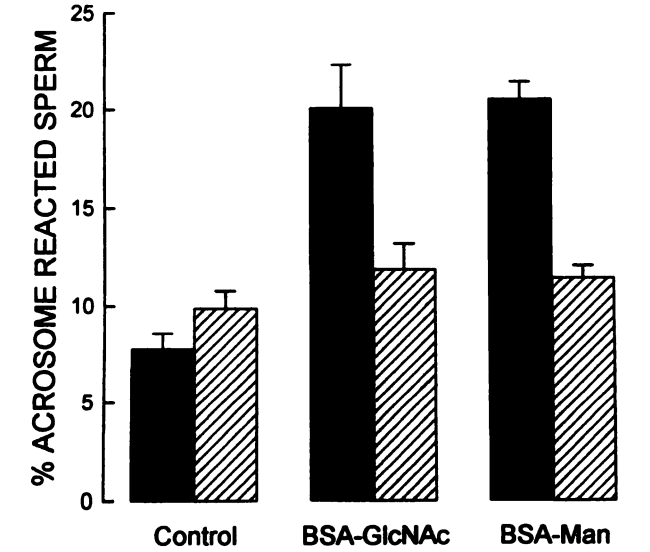

FIG. 4. Specific effect of pertussis toxin (PTx) on the acrosome reaction induced by neoglycoproteins (NGPs). Spermatozoa were capacitated in medium for 5 hours and supplemented with medium alone (■) or with 125 ng/ml PTx (▨) for 1 hour, after which different NGPs were added, as described in the Materials and Methods section. Bars show the mean ± SEM of triplicate similar experiments.

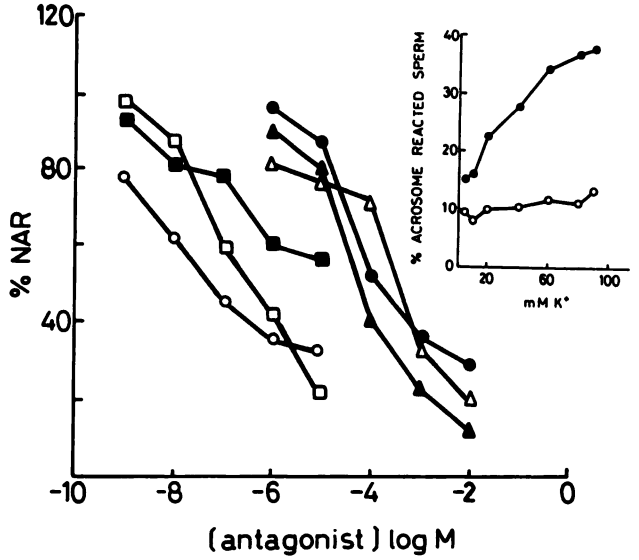

FIG. 5. Effect of VDCC antagonists and blockers in the depolarization-induced acrosome reaction (AR). Noncapacitated spermatozoa were transferred to K^+-modified (80 mM) BWW, pH 6.5, with the indicated concentrations of nitrendipine (○), verapamil (□), ω-conotoxin (■), Ni^{2+} (▲), La^{3+} (●), Cd^{2+} (△), or BWW alone as control. Cells were incubated for 10 minutes, and the pH was adjusted to 8.5 with 1 M Tris. After 30 minutes at 37°C, aliquots were taken to assess the percentage of acrosome-reacted spermatozoa. The normalized acrosome reaction (% NAR) was described in the Materials and Methods section. Results are representative of three similar experiments. Each point represents the mean of duplicate assays. Insert: The AR induced by membrane depolarization with high K^+ concentration. Capacitated spermatozoa were transferred to K^+-modified BWW, pH 8.5 (●) or pH 7.4 (○), before incubation for 30 minutes at 37°C.

for Ca^{2+}, inhibited the depolarization-induced AR at higher concentrations, and their IC_{50} values were 80 μM for both Ni^{2+} and La^{3+}, and 500 μM for Cd^{2+}.

After establishing an effective concentration at which VDCC antagonists affect the depolarization-induced AR in human spermatozoa, we examined the participation of VDCC in the AR induced by GlcNAc-BSA or Man-BSA. The effect of calcium antagonists was tested in spermatozoa incubated in BWW35 medium (low K^+), as described in the Materials and Methods section. Nitrendipine, verapamil, ω-conotoxin, and UV-inactivated nitrendipine were used at 1 μM, whereas Ni^{2+}, Cd^{2+}, La^{3+}, and AMP were used at 1 mM. The rate of AR in the presence of active VDCC antagonists was similar to that of controls, whereas the induction observed with GlcNAc-BSA and Man-BSA was not affected by inactive blockers (Table 1). The calcium antagonists Ni^{2+}, Cd^{2+}, and La^{3+} produced an inhibition similar to that observed with VDCC antagonists (Table 1).

Finally, we explored the possible sequence of events between G_i-protein and VDCC, studying the effect of PTx on the AR induced by membrane depolarization. The pretreatment with PTx did not affect the percentage of acrosome-reacted spermatozoa after incubation with high

Table 1. *Effect of voltage-dependent calcium channel inhibitors in the neoglycoprotein-induced acrosome reaction*

	BWW35	BSA-GlcNAc	BSA-Man	*N*
Control	11 ± 1*†	25 ± 1	25 ± 2	5
Nitrendipine	13 ± 2	14 ± 3*	12 ± 4†	3
Verapamil	10 ± 1	14 ± 2*	16 ± 1†	5
ω-Conotoxin	9 ± 5	12 ± 3*	11 ± 4†	3
Nitrendipine-UV	10 ± 4	20 ± 1	22 ± 4	3
AMP	10 ± 4	22 ± 1	23 ± 4	3
Lanthanum	10 ± 3	10 ± 3*	11 ± 1†	3
Nickel	8 ± 3	10 ± 3*	10 ± 1†	3
Cadmium	11 ± 1	11 ± 6*	13 ± 4†	3

* $P < 0.05$ vs. BSA-GlcNAc alone.

† $P < 0.05$ vs. BSA-Man alone.

Capacitated spermatozoa were transferred to BWW, pH 6.5, and incubated for 10 minutes with 1 μM nitrendipine, verapamil, ω-conotoxin, UV-irradiated nitrendipine (Nitrendipine-UV), or 1 mM AMP, Ni^{2+}, La^{3+}, or Cd^{2+}. Cells incubated in BWW35 were used as controls. After adjusting the pH to 7.4, GlcNAc-BSA or Man-BSA were added, to a final concentration of 1 μg/ml. After 30 minutes at 37°C, aliquots were taken to assess the AR. Results are given as the mean ± SEM of (*N*) independent experiments.

K^+ concentration (Fig. 6). This result indicates that G_i-protein modulation would occur prior to VDCC activation.

Discussion

Inhibitory G-proteins (G_i-proteins) sensitive to the perturbing action of *Bordetella pertussis* toxin (PTx) were shown to mediate the ZP-induced AR in mouse (Endo et al, 1987, 1988), bull (Florman et al, 1989, 1992), and human spermatozoa (Lee et al, 1992; Tesarik et al, 1993). In previous studies, we observed that the neoglycoproteins bearing *N*-acetylglucosamine or mannose residues induced the AR in human spermatozoa resembling the induction of ZP; there were similar kinetics, the requirement of sperm capacitation, and mobilization of extracellular calcium (Brandelli et al, 1994). In the present study, we report that both GlcNAc-BSA and Man-BSA effects were blocked when spermatozoa were pretreated with PTx at a concentration similar to that described to inhibit the ZP effect (Lee et al, 1992). Conversely, the treatment with CTx did not affect the AR induced by all of the agents tested. These results are consistent with the nonobservation of G_s-proteins in spermatozoa (Hildebrandt et al, 1985; Bentley et al, 1986; Kopf et al, 1986).

This inhibitory effect of PTx on the AR induction by GlcNAc-BSA and Man-BSA suggests the participation of a G_i-regulatory protein, similar to that described for human ZP (Tesarik et al, 1993). The different sensitivity to PTx of the AR induced by Prg would be evidence of this inducer acting through a G_i-independent mechanism, as suggested by Tesarik et al (1993), such as the direct ac-

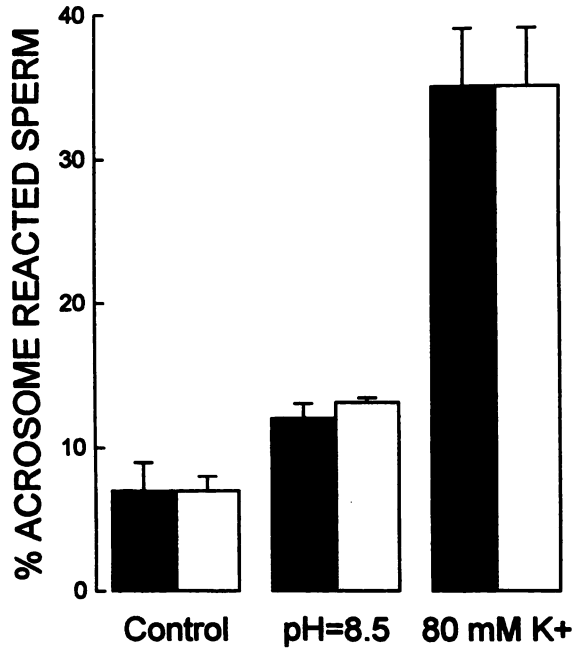

FIG. 6. Effect of pertussis toxin (PTx) in the depolarization-induced acrosome reaction (AR). Spermatozoa were capacitated in the presence (□) or absence (■) of 0.5 μg/ml PTx, and then transferred to BWW, pH 8.5; 80 mM K^+-modified BWW, pH 8.5; or BWW (Control). Cells were incubated for 30 minutes, and aliquots were taken to assess the AR. Bars represent the mean ± SEM of triplicate similar experiments.

tivation of chloride fluxes that results in the elevation of intracellular calcium (Aurell Wistrom and Meizel, 1993).

Depolarization of the plasma membrane is necessary for the ZP-induced AR in bull and mouse spermatozoa (Florman et al, 1992; Zeng et al, 1995). As in many other cell types, activation of sperm receptors by extracellular ligands alters the membrane potential modulating VDCC, which regulate the intracellular Ca^{2+} concentration. Such channels were suggested to be involved in mammalian sperm maturation (Okamura et al, 1992), capacitation (Babcock and Pfeiffer, 1987; Okamura et al, 1993), and the ZP-induced acrosome reaction (Florman et al, 1992).

The stimulatory effect of membrane depolarization by high K^+ on AR and fertilizing ability (Roblero et al, 1988, 1990) had been observed in human spermatozoa. Our results show that human spermatozoa undergo the AR after membrane depolarization, and pretreatment with VDCC blockers and calcium antagonists inhibits this effect. The depolarization-induced AR cannot be totally blocked by VDCC antagonists (Florman et al, 1992); this may indicate some direct effect of K^+, as has been reported for hamster spermatozoa (Llanos, 1994). We observed that, similarly to the ZP induction of AR in the bull (Florman et al, 1992), VDCC blockers and calcium antagonists inhibit the induction of the AR by GlcNAc- or Man-BSA in human spermatozoa, indicating the participation of this type of channel in the ligand-induced AR. However, the present data do not allow a clear identification of the type of channel involved either in the depolarization or the NGP-induced AR.

Benoff et al (1994) proposed that during capacitation, VDCC modulate the exposure of mannose binding sites and, consequently, ZP binding activity in spermatozoa. In the series of experiments presented here, channel blockers were added after capacitation, when binding sites for GlcNAc or Mannose residues are fully exposed (Benoff et al, 1993b; Brandelli et al, 1994). With this experimental design, we can state that VDCC blockers and antagonists specifically inhibit the exocytotic event triggered by the occupation of GlcNAc and mannose receptors.

Regarding the sequence of events between G_i-protein and VDCC activation, our results suggest that activation of VDCC by depolarization bypasses the regulatory step of G_i-proteins, because PTx does not inhibit the AR in high-K^+ media. This mechanism has been described previously for bull spermatozoa (Florman et al, 1992). The putative *N*-acetylglucosaminyl/mannosyl binding sites would be coupled to G_i-proteins, and the inhibitory effect of VDCC blockers and antagonists suggests that the G_i-protein regulatory step would be upstream to VDCC activation in this pathway. The mechanism of G-protein regulation of calcium channel currents is not yet clear, and physiological induction of the sperm AR may involve indirect activation of VDCC by a G-protein.

We have established that AR induction by GlcNAc-BSA in human spermatozoa correlates with their ability to fertilize oocytes, suggesting that the sperm response to this sugar is indicative of its function (Brandelli et al, 1995). The present results show that the human sperm acrosomal exocytosis induced by *N*-acetylglucosaminyl and/or mannosyl residues are mediated by PTx-sensitive G-protein and VDCC, similarly to the AR induced by ZP. Although an analogy between the exocytosis mechanisms evoked by physiological stimulus (ZP) and GlcNAc or Mannose NGPs cannot yet by fully established, their similarities lead us to propose the use of these compounds for the study of the complex events involved in the sperm–egg interaction and sperm function.

Acknowledgments

J.G.T. and P.V.M. are Senior Investigator and Research Fellow, respectively, of the CONICET of Argentina, and A.B. is a member of the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, CNPq. of Brazil. We thank Stella Delcourt from Hospital Italiano and Dr. Jorge A. Blaquier from Fertilab for semen samples, Patricia Delcourt for her technical assistance, and Ricardo Damnotti and Marcelo Lammami for graphics and photographs.

References

Aurell Wistrom C, Meizel S. Evidence suggesting involvement of a unique human sperm steroid receptor/Cl^- channel complex in the progesterone-initiated acrosome reaction. *Dev Biol* 1993;159:679–690.

Babcock DF, Pfeiffer DR. Independent elevation of cytosolic [Ca2+] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. *J Biol Chem* 1987;262:15041–15047.

Bailey JL, Storey BT. Calcium influx into mouse spermatozoa activated by solubilized mouse zona pellucida, monitored with the calcium fluorescent indicator, fluo-3. Inhibition of the influx by three inhibitors of the zona pellucida induced acrosome reaction: tyrphostic A48, pertussis toxin, and 3-quinuclidinyl benzilate. *Mol Reprod Dev* 1994;39: 297–308.

Benoff S, Cooper GW, Hurley I, Mandel FS, Rosenfeld DL, Scholl GM, Gilbert BR, Hershlag A. The effect of calcium ion channel blockers on sperm fertilization potential. *Fertil Steril* 1994;62:606–617.

Benoff S, Cooper GW, Hurley I, Napolitano B, Rosenfeld DL, Scholl GM, Hershlag A. Human sperm fertilizing potential *in vitro* is correlated with differential expression of a head-specific mannose-ligand receptor. *Fertil Steril* 1993a;59:854–862.

Benoff S, Hurley I, Cooper GW, Mandel FS, Rosenfeld DL, Hershlag A. Head-specific mannose-ligand receptor expression in human spermatozoa is dependent on capacitation-associated membrane cholesterol loss. *Hum Reprod* 1993b;8:2141–2154.

Bentley JK, Garbers DL, Domino SE, Noland TD, Van Dop C. Spermatozoa contain a guanine nucleotide-binding protein ADP-ribosylated by pertussis toxin. *Biochem Biophys Res Commun* 1986;138: 728–734.

Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos *in vitro*. Daniel JD, ed. *Methods in Mammalian Embryology*. San Francisco: Freeman; 1971:86–116.

Blackmore PF, Beebe SJ, Danforth DR, Alexander N. Progesterone and 17α-hydroxyprogesterone: novel stimulators of calcium influx in human sperm. *J Biol Chem* 1990;265:1376–1380.

Bleil JD, Wassarman PM. Sperm–egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zone pellucida glycoprotein. *Dev Biol* 1983;95:317–324.

Bleil JD, Wassarman PM. Galactose at the nonreducing terminus of 0-linked oligosaccharides of mouse egg zone pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. *Proc Natl Acad Sci USA* 1988;85:6778–6782.

Brandelli A, Miranda PV, Añon Vazquez MG, Marin Briggiler CI, Sanjurjo C, Gonzalez-Echeverria MF, Blaquier JA, Tezón JG. A new predictive test for *in vitro* fertilization based on the induction of sperm acrosome reaction by *N*-acetylglucosamine neoglycoprotein. *Hum Reprod* 1995;10:1751–1756.

Brandelli A, Miranda PV, Tezón JG. Participation of glycosylated residues in the human sperm acrosome reaction: possible role of *N*-acetylglucosaminidase. *Biochim Biophys Acta* 1994;1220:299–304.

Burkman LJ, Coddington CC, Franken DR, Kruger TF, Rosenwaks Z, Hodgen GD. The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril* 1988;49:688–697.

Chen J-S, Doncel GF, Alvarez C, Acosta AA. Expression of mannose-binding sites on human spermatozoa and their role in sperm–zona pellucida binding. *J Androl* 1995;16:55–63.

Cox T, Peterson RN. Identification of calcium conducting channels in isolated boar sperm plasma membranes. *Biochem Biophys Res Commun* 1989;161:162–168.

Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res* 1986;15: 213–226.

Cross NL, Morales P, Overstreet JW, Hanson FW. Induction of acrosome reactions by the human zona pellucida. *Biol Reprod* 1988;38:235–242.

DeJonge CJ, Mack SR, Zaneveld LJD. Synchronous assay for human sperm capacitation and the acrosome reaction. *J Androl* 1989;10:232–239.

Endo Y, Lee MA, Kopf GS. Evidence for the role of a guanine nucleotide-binding regulatory protein in the zona pellucida-induced mouse sperm acrosome reaction. *Dev Biol* 1987;119:210–216.

Endo Y, Lee MA, Kopf GS. Characterization of an islet activating protein-sensitive site in mouse sperm that is involved in the zona pellucida-induced acrosome reaction. *Dev Biol* 1988;129:12–24.

Florman HM, Corron ME, Kim TD-H, Babcock DF. Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. *Dev Biol* 1992;152: 304–314.

Florman MH, Tombes RM, First NL, Babcock DF. An adhesion-associated agonist from the zona pellucida activates G protein-promoted elevations of internal Ca^{2+} and pH that mediate mammalian sperm acrosomal exocytosis. *Dev Biol* 1989;135:133–146.

Florman HM, Wassarman PM. O-linked oligosacharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* 1985;41:313–324.

Foresta C, Rossato M, DiVirgilio F. Ion fluxes through the progesterone-activated channel of the sperm plasma membrane. *Biochem J* 1993; 294:279–283.

Hildebrandt JD, Codina J, Tash JS, Kirchick HJ, Lipschultz L, Sekura RD, Birnbaumer L. The membrane-bound spermatozoal adenylyl cyclcase system does not share coupling characteristics with somatic cell adenylyl cyclases. *Endocrinology* 1985;116:1357–1366.

Hyne RV, Garbers DL. Calcium dependent elevation of adenosine 3′,5′-monophosphate and induction of the acrosome reaction in guinea pig spermatozoa. *Proc Natl Acad Sci USA* 1979;76:5699–5703.

Jamil K, White IG. Induction of acrosomal reaction in sperm with ionophore A23187 and calcium. *Arch Androl* 1981;7:283–292.

Kopf GS, Wilde MW. Signal transduction processes leading to acrosomal exocytosis in mammalian spermatozoa. *Trends Endocrinol Metab* 1990;1:362–368.

Kopf GS, Woolkalis MJ, Gerton GL. Evidence for a guanine nucleotide-binding regulatory protein in invertebrate and mammalian sperm: identification by islet-activating protein-catalyzed ADP-ribosylation and immunochemical methods. *J Biol Chem* 1986;261:7327–7331.

Lee MA, Check JH, Kopf GS. A guanine nucleotide-binding regulatory protein in human sperm mediates acrosomal exocytosis induced by the human zona pellucida. *Mol Reprod Dev* 1992;31:78–86.

Leyton L, Saling PM. Evidence that aggregation of sperm receptors by ZP3 triggers the acrosome reaction. *J Cell Biol* 1989;108:2163–2168.

Liu DY, Baker HWG. Tests of human sperm function and fertilization *in vitro*. *Fertil Steril* 1992;58:465–483.

Liu DY, Baker HWG. A new test for the assessment of sperm–zona pellucida penetration: relationship with results of other sperm tests and fertilization *in vitro*. *Hum Reprod* 1994a;9:489–496.

Liu DY, Baker HWG. Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm–zona pellucida penetration and reduced fertilization *in vitro*. *Hum Reprod* 1994b;9:1694–1700.

Llanos MN. Evidence in support of a role for Ca^{2+}-activated K^+ channels in the hamster sperm acrosome reaction. *J Exp Zool* 1994;269:484–488.

Macek MB, Lopez LC, Shur BD. Aggregation of β-1,4-galactosyltransferase on mouse sperm induces the acrosome reaction. *Dev Biol* 1991; 147:440–444.

Mendoza C, Carreras A. Moos J, Tesarik J. Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin. *J Reprod Fertil* 1992;95: 755–763.

Okamura N, Fukuda A. Tanba M, Sugita Y, Nagai T. Changes in the nature of calcium transport systems on the porcine sperm plasma

membrane during epididymal maturation. *Biochim Biophys Acta* 1992;1108:110–114.

Okamura N, Tanba M, Fukuda A, Sugida Y, Nagai T. Forskolin stimulates porcine sperm capacitation by increasing calcium uptake. *FEBS Lett* 1993;316:283–286.

Roblero LS, Fernandez E, Guadarrama A, Zegers-Hochschild F, Ortiz ME. High potassium concentration and the cumulus corona oocyte complex stimulate the fertilizing capacity of human spermatozoa. *Fertil Steril* 1990;54:328–332.

Roblero LS, Guadarrama A, Ortiz ME, Fernandez E, Zegers-Hochschild F. High potassium concentration improves the rate of acrosome reaction in human spermatozoa. *Fertil Steril* 1988;49:676–680.

Saragüeta P, Lanuza G, Miranda PV, Tezón JG, Barañao JL. Immunoglobulins from human follicular fluid induce the acrosome reaction in human sperm. *Mol Reprod Dev* 1994;39:280–288.

Siiteri JE, Gottlieb W, Meizel S. Partial characterization of a fraction from human follicular fluid that initiates the human sperm acrosome reaction *in vitro*. *Gamete Res* 1988;20:25–43.

Tesarik J, Carreras A, Mendoza C. Differential sensitivity of progesterone- and zona pellucida-induced acrosome reactions to pertussis toxin. *Mol Reprod Dev* 1993;34:183–189.

Turner KO, Garcia MA, Meizel S. Progesterone initiation of the human sperm acrosome reaction: the obligatory increase in intracellular calcium is independent of the chloride requirement. *Mol Cell Endocrinol* 1994;101:221–225.

World Health Organization (WHO). *WHO Laboratory Manual for the Examination of Human Semen and Sperm Cervical Mucus Interaction*. 3rd ed. New York: Cambridge University Press; 1992.

Yanagimachi R, Usui N. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Exp Cell Res* 1974;89:161–174.

Zeng Y, Clark EN, Florman HM. Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. *Dev Biol* 1995;171:554–563.