Sperm N-acetylglucosaminidase is involved in primary binding to the zona pellucida

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The glycosidase-recognizing N-acetylglucosamine terminal residue, N-acetylglucosaminidase (NAG), has been repetitively implicated in fertilization. Nevertheless, its role in the multiple steps comprising this process is a matter of debate because it has been involved in zona pellucida (ZP) binding and penetration and polyspermy block. In this study, the involvement of NAG during sperm interaction with the ZP was analysed. Soluble ZP was able to inhibit sperm NAG activity, suggesting that it can be recognized as a ligand by this enzyme. Sperm–ZP binding assays were carried out under conditions where acrosome reaction (AR) could not take place (salt-stored oocytes and a modified medium where Ca\(^{2+}\) was replaced by Sr\(^{2+}\)). Different NAG-specific reagents—an inhibitor (2-acetamido-2-deoxy-D-glucono-1,5-lactone), a substrate (p-nitrophenyl-N-acetylglucosaminide) and an anti-NAG antibody—were able to impaire sperm binding to the ZP when present during these assays. The lactone was also able to inhibit oocyte penetration during IVF assays, although not when present after primary binding had taken place. This result was not related to the interference of lactone with AR or zona penetrability. Exogenous NAG also inhibited sperm–oocyte interaction when present during binding and IVF assays or used for oocyte pre-incubation. These results suggest the participation of NAG in sperm primary binding to the ZP.

Key words: N-acetylglucosaminidase/primary binding/sperm/zona binding

Introduction

Sperm enzymes are key elements during fertilization. Although historically proposed to participate in zona pellucida (ZP) penetration (Tulsiani et al., 1998), they have also been shown to be involved in sperm binding (Lopez et al., 1985; Primakoff et al., 1985; Godknecht and Honegger, 1991; Carmona et al., 2002; Hemachand et al., 2002; Montfort et al., 2002).

Glycosidases, enzymes that hydrolyse terminal sugar residues in different glycoconjugates, are particularly abundant in the mammalian epididymis as well as in spermatozoa. N-acetylglucosaminidase (NAG), also known as hexosaminidase, is the glycosidase that cleaves terminal N-acetylglucosamine (GlcNAc) residues from β-glycosidic linkages. Its activity is the highest in mammalian sperm and epididymis (Conchie et al., 1959; Miller et al., 1993b), making the epididymis the richest source for its purification.

The participation of NAG in sperm–oocyte interaction has been proposed in many species, although it is still not clear at what stage, because it has been related to cumulus dispersion, ZP binding and penetration, and polyspermy block (Farooqui and Srivastava, 1980; Prody et al., 1985; Lambert, 1989; Godknecht and Honegger, 1991; Sada et al., 1991; Miller et al., 1993a,b; Godknecht and Honegger, 1995; Martinez et al., 2000; Miranda et al., 2000; Perotti et al., 2001).

Previous results from our laboratory suggest the participation of NAG in sperm–ZP binding and acrosome reaction (AR) in humans (Brandelli et al., 1994; Miranda et al., 2000). However, owing to the scarcity of material and ethical considerations for some experimental designs, further studies into the mechanism of action were continued in the hamster. Recent studies from our laboratory confirmed that GlcNAc residues are involved in hamster sperm binding to ZP and AR (Zitta et al., 2004), similar to what we found in humans (Brandelli et al., 1994; Miranda et al., 1997). To determine whether this was related to NAG, we analysed the participation of this enzyme in hamster fertilization in the present study.

Materials and methods

Materials

All reagents used were of the highest purity or analytical grade and purchased from Sigma (St Louis, MO, USA), Fisher (Fairlawn, NJ, USA), Merck (Darmstadt, Germany) or J.T. Baker (Phillipsburg, NJ, USA). Fatty acid-free bovine serum albumin (BSA) was from Serological Corporation (Kankakee, IL, USA), the pregnant mare’s serum gonadotrophin (PMSG) used was Novormon (Sintex, Buenos Aires, Argentina), 2-acetamido-2-deoxy-D-glucurono-1,5-lactone (lactone) was from Toronto Research Chemicals Inc. (Toronto, Canada) and NAG-thiazoline (thiazoline) was kindly provided by Dr Spencer Knapp (Department of Chemistry and Chemical Biology, University of New Jersey, USA). Polyclonal antibody against NAG (anti-NAG) was from Nordic Immunological Laboratories (Tilburg, the Netherlands).

Spermatozoa

Hamster sperm were obtained from adult male epididymis by swim-out in sperm washing medium [SWM: 72 mM NaCl, 1.5 mM KCl, 3.2 mM NaHPO\(_4\), 0.6 mM KH\(_2\)PO\(_4\), 0.5 mM CaCl\(_2\), 0.4 mM MgCl\(_2\), 150 mM sucrose and 1 mg/ml of polyvinyl alcohol (PVA)] and purification through a glass bead column as described (Bavister, 1989). Sperm were incubated under capacitating conditions using TL medium (101.02 mM NaCl, 2.68 mM KCl, 1.8 mM Na\(_2\)HPO\(_4\), 0.6 mM KH\(_2\)PO\(_4\), 0.5 mM CaCl\(_2\), 0.4 mM MgCl\(_2\), 150 mM sucrose and 1 mg/ml of polyvinyl alcohol (PVA)) and purification through a glass bead column as described (Bavister, 1989). Sperm were incubated under capacitating conditions using TL medium (101.02 mM NaCl, 2.68 mM KCl, 1.8 mM Na\(_2\)HPO\(_4\), 0.6 mM KH\(_2\)PO\(_4\), 0.5 mM CaCl\(_2\), 0.4 mM MgCl\(_2\), 150 mM sucrose and 1 mg/ml of polyvinyl alcohol (PVA))
CaCl$_2$, 0.5 mM MgCl$_2$, 35.7 mM NaHCO$_3$, 0.35 mM NaH$_2$PO$_4$, 9 mM sodium lactate, 4.5 mM glucose and 1 mg/ml of PVA), supplemented with 0.25 mM sodium pyruvate, 3 mg/ml of fatty acid-free BSA and motility factors (2 mM α-penicillamine, 10 mM hypotaurine and 100 μM epinephrine) (TALP) (Bavister, 1989). Alternatively, a modified TALP was prepared replacing CaCl$_2$ with SrCl$_2$ (TALP-Sr). Medium (0.5 ml/well) was placed in 24-well dishes (Costar, Corning, Acton, MA, USA), covered with mineral oil and equilibrated for 1 h at 37°C in 5% CO$_2$ in air. Aliquots of the purified sperm suspension were added to each well (5 × 10$^5$ cells/ml) and incubated for a total period of 6 h. AR was evaluated by light microscopy (dark field, ×200) on at least 100 motile cells using the classification of Yanagimachi and Philips (1984). Aliquots were also taken for objective motility evaluation by light microscopy (×200). No effect on motility was evident for any of the agents tested. To analyse the effect of different agents on the AR, these were added to the wells at the end of the capacitation period (3 h of incubation) and AR evaluated 3 h later.

**Oocytes**

Prepubertal female hamsters were superovulated by administration of 40 IU of PMSG, followed by 40 IU of hCG 50–65 h later. Oocytes were collected from the oviduct 15–17 h post-hCG and were freed of cumulus cells by treatment with 0.1% hyaluronidase for 5 min. Oocytes were then washed by pipetting through 5 droplets of TALP supplemented with 5 mM HEPES (TALP-HEPES) and placed in TALP under 5% CO$_2$ at 37°C for IVF assays. Alternatively, oocytes were maintained up to 2 months in 0.5 M (NH$_4$)$_2$SO$_4$, 0.75 M MgCl$_2$, 0.2 mM ZnCl$_2$ and 0.1 mg/ml of PVA, pH 7.4, at 4°C, until their use in binding assays (Boatman et al., 1988). Soluble ZP was obtained from salt-stored oocytes by acid treatment after extensive washing with PBS (Bleil and Wassarman, 1986). The solution was aspirated and kept at −20°C until use.

**Enzyme activity**

Enzyme activity was assayed by measuring the fluorescent signal produced by the cleavage of N-acetylgalcosamine from 4-methylumbelliferyl-N-acetyl-[β-D-glucosaminide (Leaback and Walker, 1961). The cell suspension (100 000 sperm/tube) was supplemented with 100 μl of 0.25 M citrate pH = 4.5 and 100 μl of substrate solution (5 mM). The reaction mixture was incubated at 37°C for 3 h. The amount of the fluorescent product methylumbelliferone (MU) was measured in a Hoeffer TKO 100 fluorimeter (emission 380 nm, detection 460 nm). A calibration curve was plotted using different concentrations of MU and the enzyme activity determined by interpolation. For the inhibition studies, increasing concentrations of different agents were tested: lactone (0.033–3.3 mM), thiazolone (0.1–10 mM), GlcNAc (1–200 mM) and different aliquots of the solubilized ZP solution (representing 2–33 ZP/ml). The colorimetric substrate curve was traced by incubating sperm with p-nitrophenyl-N-acetylgalcosaminide (PNP-GlcNac, 0.01–10 mM) for 3 h and reading the absorbance at 404 nm.

**Production of human recombinant NAG**

Human recombinant NAG (hrNAG) was obtained from a stably expressing CHO cell line. Culture medium was processed by a four-step chromatography purification procedure, as previously described (Miranda et al., 2000). Medium from standard CHO cells (not expressing human NAG) purified using an identical procedure was used as control. Activity was expressed in units/mg bound sperm or the percentage of penetrated and activated oocytes. In some cases, results were normalized to the control values, which were considered as 100%. Inhibition was calculated as: (control – treated) × 100/ control. Graphics represent averages ± SEM of 3–12 different experiments. To assess normal distribution, we converted percentages to ratios and subjected all data to the arcsine square root transformation. Statistical analysis was performed using the parametric Dunnett’s test for multiple comparisons. All statistical procedures were carried out with the aid of GraphPad software.

To analyse the effect of different agents on sperm–ZP binding, we added these to the medium used for sperm–oocyte co-incubation. Lactone was used at 1 and 10 mM final concentration, and droplets with no addition were included as controls. In the case of PNP-GlcNac, a final concentration of 3 mM was present during the assay and vehicle [dimethylsulphoxide (DMSO)] was included as an additional control. For anti-NAG, a final concentration of 50 μg/ml was used and pre-immune IgG at the same concentration was the respective control.

For assays including exogenous enzyme, increasing amounts of hrNAG were added to the co-incubation drop during binding assays. An EC$_{50}$ of 13 mM (equivalent to 1.6 μg/ml) was obtained from the dose–response curve, and this amount was used for all functional assays. The same amount of total protein from regular CHO cells (CHOp) was used as control. As an additional control, hrNAG was denatured by heating for 15 min at 100°C and then used for binding assays. In pre-incubation experiments, oocytes were placed for 30 min in medium containing hrNAG or CHOp, washed by pipetting through five droplets of medium and placed for 15 min in a fresh droplet before the addition of sperm. For sperm pretreatment, hrNAG was added to the capacitation well 2.25 h after the beginning of the sperm incubation. After a further 30 min, 2.5 ml of sperm suspension was centrifuged for 5 min at 600 g. The sperm pellet was resuspended in 5 ml of fresh medium and centrifuged again. The final pellet was then resuspended in 0.5 ml of medium and used for insemination.

**IVF assays**

Fresh oocytes (10–15/droplet) were placed in 50 μl droplets of TALP medium, and an aliquot of sperm previously capacitated for 3 h was added to a final concentration of 1 × 10$^5$ cells/ml (Bavister, 1989). After 3-h co-incubation at 37°C in 5% CO$_2$, oocytes were washed and fixed as previously described. To facilitate the visualization of sperm, we stained oocytes for 6–8 min with Hoechst 33342 (30 μg/ml in 2.3% sodium citrate : ethanol 3 : 1), washed in citrate : ethanol and mounted under 25 μl of glycerol : citrate (9 : 1 v/v) containing 20 mg/ml of N-propylgalate. Oocytes were observed under a Nikon Optiphot microscope with epifluorescent illumination, and the proportion with penetrated sperm (decondensed heads) was determined. Different incubation procedures were used to analyse the effects of lactone (1 mM) or hrNAG (1.6 μg/ml) during IVF. For hrNAG, only its effect when present during the entire co-incubation period (3 h) was assayed, whereas in the case of lactone, additional experiments analysing its effect after primary binding were also carried out. Lactone was added after the first 45 min of co-incubation (addition) or, alternatively, oocytes were transferred to sperm-free lactone-containing droplets (transfer) after an initial 45 min under control conditions. Control assays for this last procedure consisted of the transfer of the sperm–oocyte complexes to droplets free of both sperm and inhibitor.

**Oocyte activation followed by IVF**

Oocyte activation was induced using two treatments: incubation in TALP-Sr (Fraser, 1987) or in regular TALP supplemented with 10 μM calcium ionophore A23187 (Miller et al., 1993a). These activation procedures were carried out in the presence or absence of 1 mM lactone. As controls, oocytes were subjected to a similar manipulation in regular TALP (no activation). After a 30-min incubation under 5% CO$_2$ at 37°C, oocytes were washed by pipetting through five droplets of TALP-HEPES. These oocytes were then used to carry out IVF assays under control conditions. Activation was verified by the presence of at least one female pronucleus after staining with Hoechst (usually two female pronuclei or one plus the second polar body were visible) (Rougier and Werb, 2001).

**Expression of results and statistical analysis**

Enzyme activity was expressed in arbitrary units [fluorescent units (FU)] assigned to the signal by the fluorimeter. Other results were expressed as the percentage of acrosome-reacted sperm (%AR); the percentage of oocytes with bound sperm or the percentage of penetrated and activated oocytes. In some cases, results were normalized to the control values, which were considered as 100%. Inhibition was calculated as: (control – treated) × 100/ control. Graphics represent averages ± SEM of 3–12 different experiments. To assess normal distribution, we converted percentages to ratios and subjected all data to the arcsine square root transformation. Statistical analysis was performed using the parametric Dunnett’s test for multiple comparisons. All statistical procedures were carried out with the aid of GraphPad software.
Results

A previous study from this laboratory reported that GlcNAc was able to inhibit sperm primary binding to ZP, suggesting that these residues on the zona would be involved in the association with sperm (Zitta et al., 2004). Taking into account that terminal GlcNAc residues are substrates for NAG, we analysed the involvement of this enzyme during hamster sperm–ZP interaction.

To determine whether the ZP could be a ligand for NAG, we analysed the ability of soluble ZP to inhibit enzyme activity. A dose-dependent inhibition of NAG activity by soluble ZP was detected, suggesting that the sperm enzyme is able to recognize the oocyte extracellular matrix (Figure 1).

NAG in sperm–ZP binding

To directly check the participation of a sperm NAG during interaction with the ZP, we evaluated the ability of different agents to inhibit the hamster sperm enzyme. Sperm NAG activity was measured in the presence of increasing concentrations of its monosaccharide substrate (GlcNAc) and two specific inhibitors lactone and thiazoline (Findlay and Levvy, 1960; Knapp et al., 1996). Lactone was the most powerful inhibitor, with a maximum effect at 0.3 mM (Figure 2), whereas the other agents reached significant inhibition at much higher concentrations (EC50: 0.02, 7.5 and 32 mM for lactone, thiazoline and GlcNAc, respectively).

When the inhibitors lactone and thiazoline were tried during IVF assays, not only sperm–ZP interaction was affected but also spontaneous AR was inhibited (see below). To avoid this latter effect, we analysed sperm–ZP binding using salt-stored oocytes and a medium where Ca2+ was replaced by Sr2+, which allows binding to ZP but not AR (Marín-Briggler et al., 1999; Zitta et al., 2004). Under these conditions, lactone significantly reduced sperm binding (Figure 3).

The effect of a specific NAG substrate on sperm binding to the ZP was also determined. The presence of a saturating concentration of p-nitrophenyl-N-acetylglucosaminide (PNP-GlcNAc, 3 mM) reduced sperm binding when compared with controls with no addition or vehicle alone (Figure 4).

As an alternative approach, exogenous NAG was included in functional assays to determine whether it was able to compete with the sperm enzyme and affect its interaction with the ZP. Human recombinant

Figure 1. Hamster sperm N-acetylglucosaminidase is inhibited by solubilized zona pellucida (ZP). Soluble ZP was obtained by acid treatment of salt-stored hamster oocytes. Increasing concentrations of soluble ZP were added during enzyme assays. Results are expressed as the activity (in arbitrary fluorescent units) relative to control (vehicle) and represent the mean ± SEM of three experiments.

Figure 2. Dose–response of hamster sperm N-acetylglucosaminidase (NAG) inhibition by different agents. Increasing concentrations of lactone (◦), thiazoline (○) or N-acetylglucosamine (●) were included during hamster sperm NAG enzyme assays. Results were normalized to control values (no addition) and represent the mean ± SEM of three experiments.

Figure 3. A specific N-acetylglucosaminidase inhibitor affects sperm primary binding to zona pellucida. Salt-stored hamster oocytes were incubated with capacitated sperm in TALP-Sr in the absence (control) or the presence of lactone. After 45 min, oocytes were washed and those with bound sperm were quantified. Results were expressed as the percentage of oocytes with bound sperm and represent the mean ± SEM of four experiments. *P < 0.05 versus control.

Figure 4. A specific N-acetylglucosaminidase substrate inhibits sperm binding to the zona pellucida. Salt-stored hamster oocytes were incubated with capacitated sperm in TALP-Sr in the absence (control) or the presence of 3 mM PNP-GlcNAc (PNP). The addition of vehicle alone (dimethylsulphoxide) was used as an additional control (DMSO). Results are expressed as the percentage of oocytes with bound sperm and represent the mean ± SEM of three experiments. *P < 0.02.
NAG (hrNAG) produced by a stably expressing CHO cell line was purified from culture medium (Miranda et al., 2000). Sperm binding to the ZP was inhibited by the presence of hrNAG during the assay (Figure 5), whereas proteins from regular CHO cells produced no effect (see bar CHOp). This inhibitory effect was also obtained when hrNAG was used to pre-incubate the oocytes (see Pre-inc). No effect was observed when sperm were pre-incubated with hrNAG or when denatured hrNAG was used during the assays (data not shown).

Finally, to verify the identity of the molecule involved in the GlcNAc-mediated event, binding assays were carried out in the presence of a specific anti-NAG antibody. As shown in Figure 6, a significant reduction in sperm binding to the ZP was observed in the presence of the specific antibody, whereas no effect was produced by pre-immune IgG.

NAG and ZP penetration

Although our previous study results do not support the participation of GlcNAc residues beyond primary binding of hamster sperm to the zona (Zitta et al., 2004), NAG has been implicated in ZP penetration in mice (Miller et al., 1993b). Consequently, the putative participation of NAG during the penetration of hamster ZP by sperm was analysed by including a specific enzyme inhibitor during IVF assays.

Taking into account that NAG inhibitors also affect acrosomal exocytosis, we investigated conditions under which enzyme activity but not AR was affected. Because this had not proved possible when using GlcNAc (Zitta et al., 2004), both lactone and thiazoline were assayed. Following incubation of capacitated sperm for 3 h with these NAG inhibitors, 1 mM lactone was selected for the subsequent assays, because it was found not to affect AR (Table I) while efficiently reducing enzyme activity (Figure 2). When lactone was included in the medium during IVF assays, it produced a 52 ± 5% inhibition in fertilization. The magnitude of this effect was not greater than that observed in the binding assay, suggesting that the only step impaired would be the primary binding to the ZP. To verify that no other event of sperm–oocyte interaction was being affected, we added lactone 45 min after the beginning of gamete co-incubation, once primary binding had taken place. Either the inhibitor was added to the incubation droplet or the oocytes were transferred to a new drop containing the inhibitor but not sperm. Results showed that the presence of lactone after co-incubation did not reduce penetration rates, whereas its presence during the entire assay inhibited fertilization (Figure 7).

The specific NAG substrate PNP-GlcNAc could not be used in IVF assays because it inhibited spontaneous AR (68 ± 8% versus control). On the contrary, hrNAG did not affect the AR (83 versus 80% for hrNAG and control, respectively), but its presence in the assay medium or during oocyte pre-incubation reduced oocyte penetration (Figure 8).

Table I. Effect of N-acetylglucosaminidase inhibitors on hamster sperm spontaneous acrosome reaction

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<tr>
<th>Concentration (mM)</th>
<th>Acrosome reaction (% control)</th>
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<tbody>
<tr>
<td>1</td>
<td>Lactone 91 ± 3</td>
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<tr>
<td></td>
<td>Thiazoline 83 ± 5</td>
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<tr>
<td>10</td>
<td>61 ± 4*</td>
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<td>51 ± 4*</td>
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Different concentrations of lactone and thiazoline were added to capacitated (2.5 h incubated) hamster sperm. The percentage of reacted cells was scored 3 h later and normalized against control values (considered as 100%). *P < 0.05 versus control.

Figure 5. Exogenous N-acetylglucosaminidase (NAG) reduces sperm binding to zona pellucida. Human recombinant NAG (hrNAG) expressed in CHO cells was used during binding assays (Co-inc) or for oocyte pre-incubation (Pre-inc). The same amount of total protein from regular CHO cells was used as control (CHOp). Results represent the mean ± SEM of four experiments. *P < 0.05 versus control.

Figure 6. Effect of anti-N-acetylglucosaminidase (anti-NAG) on sperm binding to the zona pellucida. Salt-stored hamster oocytes were incubated with capacitated sperm in TALP-Sr in the presence of anti-NAG (aNAG, 50 μg/ml) or pre-immune immunoglobulin G (IgG). A drop with no additions is also shown (control). Results are expressed as the percentage of oocytes with bound sperm and represent the mean ± SEM of three experiments. *P < 0.05.

Figure 7. Effect of lactone on the different steps of sperm-zona pellucida interaction. IVF assays were carried out under different conditions: sperm were co-incubated with oocytes in the presence of 1 mM lactone, either during the entire assay (All) or after the first 45 min of co-incubation (Addition). A droplet containing no lactone was used as control. In a different approach, after 45 min of co-incubation, oocytes were transferred to a sperm-free droplet supplemented with lactone (Transfer). For this latter treatment, a second lactone-free droplet was used as control. Results are expressed as the percentage of penetrated oocytes and normalized against the respective control (considered as 100%). Numbers represent the mean ± SEM of six experiments. *P < 0.05 versus control.
activation did not modify these results. The absence of remnant inhibitor that could be masking any effect was verified when oocytes pre-incubated with lactone under non-activating conditions produced results similar to controls (Figure 9).

Discussion

The participation of NAG during the fertilization process in several species has been supported by different groups (Farooqui and Srivastava, 1980; Prody et al., 1985; Racan, 1986; Godknecht and Honegger, 1991; Miller et al., 1993b; Martinez et al., 2000; Miranda et al., 2000; Perotti et al., 2001). It has been reported to be involved not only in ZP penetration mediated by enzymatic digestion (Farooqui and Srivastava, 1980; Miller et al., 1993b) but also in sperm binding to the oocyte extracellular coat (Godknecht and Honegger, 1991; Sada et al., 1992; Godknecht and Honegger, 1995; Miranda et al., 2000). Considering our previous studies carried out in humans (Brandelli et al., 1994; Brandelli et al., 1995; Miranda et al., 1997; Miranda et al., 2000), and the last evidence supporting the relevance of GlcNAc in hamster sperm binding to the ZP (Zitta et al., 2004), we analysed the involvement of NAG in this GlcNAc-mediated event.

The putative participation of NAG in sperm–ZP interaction was initially suggested by the ability of solubilized ZP to inhibit enzyme activity. Contrary to what happened in the presence of synthetic inhibitors, only a partial effect could be achieved with soluble ZP. This could be related to the more complex chemical composition of ZP, which could reduce its accessibility to the enzyme active site. The specific involvement of NAG in primary binding to ZP was analysed by performing binding assays under conditions where AR could not take place. This can be achieved by replacing Ca^{2+} by Sr^{2+} in the incubation medium, and using salt-stored oocytes to avoid activation (Fraser, 1987), conditions previously validated (Yanagimachi et al., 1979; Boatman et al., 1988; Yoshimatsu et al., 1988; Marin-Briggiler et al., 1999; Zitta et al., 2004). In the present study, the specific NAG inhibitor 2-acetamido-2-deoxy-D-glucono-1,5-lactone and the substrate PNP-GlcNAc were able to reduce sperm binding to ZP. To determine the effect of the NAG substrate, the colorimetric reagent was required because the use of saturating concentrations of the fluorometric substrate was not possible owing to solubility restrictions.

The possible participation of NAG in an additional step during sperm–oocyte interaction was also analysed. The presence of lactone during IVF produced an inhibitory effect only when present during the entire assay but not when added after primary binding. This result confirmed the one obtained with salt-stored oocytes concerning the involvement of NAG in the early steps of sperm–ZP interaction. Moreover, it is interesting to note that the inhibition produced by lactone during IVF was similar to the one produced in binding assays. This is likely to be related to the fact that, unlike what occurred with GlcNAc (Zitta et al., 2004), it was possible to use a lactone concentration capable of affecting NAG without inhibiting AR. On the contrary, the NAG substrate PNP-GlcnAc could not be used in IVF assays because it inhibited AR.

Interestingly, several GlcNAc-related compounds (GlcNAc, lactone, thiouizolone and PNP-GlcnAc) have the ability to inhibit AR. This could be related to a signal transduction pathway relying on a GlcNAc–dependent interaction between two molecules located within the same plasma membrane, as recently reported for neutrophils (Huang et al., 2004). This interaction would be blocked by exogenous GlcNAc-related ligands. Nevertheless, the involvement of NAG in AR or the interference of GlcNAc-related compounds in some metabolic or signalling process cannot be ruled out. These possibilities are currently under study in our laboratory.

Taking into consideration that lactone did not modify the rate of fertilization when present after primary binding occurrence, it could
be inferred that NAG is not involved in ZP penetration or sperm–oocyte fusion. However, if the NAG inhibitor was interfering with the polyspermy block, as stated for mice (Miller et al., 1993a), opposing effects would be taking place and this could explain the lack of inhibition when the lactone was added late during the assay. To discard this possibility, oocytes were activated in the presence of lactone before using them for IVF assays. Given that this treatment did not modify oocytes penetrability, it can be concluded that NAG seems not to be involved in ZP penetration in the hamster.

The participation of NAG in sperm–oocyte interaction has been refuted because enzyme-deficient mice are fertile at a young age (Juneja, 2002). However, the disruption of NAG function has been reported to affect fertilization in different systems (Godknecht and Honegger, 1991; Miller et al., 1993b; Godknecht and Honegger, 1995; Perotti et al., 2001). Therefore, results coming from mutant mice should be analysed considering the possible existence of compensatory mechanisms in genetically modified animals.

The addition of exogenous NAG during functional assays also produced an inhibitory effect. Moreover, oocyte pre-incubation with hrNAG reproduced this inhibition, suggesting that oocytes were the target of the enzyme action, as previously reported for humans (Miranda et al., 2000). This result would be instinctively attributed to a catalytic action of hrNAG on the ZP. However, it could also be explained whether hrNAG simply binds to GlcNAc residues of the zona. As most glycosidases, NAG catalytic activity is optimum at acidic pH (4.5) and negligible at physiological values (data not shown). However, this enzyme is able to bind to its substrate at a neutral pH (Geiger et al., 1974) and has been directly involved in cellular adhesion events (Rauvala et al., 1981). If hrNAG would act catalytically, any GlcNAc-binding molecule could be the one involved in hamster sperm–ZP interaction. Nevertheless, the inhibitory effect produced by the specific anti-NAG antibody supports NAG as the GlcNAc-binding molecule involved in sperm–ZP binding in the hamster. Therefore, the participation of other molecules with the ability to bind this sugar cannot be ruled out. Preliminary results obtained in our laboratory suggest that galactosyltransferase would not be involved in sperm–ZP binding in the hamster (data not shown). This result together with the fact that the NAG substrate that inhibited sperm–ZP binding in the hamster did not produce the same effect in mice (Lopez et al., 1985) could be indicating that different molecular mechanisms would be acting during sperm–ZP interaction in both species.

Although NAG is assumed to be an acrosomal enzyme, results from our laboratory suggested its presence in sperm plasma membrane (Brandelli et al., 1994; Miranda et al., 1995; Miranda et al., 2000). This possibility has been supported by other groups (Cattaneo et al., 1997; Flesch et al., 1998; Hutchinson et al., 2002) and recent results from our laboratory (data not published). The evidence suggests that, besides the NAG located inside the acrosome, there would be a population weakly associated with the sperm plasma membrane (Hutchinson et al., 2002 and data not published). This would be the one involved in primary binding to the ZP.

The optimum pH required for NAG activity could appear paradoxical for the involvement of this enzyme in sperm–oocyte interaction. However, it could be speculated that the absence of an appropriate environment for the expression of the catalytic activity could sustain NAG to function as a lectin-like receptor. This possibility has been suggested for other enzymes involved in sperm–ZP interaction (Jones and Brown, 1987; Benau and Storey, 1988; Macek and Shur, 1988; Hunnicutt et al., 1996).

In summary, the results presented in this article suggest the participation of NAG in hamster sperm primary binding to the ZP.

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N-acetylglucosaminidase in sperm-ZP primary binding


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