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An intact acrosome is required for the chemotactic response to progesterone in mouse spermatozoa

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Abstract

Mammalian sperm become fertilization-competent in the oviduct, during a process known as capacitation that involves the acquisition of the ability to exocytose the acrosome but also the chemotactic responses – both of which contribute to successful fertilization. Chemotaxis is used by spermatozoa to orient and to locate the egg; the acrosome reaction facilitates sperm binding to and fusing with the egg membrane. Mammalian spermatozoa are able to sense picomolar concentrations of progesterone, which drives chemotactic behavior. The state of the acrosome during the chemotactic response, however, is unknown. Genetically modified mouse spermatozoa were employed in a chemotaxis assay under fluorescence microscopy to evaluate the acrosome status while swimming, allowing us to elucidate the acrosome integrity of sperm responding to progesterone-induced chemotaxis. We first showed that wild-type mouse spermatozoa chemotactically respond to a gradient of progesterone, and that the genetic modifications employed do not affect the chemotactic behavior of sperm to progesterone. Next, we found that acrosome-intact, but not acrosome-reacted, spermatozoa orient and respond to picomolar concentrations of progesterone and that chemotaxis normally occurs prior to the acrosome reaction. Our results suggest that premature commitment to acrosome exocytosis leads to navigation failure, so proper control and timing of the acrosome reaction is required for fertilization success and male fertility.

Keywords

chemotaxis; progesterone; acrosome; sperm

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Introduction

A sperm's arrival at the egg surface requires more than sperm motility. Several mechanisms have been proposed to transport spermatozoa from the isthmus of the oviduct to the site of fertilization, such as peristalsis and several different forms of navigation, or "taxis" (Giojalas et al., 2015). Chemotaxis, for example, involves sperm movement along a defined gradient of an attractant molecule (Giojalas et al., 2015), which guides spermatozoa to and retains them at the site of fertilization (Guidobaldi et al., 2012). Spermatozoa are attracted to molecules with diverse chemical nature, such as peptides, odorants, and steroids (Giojalas et al., 2015). Progesterone, in particular, has been studied extensively with human and rabbit spermatozoa (Teves et al., 2006, 2009, 2010; Guidobaldi et al., 2008; Oren-Benaroya et al., 2008; Blengini et al., 2011; Gatica et al., 2013; Uñates et al., 2014). This hormone is secreted by the cumulus cells that surround the egg after ovulation (Bar-Ami et al., 1989; Vanderhyden and Tonary, 1995; Chian et al., 1999; Yamashita et al., 2003; Guidobaldi et al., 2008), and may form a chemical gradient from the centre towards the periphery of the cumulus layer and beyond (Teves et al., 2006; Guidobaldi et al., 2008). Spermatozoa of humans and rabbits are attracted by very low doses of progesterone, exhibiting clear chemotaxis towards this hormone; however, such directed behaviour first requires that sperm undergo capacitation (Giojalas et al., 2015).

Spermatozoa must exocytose its acrosome in order to bind to and fuse with the egg plasma membrane (Cuasnicú et al., 2016). Most fertilizing mouse spermatozoa complete this acrosome reaction before reaching the zona pellucida (Jin et al., 2011), probably while travelling through the upper isthmus (Hino et al., 2016; Muro et al., 2016; Spina et al., 2016). Therefore, an intriguing question is whether or not the responsiveness to a chemoattractant can change depending on acrosome's integrity. Considering early observations reporting that pharmacological induction of the acrosome reaction leads to a loss of the capacity of spermatozoa to be attracted towards follicular fluid (Cohen-Dayag et al., 1995; Fabro et al., 2002), we hypothesized that an intact acrosome is required for sperm chemotaxis to act towards progesterone.

Herein, the acrosome status of swimming spermatozoa exposed to a gradient of a picomolar range of progesterone was evaluated by fluorescence video microscopy and image analysis. We used transgenic mice producing spermatozoa with dual fluorescence tags (enhanced green fluorescence protein [EGFP] in the acrosome and DsRed2 in mitochondria) (Hasuwa *et al.*, 2010), which allowed us to clearly distinguish sperm with intact versus reacted acrosomes based on the presence or absence of EGFP. Thus, the acrosome status and chemotactic orientation were simultaneously evaluated for individual spermatozoa.

Results

We first verified that mouse spermatozoa chemotactically respond to a progesterone gradient, as reported for human and rabbit sperm (Teves et al., 2006). Video microscopy followed by image analysis was used to assess the classic chemotactic dose-response curve, based on varying progesterone-dosage gradients, as previously reported (Guidobaldi et al., 2008). A significant increase in the percentage of oriented spermatozoa was observed under

a 0-to-100 pM gradient of progesterone compared to negative control, in which only culture medium was used (Fig. 1A).

Recently, we developed a method, the Sperm Selection Assay (SSA), by which only capacitated spermatozoa can be selected by means of sperm chemotaxis towards progesterone (Gatica et al., 2013). Using the Sperm Selection Assay, we determined the proportion of spermatozoa recruited to a well that contains progesterone. Significantly more mouse sperm were found in a well containing 100 pM progesterone than in the absence of this hormone (Fig. 1 B). Similar to human and rabbit spermatozoa (Teves et al., 2006), a progesterone gradient (0–100 pM) did not affect sperm velocity or the pattern of wild-type mouse sperm movement (Fig. 1 C and D). Thus, mouse spermatozoa are also chemotactically oriented by a picomolar gradient of progesterone.

We next tested if transgenic mouse spermatozoa expressing acrosome EGFP and mitochondria DsRed2 also exhibit chemotactic behavior towards progesterone. A progesterone dose-response curve similar to wild-type mice was observed, with a maximum proportion of oriented spermatozoa obtained using a 100-pM gradient (Fig. 2A). This result was confirmed by the higher number of spermatozoa recruited by progesterone in the Sperm Selection Assay (Fig. 2B).

Sperm from these transgenic mice provide the possibility to analyze acrosome-intact and -reacted spermatozoa as distinct subpopulations, so we next assessed the kinetic parameters of these subpopulations. Curvilinear sperm velocity did not depend on acrosome status or the presence of progesterone stimulation (Fig. 2C). An increase in the quantity of transitional spermatozoa (those exhibiting a higher lateral amplitude of the head, but still moving progressively) was observed in the acrosome-reacted sperm subpopulation; this response was independent of progesterone (Fig. 2D). Together, these results suggest that the genetic knock-in does not affect the sperm chemotactic response towards progesterone.

Finally, the percentage of oriented spermatozoa with intact or reacted acrosomes was determined for a sperm population traveling towards a 100-pM progesterone gradient. The percentage of oriented, acrosome-intact spermatozoa was significantly augmented in the presence of a chemotactic progesterone gradient, whereas the percentage of oriented, acrosome-reacted spermatozoa did not change (Fig. 3A). Representative sequential images of swimming spermatozoa, for which the acrosome status can be easily observed, are shown in Figure 3B and in the Supplemental Video (Video S1). These results suggest that only spermatozoa with an intact acrosome can exhibit chemotactic behavior.

Discussion

Simultaneous observation of the chemical orientation and acrosome status in spermatozoa of transgenic mice possessing a fluorescent acrosome and mitochondria allowed us to concluded that only acrosome-intact spermatozoa can be guided by a chemical attractant gradient. This observation is consistent with previous reports that the pharmacological induction of the acrosome reaction abolishes the chemotactic response towards follicular fluid in human (Cohen-Dayag et al., 1995) and rabbit (Fabro et al., 2002) spermatozoa.

We also provided evidence for the occurrence of chemotactic behavior towards progesterone for wild-type and transgenic mouse spermatozoa. The chemotactic response of mouse sperm was observed at picomolar concentrations of progesterone, similar to human and rabbit (Teves et al., 2006) and bovine and equine spermatozoa (unpublished). Furthermore, the chemotactic response to progesterone by mammalian sperm appears to be elicited independently of their origin (from epididymis or ejaculated samples) or preservation status (fresh or cryopreserved semen samples) (Giojalas et al., 2015).

For many years, the acrosome reaction was postulated to occur upon sperm interaction with the zona pellucida of ovulated eggs (Buffone et al., 2014; Hirohashi, 2016). Yet, recent studies reported that acrosomal exocytosis is triggered before reaching the ampulla, where fertilization occurs in the mouse (Hino et al., 2016; Muro et al., 2016; Spina et al., 2016). Our current study suggests that spermatozoa require an intact acrosome to sense a chemical guidance cue, leading to the hypotheses that chemotaxis precedes acrosomal exocytosis and that both processes sequentially occur en route to the egg. These observations also give rise to several questions: (i) Where is the progesterone receptor located in mouse spermatozoa? Since an intact acrosome is needed for chemotaxis to occur, it might be hypothesized that the progesterone receptor or related signaling components may reside on the plasma membrane over the acrosome. This hypothesis is consistent with the recent identification of a sperm membrane protein that binds progesterone, and is distributed over the acrosome in mouse spermatozoa (Miller et al., 2016). (ii) Where in the oviduct is chemotaxis to progesterone occurring? Considering that chemotaxis may precede the acrosome reaction and that seems to occur in the upper isthmus of the mouse (Spina et al., 2016), chemotaxis may take place somewhere along the isthmus. (iii) Does progesterone stimulate both sperm processes? In humans, a picomolar gradient of progesterone stimulates different sperm subpopulations to undergo chemotaxis, primes sperm for the acrosome reaction, or triggers the acrosome reaction itself (Uñates et al., 2014). (iv) What molecule(s) guide the acrosome-reacted spermatozoon to the oocyte-cumulus complex? A chemotactic response towards CRISP1, a protein secreted by the cumulus cells, was observed in the mouse (Ernesto et al., 2015), so other molecules released by the cumulus layer may chemically attract sperm towards the egg.

Our results lead to the notion that premature acrosome exocytosis will limit or prevent progesterone-mediated sperm navigation. Thus, precise control of the acrosome reaction timing and location is required for fertilization success.

Materials and Methods

Animals

The experiments were performed with spermatozoa from two mouse strains: wild-type Balb-c and a transgenic line possessing a double gene-knock-in [BDF1-Tg (CAGmtDsRed2, Acr-EGFP) RBGS0020sb], whose males produce spermatozoa expressing soluble EGFP in the acrosome and DsRed2 in the midpiece mitochondria (Hasuwa et al., 2010). Animals of about three-months-old were used in this study, and were treated in accordance with the Guides of Animal Care (NIH), with the approval of the Institutional Committee of Animal

Care (#10/2015, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba).

Sperm preparation

Spermatozoa from wild-type and transgenic mice were surgically obtained from the cauda epididymis, and then incubated under capacitating conditions by suspending them in HTF (Irvine Scientific, California, USA) supplemented with 15% Synthetic Serum Substitute (Irvine Scientific, California, USA). Sperm populations were incubated for 90 min at 37°C under an atmosphere of 5% CO_2 in air, at a concentration of 6×10^6 cells/mL.

Sperm chemotaxis and determination of other kinetic parameters

Chemotaxis assays were performed according to Fabro et al. (2002). Briefly, 6×10^6 cells/mL, previously incubated under capacitating conditions, were placed in one compartment of a chemotaxis chamber while progesterone was placed in the other, forming a concentration gradient in the capillary space between the two wells, where sperm movement was analyzed (Fabro et al., 2002).

The sperm tracks were recorded by following the sperm head under phase-contrast microcopy, for wild-type mouse sperm, or the nucleus-oriented end of the DsRed-positive midpiece recorded by fluorescence-microscopy alternating with phase contrast illumination, for transgenic mouse sperm. A super-sensitive video camera (NC-R550b; NEC) and a blue/green dual band-pass filter set enabled simultaneous visualization of EGFP and DsRed2 fluorescence in swimming spermatozoa. The cells were recorded at 30 Hz, and the trajectories of 150 randomly selected spermatozoa per treatment were determined with ImageJ software (ver. 1.41, NIH, USA) and the MtrackJ plugin (ver. 1.1.0; Meijering *et al.*, 2012). The percentage of oriented spermatozoa (those facing the ascending gradient of progesterone) was determined using SpermTrack software (ver. 4.2.5, UNC, Argentina).

A dose-response assay was performed with several progesterone concentrations to assess sperm chemotaxis; culture medium without progesterone was used as negative control. Sperm curvilinear velocity and pattern of movement was determined in the same cells evaluated for chemotaxis, according to previous work (Fabro et al., 2002).

Sperm Selection Assay

This accumulation assay was also used to evaluate sperm chemotaxis towards progesterone (Gatica et al., 2013). Briefly, the sperm suspension, at a concentration of 6×10^6 cells/mL, was loaded in one compartment (W1) while 100 pM progesterone (or culture medium, as negative control) was loaded in another compartment (W2). After 20 min of incubation, the sperm population was recovered from W2 for counting. The percentage of sperm accumulation in W2 was calculated as the difference between the percentage of spermatozoa recovered from W2 in the presence or absence of progesterone.

Statistical analysis

Statistically significant differences among treatments were determined by one-way ANOVA and the DGC (Di Rienzo et al., 2002) post-hoc test, by means of the Infostat software (Universidad Nacional de Córdoba) (Di Rienzo et al., 2011). Differences were considered statistically significant at $p \leq 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EGFP enhanced green fluorescent protein

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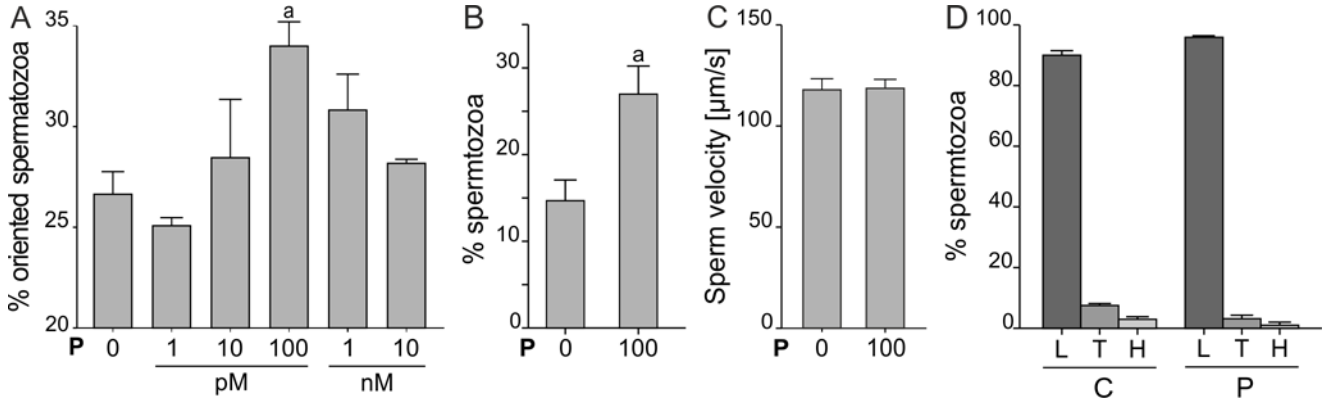

Figure 1. Wild-type mouse spermatozoa chemotactically respond to a picomolar gradient of progesterone

A, Percentage of oriented mouse spermatozoa exposed to picomolar and nanomolar progesterone concentration gradients or culture medium without the steroid. **B**, Percentage of spermatozoa accumulated in the well containing 100 pM progesterone versus culture medium alone, in the Sperm Selection Assay. **C–D**, Curvilinear velocity (C) and percentage of spermatozoa with different patterns of movement (L, linear; T, transitional; H, hyperactivated) (D) following exposure to a 100-pM gradient of progesterone or culture medium alone. Data are expressed as mean ± standard error of 3–6 independent experiments. [a], significant differences vs. without progesterone ($p \leq 0.05$).

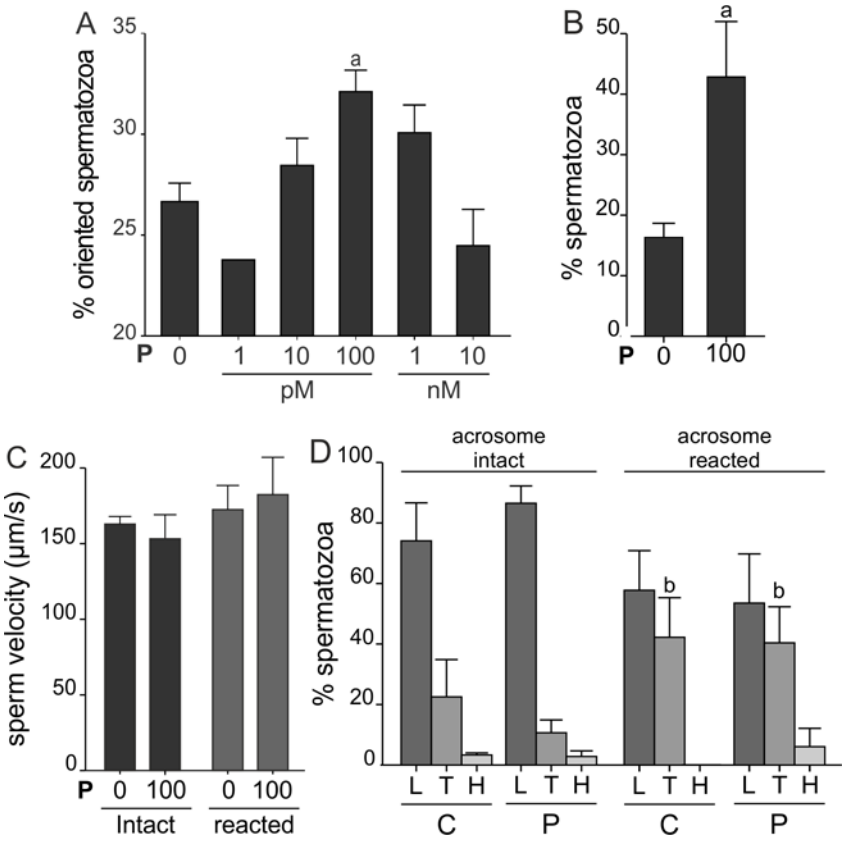

Figure 2. Transgenic mouse spermatozoa chemotactically respond to a picomolar gradient of progesterone

A, Percentage of oriented spermatozoa exposed to picomolar and nanomolar progesterone concentration gradients, or culture medium alone. **B**, Percentage of accumulated spermatozoa in the well containing progesterone or culture medium alone, in the Sperm Selection Assay. **C**, Curvilinear velocity in acrosome-intact and -reacted spermatozoa exposed to 100 pM progesterone gradients. **D**, Percentage of spermatozoa showing different patterns of movement (L, linear, T, transitional, HA, hyperactivated), in intact and acrosome-reacted cells exposed to a 100-pM gradient of progesterone or culture medium alone. Data are expressed as mean ± standard error of 3–6 independent experiments. [a], significant difference *vs.* without progesterone ($p \leq 0.05$); [b], significant differences between acrosome-intact and -reacted spermatozoa ($p \leq 0.05$).

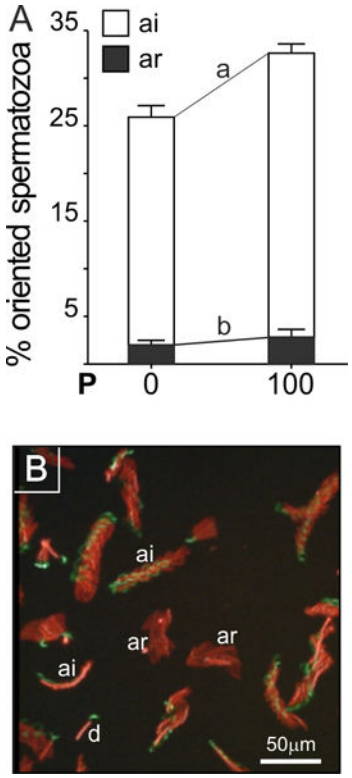

Figure 3. Transgenic mouse spermatozoa need an intact acrosome for the chemotactic response to progesterone

A, Percentage of oriented spermatozoa according to acrosome status (ai, acrosome-intact; ar, acrosome-reacted), in the presence or absence of 100 pM progesterone. Data are expressed as mean ± standard error of 6 independent experiments. **a**, significant differences *vs.* without progesterone ($p \leq 0.004$); **b**, no significant differences *vs.* without progesterone ($p = 0.413$). **B**, Sequential images of moving spermatozoa showing examples of acrosome-intact (ai), acrosome-reacted (ar), and immobile (d) spermatozoa.