Sperm from β1,4-galactosyltransferase I-null mice exhibit precocious capacitation

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Accepted 29 September 2003

Development 131, 491-501
Published by The Company of Biologists 2004
doi:10.1242/dev.00885

Summary

Mammalian sperm must undergo a physiological maturation, termed capacitation, before they are able to fertilize eggs. Despite its importance, the molecular mechanisms underlying capacitation are poorly understood. In this paper, we describe the capacitation phenotype of sperm lacking the long isoform of β1,4-galactosyltransferase I (GalT I), a sperm surface protein that functions as a receptor for the zona pellucida glycoprotein, ZP3, and as an inducer of the acrosome reaction following ZP3-dependent aggregation. As expected, wild-type sperm must undergo capacitation in order to bind the zona pellucida and undergo a Ca^{2+} ionophore-induced acrosome reaction. By contrast, GalT I-null sperm behave as though they are precociously capacitated, in that they demonstrate maximal binding to the zona pellucida and greatly increased sensitivity to ionophore-induced acrosome reactions without undergoing capacitation in vitro. The loss of GalT I from sperm results in an inability to bind epididymal glycoconjugates that normally maintain sperm in an 'uncapacitated' state; removing these decapacitating factors from wild-type sperm phenocopies the capacitation behavior of GalT I-null sperm. Interestingly, capacitation of GalT I-null sperm is independent of the presence of albumin, Ca^{2+} and HCO_3^-; three co-factors normally required by wild-type sperm to achieve capacitation. This implies that intracellular targets of albumin, Ca^{2+} and/or HCO_3^- may be constitutively active in GalT I-null sperm. Consistent with this, GalT I-null sperm have increased levels of cAMP that correlate closely with both the accelerated kinetics and co-factor-independence of GalT I-null sperm capacitation. By contrast, the kinetics of protein tyrosine phosphorylation and sperm motility are unaltered in mutant sperm relative to wild-type. These data suggest that GalT I may function as a negative regulator of capacitation in the sperm head by suppressing intracellular signaling pathways that promote this process.

Key words: Capacitation, Fertilization, Sperm, Acrosome reaction, cAMP

Introduction

Gamete interaction is a crucial event during fertilization when receptors on sperm bind to ligands on the extracellular coat of the egg, called the zona pellucida in mammals. Binding to the zona pellucida clusters the sperm receptor, activating intracellular signaling cascades that stimulate the exocytosis of the acrosome. The contents of the acrosome enable sperm to penetrate the zona pellucida and subsequently bind to the egg plasma membrane where gamete fusion occurs (Talbot et al., 2003). Before ejaculated sperm can complete any of these steps, they must undergo a series of biochemical, morphological and behavioral changes, collectively called capacitation. Although capacitation is obligatory for successful fertilization, the underlying molecular mechanisms are not fully understood.

One of the sperm proteins thought to function during fertilization is β1,4-galactosyltransferase I (GalT I; B4galt1 – Mouse Genome Informatics). GalT I normally serves as a biosynthetic enzyme in the Golgi apparatus; however, in certain cell types, it also functions as a cell-surface receptor for extracellular glycoside ligands (reviewed by Rodeheffer and Shur, 2002). Mouse sperm are unusual in that all of their GalT I is present on the plasma membrane overlying the acrosome, where it functions as a receptor for the major sperm-binding ligand in the zona pellucida, ZP3. ZP3-dependent aggregation of GalT I initiates pertussis toxin-sensitive signaling pathways that contribute to induction of the acrosome reaction (Gong et al., 1995; Lopez et al., 1985; Miller et al., 1992; Scully et al., 1987; Shi et al., 2001; Shur and Hall, 1982a; Shur and Neely, 1988). Consistent with this, sperm that overexpress GalT I bind more ZP3, have higher rates of G-protein activation, and undergo accelerated acrosomal exocytosis, relative to normal sperm (Youakim et al., 1994). Similarly, sperm from males devoid of all GalT I, or devoid of just the surface isoform of GalT I, fail to bind ZP3 or undergo a ZP3-induced acrosome reaction. However, despite their inability to bind ZP3, GalT I-null sperm are still able to bind to the egg coat and fertilize eggs in vitro, albeit at very low efficiency (Lu and Shur, 1997). The fertility of GalT I-null mice suggests that sperm binding to the zona pellucida requires a GalT-ZP3-independent interaction (Rodeheffer and Shur, 2004). Furthermore, there is reason to believe that the process of capacitation may be accelerated in GalT I-null sperm, relative to wild-type sperm. The capacitation phenotype of GalT I-null sperm is the subject of this report.

Capacitation is defined as the complement of physiological changes that sperm undergo in the female reproductive tract before gaining the competence to fertilize the egg (Austin, 1952; Chang, 1951). Capacitation can also occur in vitro in medium mimicking the fluid of the female reproductive tract (Oliphant and Brackett, 1973). In vitro capacitation results in increased sperm metabolism (Fraser and Herod, 1990; Hoppe, 1976), alterations in plasma membrane fluidity (Wolf et al., 1986) and lectin reactivity (Johnson and Hunter, 1972; Talbot and Franklin, 1978), hyperactivated motility (Ho and Suarez, 2001), elevated intracellular pH (Zeng et al., 1996), membrane hyperpolarization (Demarco et al., 2002) and protein tyrosine phosphorylation (Visconti et al., 1995a). In many species, these events are negatively and positively regulated by factors in seminal plasma and in the fluid of the female reproductive tract, respectively. Membrane fluidity is dependent on the proportion of sterols (cholesterol and desmesterol) in the lipid bilayer, which is regulated by lipid vesicles in seminal plasma and cholesterol sinks, such as albumin, in the reproductive tract (Davis, 1981; Nimmo and Cross, 2003; Visconti et al., 1999). Ca^{2+} is essential for capacitation (Yanagimachi, 1982) and controls the maintenance of sperm motility, the display of hyperactivation (Ho et al., 2002; Ren et al., 2001) and the membrane fusion that occurs during the acrosome reaction (Yanagimachi and Usui, 1974). HCO_3^- is also required for capacitation (Lee and Storey, 1986) and functions with Ca^{2+} to regulate cAMP production (Garbers et al., 1982; Litvin et al., 2003). Additionally, HCO_3^- appears to mediate a wide range of processes via its direct stimulation of adenylyl cyclase (Chen et al., 2000; Okamura et al., 1985). HCO_3^-, and possibly the cAMP produced by this enzyme, leads to increased protein tyrosine phosphorylation (Visconti et al., 1995b), phospholipid disorder on the plasma membrane (Gadella and Harrison, 2002) and changes in motility (Wennemuth et al., 2003).

We suspected that GalT I-null sperm may undergo precocious capacitation based on three observations: (1) on wild-type sperm, epididymal glycoconjugates normally occupy or 'mask' the GalT I active site and maintain sperm in an 'uncapacitated' state (Shur and Hall, 1982b); (2) washing wild-type sperm releases these glycoconjugates and results in precocious binding to the zona pellucida without a requirement for in vitro capacitation; and (3) GalT I-null sperm appear as single, motile cells upon release from the epididymis, whereas wild-type sperm are initially clumped and relatively immotile (Lu and Shur, 1997). As GalT I-null sperm should be unable to bind these epididymal 'decapacitating' glycoconjugates, their rate of capacitation may be accelerated relative to wild-type sperm in which capacitation is dependent upon the removal of these epididymal factors.

The goal of this study is to test the hypothesis that GalT I-null sperm are precociously capacitated relative to wild-type sperm. By two distinct criteria, GalT I-null sperm appear to exhibit precocious capacitation: (1) GalT I-null sperm bind maximally to the zona pellucida independent of capacitation in vitro and (2) they are more sensitive to Ca^{2+} ionophore-induced acrosome reactions prior to and throughout capacitation, than are wild-type sperm. The accelerated rate of capacitation in GalT I-null sperm is correlated with both their inability to bind 'decapacitating' factors and to constitutively activated Ca^{2+}- and HCO_3^--dependent signaling cascades. The precocious capacitation phenotype of GalT I-null sperm is specific to events occurring in the sperm head, whereas tail-specific events, such as motility, are unaltered.

Materials and methods

Materials

All chemicals, unless otherwise noted, were purchased from Sigma (St Louis, MO). The gametes were collected and incubated in a modified Krebs-Ringer buffer, referred to as 'complete' medium in the text {120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 10 mM $NaHCO_3$, 0.36 mM $NaH_2PO_4{\cdot}H_2O$, 1.2 mM $MgSO_4{\cdot}H_2O$, 5.6 mM glucose, 1.1 mM sodium pyruvate, 25 mM TAPSO [3-[N-tris(hydroxymethy)methylamino]-2-hydroxypropanesulfonic acid], 18.5 mM sucrose, 1×penicillin/streptomycin (Gibco, Carlsbad, CA), and 6 mg/ml BSA [fatty acid free], pH 7.4}. When the requirement for albumin, Ca^{2+} or HCO_3^- was being tested, sperm were collected and incubated in 'incomplete' medium. Albumin or HCO_3^- were replaced with equal amounts of polyvinylpyrrolidone (PVP) [or polyvinyl alcohol (PVA)] or NaCl, respectively. Ca^{2+} was eliminated from the medium with no further changes. The incubations were carried out in atmospheric CO_2, but the pH of all solutions remained constant at 7.4.

Sperm collection

One cauda epididymis of strain-matched wild-type and long isoform GalT I-null mice (Lu and Shur, 1997) was dissected into 2 ml complete medium, and the other into 2 ml incomplete medium and shredded. The epididymides were incubated for 15 minutes at 37°C and the sperm were collected after filtration (3-35/27 Nitex, Sefar America, Kansas City, MO). The sperm suspension was centrifuged at 66 ***g*** for 5 minutes at 24°C and resuspended in fresh media (complete or incomplete) at a final concentration of $2{\times}10^6$ sperm/ml. Sperm were capacitated at 37°C for varying time periods in complete or incomplete medium before assaying in complete medium (Fig. 1). To determine whether decapacitating factors in sperm fluid alter capacitation, sperm were released from the epididymis by trituration (not swim out), filtered, centrifuged and resuspended in fresh media. The suspension was divided into two equal volumes and both pelleted a second time at 66 ***g*** for five minutes. One sample was resuspended in fresh media ('washed') while the second sample was resuspended in the original supernatant ('unwashed').

Zona pellucida-binding assays

Cumulus-oophorous masses were collected from the oviducts of superovulated 8-week-old CD-1 females (Charles River) into 0.2% hyaluronidase. Cumulus-free eggs were washed through 6 drops of complete medium via a glass pipette approximately twice the diameter of the egg. Two-cell embryos were collected into complete medium (but not washed) from the oviducts of superovulated females that were mated 15 hours earlier. 40,000 sperm (capacitated in complete or incomplete medium) were co-incubated with 30-40 ovulated eggs and 5-10 embryos (as a negative control) in 50 μl drops of complete medium for 30 minutes at 37°C. The eggs and embryos were washed through three drops of complete medium to remove unattached and loosely bound sperm and fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). The number of sperm bound to each egg and embryo was counted at 200× magnification using phase-contrast optics. The average number of sperm bound to the embryos was subtracted from the average number of sperm bound to eggs. The average of triplicate drops for each time point was determined and the data presented are the average of at least three experiments. As the number of sperm bound varied between experiments, 100% binding was defined for each experiment as the number of fully capacitated (60 minutes) sperm bound.

A23187-induced acrosome reaction

Sperm were collected, and at specific points during in vitro

capacitation (Fig. 1) a 100 μl aliquot of sperm was added to 0.5 mM A23187 in DMSO to give a final concentration of 10 μM A23187. An equal volume of DMSO alone was used as a control. For sperm capacitated in incomplete medium, the suspension was 'spiked' with the appropriate cofactor at the same time A23187 was added. The sperm were further incubated at 37°C for 10 minutes and fixed with 4% paraformaldehyde at 24°C. The acrosomes of the sperm were stained with Coomassie Blue following the procedure described (Larson and Miller, 1999). Three drops of 200 sperm each were counted for each time point and averaged. The data presented are the average of at least three experiments (±s.e.m.).

Measurement of cAMP

Sperm were collected as described. After filtration and centrifugation, the sperm were resuspended at a final concentration of 1×10^7 sperm/ml, and capacitated in vitro. The sperm were then centrifuged at 1000 ***g*** for 4 minutes and the supernatant was discarded. The cAMP was extracted from sperm with 100 μl 65% ice-cold ethanol for 15 minutes and then at –20°C for 1 hour. The insoluble material was washed with 100 μl 65% ethanol and the extracts were combined and centrifuged at 2000 ***g*** for 15 minutes at 4°C. The supernatants were dried under vacuum and the cAMP was detected using the cAMP Biotrak Enzyme immunoassay kit (Amersham Biosciences, Piscataway, NJ). Each time point was analyzed in duplicate samples and the data shown reflects the mean±s.e.m. for four experiments.

Anti-phosphotyrosine immunoblot

Sperm were collected as described, with the exception that the 'swim-out' period lasted for only 5 minutes instead of 15 minutes. Sperm lysates were prepared and analyzed as described (Visconti et al., 1995a). After the lysates were fractionated by SDS-PAGE, the proteins were transferred to nitrocellulose and blocked in 3% milk, 0.01% Tween-20, 0.9% NaCl, Tris pH 7.4. The membranes were probed with 1 μg/ml of anti-phosphotyrosine mouse monoclonal antibody, washed and subsequently incubated in a 1:10,000 dilution of goat anti-mouse IgG-HRP (both from Upstate Biotech, Lake Placid, NY). The membranes were washed again and developed with ECL Plus (Amersham Biosciences, Piscataway, NJ). After each experiment, the membranes were stripped and reprobed with 1 μg/ml of anti-α-tubulin mouse monoclonal antibody (Sigma) to assess consistency of protein loading. The blot shown is representative of six experiments.

Measurement of protein kinase A (PKA) activity

Sperm were collected as described and PKA activity was measured following the procedure described (Visconti et al., 1997) with a few exceptions. Owing to high contaminating PKA activity in epididymal fluid, sperm were capacitated for varying periods of time and subsequently washed three times with ice-cold medium by centrifugation at 200 ***g*** at 4°C followed by resuspension in fresh buffer. After the final wash, the sperm were resuspended at a concentration of 1×10^7 cells/ml for the kinase assay. Owing to potential loss of sperm during washing, each result (pmol ^{32}P-labeled kemptide) was normalized to the quantity of protein in each sample. Each time point was analyzed in triplicate, and the data shown reflects the mean±s.e.m. for three separate experiments.

Computer-assisted semen (epididymal sperm) analysis

Sperm were collected and capacitated in complete medium for 10 or 60 minutes at 37°C. Each sperm suspension (10 μl) was loaded onto a 20 μm MicroCell slide (Conception Technologies, San Diego, CA) and analyzed in duplicate using an IVOS sperm analyzer (Hamilton Thorne, Beverly, MA). The parameters of the CASA analysis were: frames acquired, 30; frame rate, 60 Hz, minimum contrast, 80; minimum cell size, three pixels; minimum static contrast, 80; straightness (STR) threshold, 8.0%; low VAP cutoff, 5.0 μm/s; low VSL cutoff, 11 μm/s; head size, non-motile, six pixels; head intensity, non-motile, 160; static head size, 1.0-2.9; static head intensity, 0.6-1.4; static elongation, 0-80; slow cells motile, no; magnification, 10×. The data presented are the average of data (±s.e.m.) collected from three males.

Statistical analysis

The probability that the data are statistically significant was calculated using a paired *t*-test through the www.graphpad.com website. Data in which $P<0.01$ are indicated with an asterisk.

Results

GalT I-null sperm show increased binding to the zona pellucida and ability to undergo the acrosome reaction prior to and during in vitro capacitation

Cauda epididymal sperm were collected and capacitated in complete medium for varying periods of time (Fig. 1), after which they were mixed with ovulated eggs and two-cell embryos. The gametes were incubated to allow binding to occur, unbound sperm were removed by washing, and the number of sperm bound to eggs was calculated. At 0 minutes of in vitro capacitation, wild-type sperm bind to ovulated eggs at 44% the level of fully capacitated sperm (Fig. 2). This value gradually increases to 100% binding by 60 minutes of capacitation. By contrast, GalT I-null sperm exhibit maximal binding (greater than or equal to 100%) prior

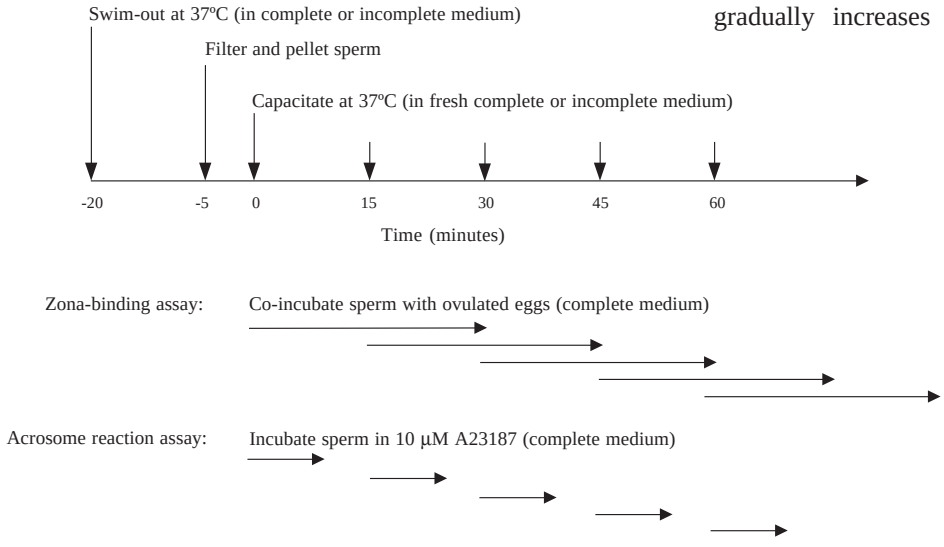

Fig. 1. Experimental protocols used to assay for capacitation in wild-type and GalT I-null sperm. Sperm were isolated, collected by centrifugation, and capacitated for various periods of time (starting at time 0) in either complete medium or medium deficient for specific components as described in the text. At the indicated time, aliquots of sperm were incubated either with eggs for 30 minutes (to monitor for zona binding activity), or with A23187 for 10 minutes (to monitor induction of the acrosome reaction).

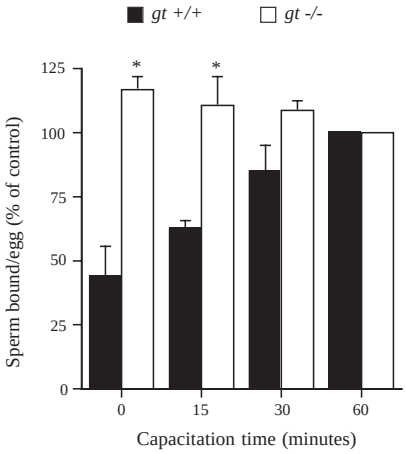

Fig. 2. GalT I-null sperm do not require capacitation in vitro to achieve maximal binding to the zona pellucida. Wild-type (*gt* +/+) and GalT I-null (*gt* –/–) sperm were capacitated for varying amounts of time before incubation with ovulated eggs. The binding of GalT I-null sperm to the zona pellucida is independent of capacitation in vitro, whereas wild-type sperm show a capacitation-dependent increase in their ability to bind the egg. Each bar represents the mean±s.e.m.; *n*=3 experiments. In a single experiment, the value for each data point represents the average of three determinations. Thus, each bar reflects data from nine assays, each containing 30-40 eggs. The average number of sperm bound to eggs at 0 minutes was: wild type, 8±0; GalT I-null, 25±2; at 15 minutes: wild-type, 14±2; GalT I-null, 25±4; at 30 minutes: wild-type, 19±5; GalT I-null 25±2; and at 60 minutes: wild type, 19±2; GalT I-null, 21 ± 2. In each experiment, this value was normalized to fully capacitated sperm (60 minutes of capacitation is equivalent to 100% bound), so that results from replicate assays could be compared. **P*<0.01.

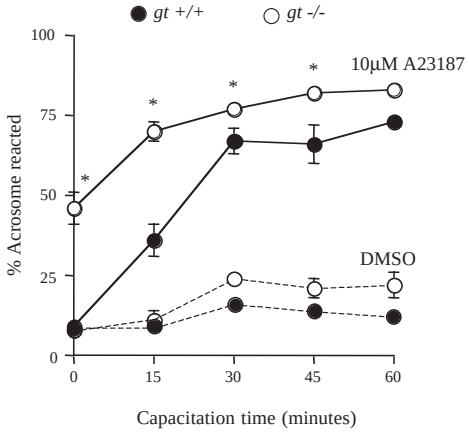

Fig. 3. GalT I-null sperm are more sensitive to Ca^{2+} ionophore than wild-type sperm during in vitro capacitation. Wild-type (*gt* +/+) and GalT I-null (*gt* –/–) sperm were capacitated for varying periods of time before incubation with 10 μM A23187 or an equal volume of DMSO. GalT I-null sperm show higher basal levels of sensitivity to ionophore-induced acrosome reactions, as well as greater sensitivity during capacitation in vitro. Each point represents the mean±s.e.m.; *n*=3 experiments. In a single experiment, the value of each data point represents three determinations. Thus, each data point reflects data collected from nine assays, each containing 200 sperm counted. **P*<0.01.

to, and at all times during, in vitro capacitation. According to this criterion, GalT I-null sperm exhibit a precociously capacitated phenotype.

As an independent measurement, we assayed the rate of capacitation in wild-type and GalT I-null sperm by measuring their ability to undergo an acrosome reaction. Normally, capacitated sperm undergo a ligand-induced acrosome reaction in response to binding ZP3 (Florman and Babcock, 1991) or progesterone (Osman et al., 1989); however, GalT I-null sperm do not bind ZP3 or undergo a ZP3-induced acrosome reaction (Lu and Shur, 1997). Furthermore, progesterone did not induce the acrosome reaction to a greater extent than did the DMSO control, in either wild-type or GalT I-null sperm (data not shown). However, we were able to monitor the ability of sperm to undergo acrosomal exocytosis, in a capacitation-dependent manner, following brief exposure to the calcium ionophore, A23187 (Fig. 1). A 10-minute exposure to 10 μM A23187 is sufficient to induce the acrosome reaction in wild-type sperm capacitated for at least 30 minutes, whereas uncapacitated sperm (0 minutes) are not responsive to A23817 (Fig. 3). Unlike that seen in wild-type, a significant proportion (~50%) of GalT I-null sperm undergo the acrosome reaction in response to A23187 at 0 and 15 minutes of in vitro capacitation. The increased sensitivity of GalT I-null sperm to ionophore-induced acrosome reactions is not caused by differences in the degree of spontaneous acrosome reactions, as neither wild-type or GalT I-null sperm undergo acrosomal exocytosis in the absence of A23187. Thus, by a second independent measure, GalT I-null sperm exhibit a precociously capacitated phenotype.

Removing epididymal glycoconjugates recapitulates the GalT I-null phenotype

The accelerated capacitation of GalT I-null sperm is correlated with their inability to bind epididymal-derived decapacitation factors. It is hypothesized that the presence of decapacitating factors on wild-type sperm prevents the initiation of plasma membrane and intracellular events that promote capacitation. Consequently, the loss of these factors from GalT I-null sperm may lead to the premature activation of these signaling cascades. This predicts that forcing wild-type sperm to release these factors would cause them to undergo precocious capacitation, and therefore phenocopy the GalT I-null sperm. In fact, this has been shown to be the case for zona pellucida-binding (Shur and Hall, 1982a), but these earlier studies did not address effects on capacitation rate or compare wild-type and GalT I-null sperm. Therefore, wild-type and GalT I-null epididymal sperm were collected, washed to completely remove epididymal decapacitating factors (labeled as ‘washed’), and assayed for their sensitivity to A23187 as a function of capacitation. To control for the effects of washing, sperm were treated in parallel but resuspended in the original supernatant containing the decapacitating factors (labeled as ‘unwashed’).

As shown in Fig. 4, wild-type and GalT I-null sperm continue to exhibit a time-dependent increase in ionophore-induced acrosome reactions after washing. The sperm are significantly less sensitive to ionophore after this procedure (compare Fig. 4 with Fig. 3) presumably because trituration is more disruptive than allowing the sperm to swim out of the epididymis. Nevertheless, washing wild-type sperm results in increased sensitivity to A23187 during in vitro capacitation. By

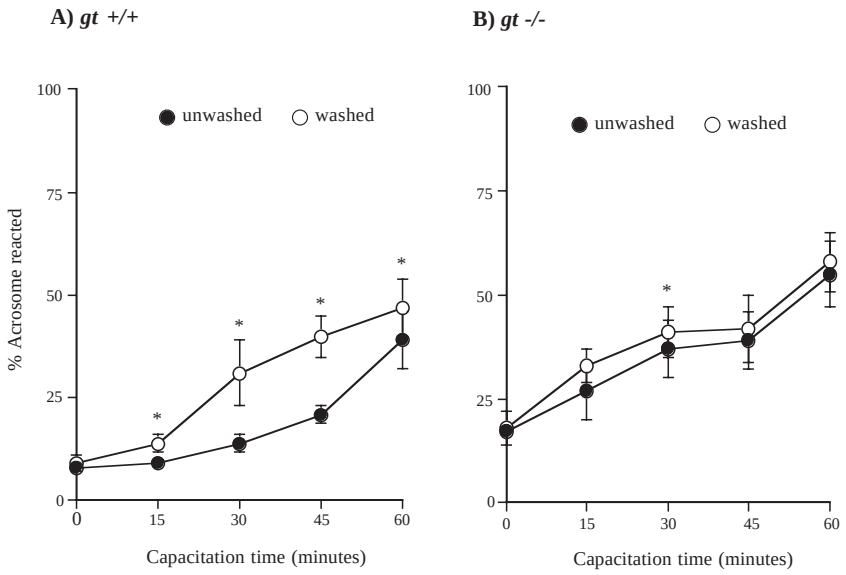

Fig. 4. Removing epididymal decapacitation factors increases the sensitivity of wild-type sperm, but not GalT I-null sperm, to Ca^{2+} ionophore. Wild-type (*gt* +/+, A) and GalT I-null (*gt* –/–, B) sperm were isolated from the cauda epididymis by trituration and resuspended in fresh media, centrifuged and resuspended again (washed). Control sperm were triturated and centrifuged in parallel but resuspended in the original supernatant (unwashed). The sperm were then capacitated for varying periods of time before incubation with 10 μM A23187 or an equal volume of DMSO (data not shown). Each point represents the mean±s.e.m.; *n*=3 experiments. In a single experiment, the value of each data point represents three determinations for a total of nine individual assays. A minimum of 200 sperm was counted for each determination. **P*<0.01.

contrast, washing GalT I-null sperm has no apparent effect, because as expected; they are already genetically 'washed' owing to the loss of GalT I, the binding site for the decapacitating factors (Shur and Hall, 1982b). Therefore, decapacitating factors bound to wild-type sperm appear to inhibit the initiation of capacitation; removal of these factors during normal capacitation, by centrifugal washing or by elimination of GalT I initiates at least some aspects of capacitation. These results suggest that GalT I maintains sperm in an uncapacitated state until they enter the female reproductive tract or are incubated in capacitating medium.

Capacitation of GalT I-null sperm occurs in the absence of the cofactors albumin, Ca^{2+} and HCO_3^-

If GalT I is functioning as a negative regulator of capacitation, we would predict that at least some of the signaling pathways that promote capacitation are constitutively activated in GalT I-null sperm, relative to wild-type sperm. Although the intracellular signaling pathways regulating capacitation have not been fully characterized, it is well documented that successful capacitation in vitro requires two extracellular co-factors thought to initiate intracellular signaling cascades: Ca^{2+} and HCO_3^- as well as albumin (or a cholesterol sink). We tested the prediction that these signaling cascades are constitutively activated in GalT I-null sperm by comparing the dependence of wild-type and GalT I-null sperm capacitation on each of these co-factors.

As before, capacitation was monitored by two criteria; sensitivity to A23187-induced acrosome reactions (Fig. 5) and the ability to bind the zona pellucida (Fig. 6). Sperm were capacitated in incomplete medium (i.e. absence of albumin, Ca^{2+} or HCO_3^-) for varying periods of time, after which sensitivity to A23187 was determined in complete medium (i.e. containing all co-factors). This ensures that the only co-factor-dependent effects being assayed were during the capacitation period, and not during induction of the acrosome reaction. For

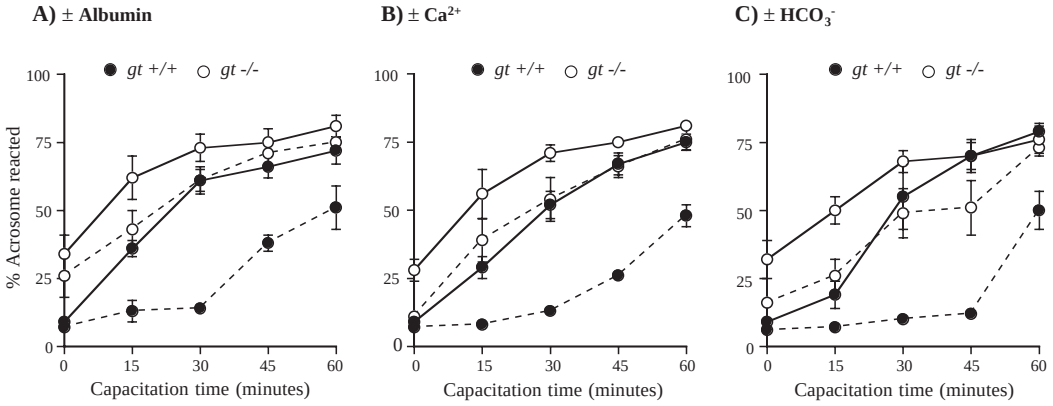

Fig. 5. GalT I-null sperm do not require albumin, Ca^{2+}, or HCO_3^- during capacitation to undergo an ionophore-induced acrosome reaction. Wild-type (*gt* +/+) and GalT I-null (*gt* –/–) sperm were capacitated for varying periods of time in complete media (unbroken line) or in media lacking (broken line)either albumin (A), Ca^{2+} (B) or HCO_3^- (C). Sperm were subsequently incubated with 10 μM A23187 and the appropriate co-factor (to reconstitute complete media) and the percentage of acrosome-reacted sperm was determined. Each point represents the mean±s.e.m.; *n*=3 experiments. In a single experiment, the value of each data point represents three determinations for a total of nine assays. A minimum of 200 sperm was counted for each of the assays.

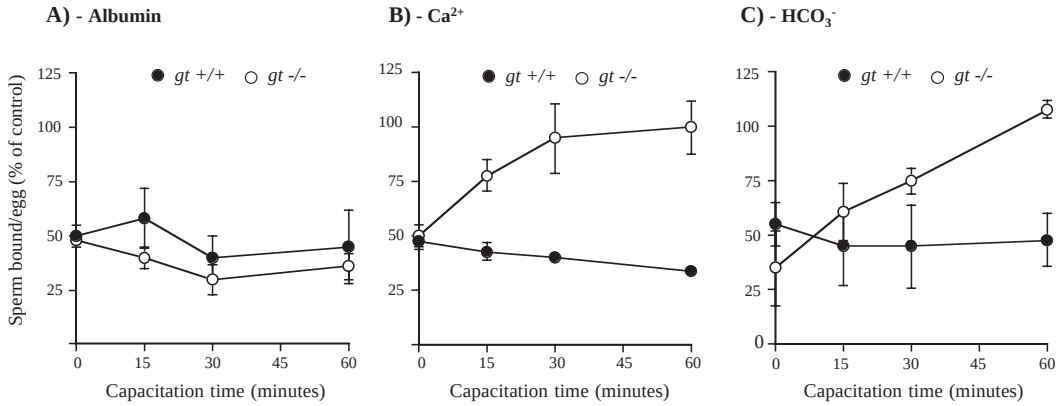

Fig. 6. GalT I-null sperm do not require Ca^{2+} or HCO_3^- during capacitation to achieve maximal binding to the zona pellucida. Wild-type (*gt* +/+) and GalT I-null sperm (*gt* –/–) were capacitated in either albumin-free (A), Ca^{2+}-free (B), HCO_3^--free (C) or complete media (control) for varying amounts of time before incubation with ovulated eggs in complete media. Each bar represents the mean±s.e.m.; *n*=3 experiments. In a single experiment, the value for each data point represents the average of three determinations (nine assays in total). The average number of sperm bound to eggs at each time point was normalized to fully capacitated sperm (60 minutes of capacitation in complete media is equivalent to 100% bound).

wild-type sperm, eliminating each of these co-factors from the capacitation medium significantly reduces the rate of capacitation, such that sperm do not respond to A23187 until late in capacitation (i.e. 45 minutes). For GalT I-null sperm, the rate of capacitation is also somewhat reduced in the absence of these co-factors. However, the rate at which GalT I-null sperm undergo capacitation in the absence of each co-factor is similar to the rate of wild-type sperm capacitation in complete medium. In other words, GalT I-null sperm phenocopy wild-type sperm when capacitated in the absence of albumin, Ca^{2+} or HCO_3^-, suggesting they are primed to undergo capacitation independent of these co-factors.

We next determined the requirement of these co-factors during in vitro capacitation on the ability of sperm to bind the zona pellucida. Sperm were capacitated in incomplete medium and then allowed to bind ovulated eggs in complete medium (Fig. 6). As expected, wild-type sperm fail to achieve maximal binding to the zona pellucida when capacitated in the absence of albumin, Ca^{2+} or HCO_3^-. GalT I-null sperm also fail to achieve maximal binding when capacitated in the absence of albumin (Fig. 6A). However, GalT I-null sperm show maximal binding to the zona pellucida when capacitated in the absence of either Ca^{2+} (Fig. 6B) or HCO_3^- (Fig. 6C). Again, the rate of capacitation of GalT I-null sperm in Ca^{2+}- or HCO_3^--deficient medium is similar to the rate of wild-type sperm capacitation in complete medium (compare Fig. 6 with Fig. 2). The results in Figs 5 and 6 strongly suggest that capacitation of GalT I-null sperm is largely independent of Ca^{2+} and HCO_3^-, unlike wild-type sperm where capacitation is completely dependent on these co-factors. Furthermore, these data suggest that Ca^{2+}- and HCO_3^--dependent targets are constitutively active in GalT I-null sperm.

The precocious capacitation of GalT I-null sperm correlates with elevated cAMP levels

The downstream targets of Ca^{2+} and HCO_3^- during capacitation have not been extensively characterized. However, both Ca^{2+} and HCO_3^- directly modulate the intracellular levels of cAMP (Garbers et al., 1982; Litvin et al., 2003). cAMP is necessary and sufficient for multiple events during capacitation, including tyrosine phosphorylation (Visconti et al., 1995b). Sperm adenylyl cyclases are regulated by Ca^{2+}-binding proteins, such as calmodulin (Gross et al., 1987), as well as directly by Ca^{2+} and HCO_3^- (Garbers et al., 1982; Garty and Salomon, 1987; Litvin et al., 2003).

Owing to the Ca^{2+} and HCO_3^--independent nature of capacitation in GalT I-null sperm, we predicted that they may have elevated levels of cAMP, and therefore compared cAMP levels during in vitro capacitation of wild-type and GalT I-null sperm (Fig. 7). Four points deserve emphasis. First, GalT I-null sperm have constitutively higher cAMP levels than do wild-type sperm. Second, cAMP levels are highest in GalT I-null sperm at 0-15 minutes of capacitation when GalT I-null

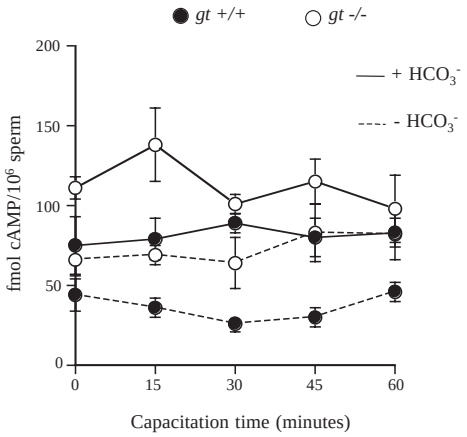

Fig. 7. GalT I-null sperm exhibit constitutively increased levels of cAMP during capacitation in HCO_3^--containing or HCO_3^--free media. Wild-type (*gt* +/+) and GalT I-null (*gt* –/–) were capacitated for varying periods of time in HCO_3^--containing or HCO_3^--free media. The cAMP in 1×10^6 sperm was extracted with ice-cold ethanol and detected by enzyme immunoassay. Each bar represents the mean±s.e.m.; *n*=4 experiments. For a single experiment, the value for each data point represents the average of two determinations (for a total of eight assays).

sperm display the greatest difference from wild-type sperm with respect to zona binding and sensitivity to acrosomal exocytosis. Third, eliminating HCO_3^- from the capacitation medium reduces cAMP levels in both wild-type and GalT I-null sperm, but GalT I-null sperm retain approximately twice the levels of cAMP. Fourth, the quantity of cAMP in GalT I-null sperm capacitated in the absence of HCO_3^- is almost identical to cAMP levels in wild-type sperm capacitated in complete medium. Thus, the relative cAMP levels closely mirror the capacitative state of GalT I-null sperm.

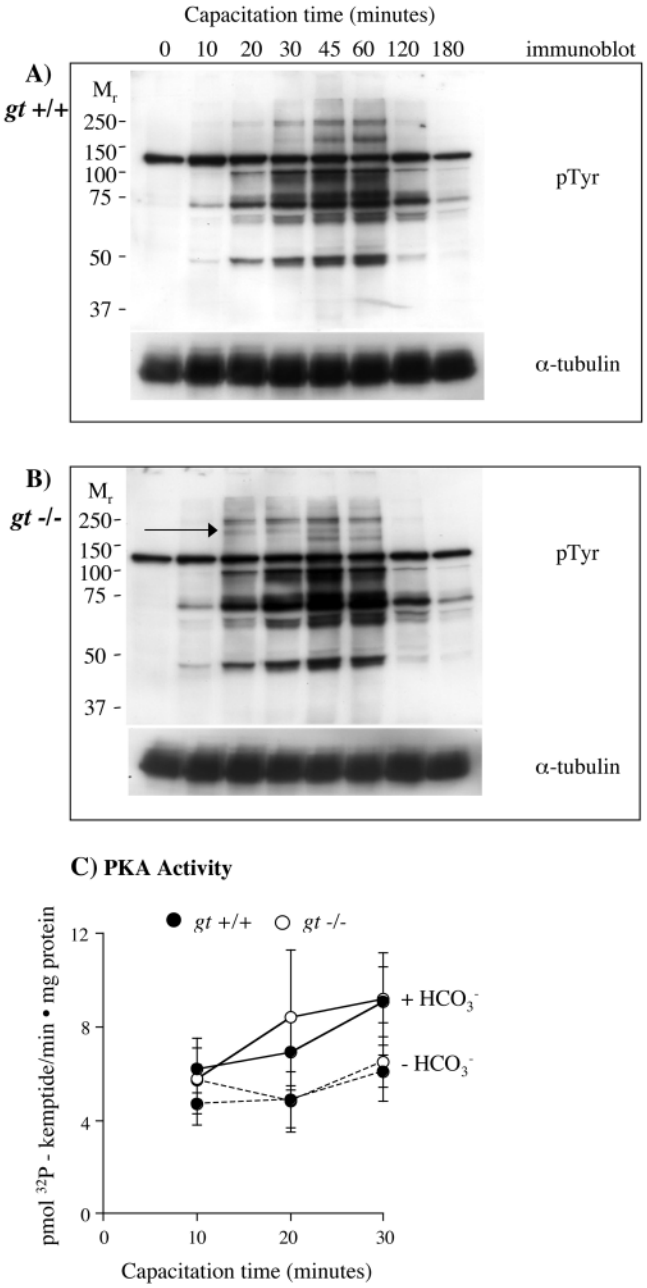

Fig. 8. Wild-type and GalT I-null sperm show similar profiles of capacitation-dependent protein tyrosine phosphorylation. Wild-type (*gt* +/+) and GalT I-null (*gt* –/–) sperm lysates were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine and anti-α-tubulin antibodies. The arrow indicates a protein of ~200 kDa that is present only in GalT I-null sperm lysates. This blot is representative of at least six experiments. (C) Protein kinase A activity in wild-type (*gt* +/+) and GalT I-null (*gt* –/–) sperm capacitated for varying periods of time in HCO_3^--containing or HCO_3^--free media. Each point represents the mean±standard error; *n*=3 experiments. In a single experiment, the value of each data point represents three determinations. Thus, each data point reflects data collected from nine assays.

The onset of protein tyrosine phosphorylation is not altered in GalT I-null sperm

In somatic cells, cAMP acts on a variety of molecular targets, including PKA (Harrison, 2003). In mammalian sperm, one well-defined PKA-dependent event during capacitation is the appearance of tyrosine-phosphorylated proteins. As cAMP levels are elevated in GalT I-null sperm throughout capacitation, we examined whether protein tyrosine phosphorylation is similarly accelerated (Fig. 8).

Phosphorylated proteins appear in both wild-type and GalT I-null sperm very early in capacitation (within 10 minutes), and there are no gross differences in the kinetics of tyrosine phosphorylation between wild-type and GalT I-null sperm. The pattern of protein phosphorylation is also nearly identical, except for a protein of ~200 kDa that appears in GalT I-null sperm at 20-60 minutes of capacitation. This protein is not detectable in wild-type sperm lysates, and its significance is unknown.

Because the profiles of protein tyrosine phosphorylation are similar in wild-type and GalT I-null sperm, we suspected that PKA activity would also be similar between wild-type and GalT I-null sperm. As shown in Fig. 8C, PKA activity increases during capacitation in a HCO_3^--dependent manner to the same extent in wild-type and GalT I-null sperm lysates.

Sperm motility is unaltered in GalT I-null sperm

The appearance of a specific pattern of motility, called hyperactivation, has been associated with the capacitated state (Ho and Suarez, 2001). To determine whether wild-type and GalT I-null sperm exhibit different motility patterns during the course of in vitro capacitation, the progressive velocity of sperm was measured by computer-assisted sperm analysis (CASA) (Fig. 9). Motility values were similar for both wild-type and GalT I-null sperm during early (10 minutes) and late (60 minutes) stages of in vitro capacitation. When individual sperm were observed following the procedure of Tessler et al. (Tessler et al., 1981), no differences in motility were observed (data not shown). Individual sperm within each population exhibited hyperactivated motility (figure-of-eight swimming pattern), but there was no difference in the number of hyperactivated wild-type or GalT I-null sperm. This result, along with those in Fig. 8, suggests that not all aspects of capacitation are globally accelerated in GalT I-null sperm. Rather, processes dependent on GalT I function (zona pellucida-binding and the acrosome reaction) are accelerated, whereas GalT I-independent events (tyrosine phosphorylation and motility) are unaltered.

Discussion

GalT I-null sperm exhibit precocious capacitation

In this study, we demonstrate by two independent criteria that GalT I-null sperm are precociously capacitated; they are able

to bind the zona pellucida and undergo ionophore-induce acrosomal exocytosis independent of in vitro capacitation. GalT I is normally masked on wild-type sperm by epididymally derived glycoconjugates that maintain sperm in an uncapacitated state (Shur and Hall, 1982b). Initial characterization of these 'decapacitating' factors shows them to be large molecular weight polylactosaminyl-containing glycoconjugates; traditional complex-type oligosaccharides isolated from epididymal fluid are unable to function in this capacity (Shur and Hall, 1982b). If these epididymal 'decapacitating' factors are intentionally removed from wild-type sperm, their capacitation rate increases and phenocopies that of GalT I-null sperm. These results indicate that GalT I functions to maintain sperm in an uncapacitated state. In addition, GalT I-null sperm undergo capacitation in a Ca^{2+}- and HCO_3^--independent manner, further suggesting that GalT I normally functions to suppress these signaling pathways. The precocious capacitation of GalT I-null sperm correlates with increased levels of cAMP, even in the absence of HCO_3^-. However, cAMP-dependent events primarily localized in the tail, such as tyrosine phosphorylation and motility, are not affected in GalT I-null sperm. We predict that the augmented pools of cAMP in GalT I-null sperm act locally in the sperm head, where GalT I functions during zona binding and induction of the acrosome reaction.

Capacitation is often assayed by successful fertilization in vitro. We did not use this assay here as GalT I-null sperm fertilize eggs at only 7% of the efficiency of wild-type sperm because of to their inability to undergo zona-induced acrosomal exocytosis (Lu and Shur, 1997). Zona pellucida binding is an obligatory step during mammalian fertilization and this process is capacitation dependent (Si and Olds-Clarke, 1999). Using this criteria, we determined that wild-type sperm show a time-dependent increase in zona-binding during capacitation. By contrast, GalT I-null sperm bind to eggs without any requirement for capacitation (Fig. 2). Thus, by this measure, GalT I-null sperm are precociously capacitated.

A second method to monitor capacitation status is to assay for the ability to undergo a ligand-induced acrosome reaction. Neither of the two physiological inducers of the acrosome reaction (ZP3 or progesterone) can induce acrosome reactions in GalT I-null sperm. Therefore, we determined whether the competence to undergo an acrosome reaction in response to Ca^{2+} influx was also a capacitation-dependent event. For wild-type sperm, short exposures to the Ca^{2+} ionophore A23187 induced the acrosome reaction only in sperm incubated for at least 30 minutes in capacitating medium. Sperm incubated with A23187 prior to capacitation, or capacitated in medium lacking albumin, Ca^{2+} or HCO_3^-, failed to respond to A23187 (Figs 3 and 5). These results suggest that A23187 induces the acrosome reaction in a capacitation-dependent manner. In this assay, GalT I-null sperm showed both a higher basal level of sensitivity to A23187 (at 0 minutes) as well as an increased rate of A23187-induced acrosome reactions within the first 30 minutes of capacitation (Fig. 3). Thus, by a second, independent assay, GalT I-null sperm display a precociously capacitated phenotype.

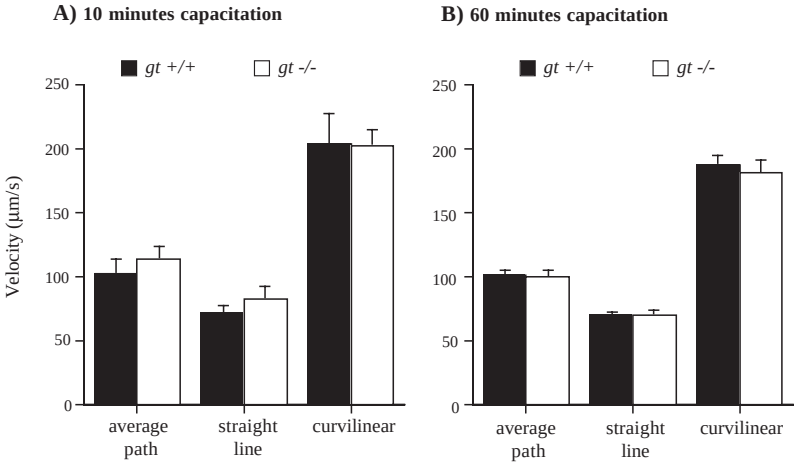

Fig. 9. Wild-type and GalT I-null sperm exhibit similar motility during in vitro capacitation. Wild-type (*gt* +/+) and GalT I-null sperm (*gt* –/–) were incubated in capacitating media for either 10 (A) or 60 (B) minutes before CASA. Each bar represents the mean±s.e.m.; *n*=3 experiments for each time point. Each experiment was carried out in duplicate.

Capacitation of GalT I-null sperm is independent of cofactors normally required for capacitation

We explored the mechanisms that contribute to the precocious capacitation phenotype of GalT I-null sperm. First, we hypothesized that GalT I-null sperm undergo precocious capacitation because they are uninhibited by decapacitating epididymal glycoconjugates normally bound to GalT I on the surface of wild-type sperm (Oliphant et al., 1985; Shur and Hall, 1982b). Consistent with this, washing sperm to completely remove these factors increases the capacitation rate of wild-type sperm, but has no appreciable effect on capacitation in GalT I-null sperm (Fig. 4). This suggests that washing wild-type sperm causes them to approach the behavior of GalT I-null sperm, which are genetically 'washed' and maximally capacitated. At least some decapacitating factors are known to function as substrates for GalT I; however, they may also be vesicular in nature, and washing sperm may promote capacitation by altering the sterol:phospholipid ratio in the sperm plasma membrane (Davis, 1981).

Second, we hypothesized that GalT I inhibits the activation of signaling pathways normally required for capacitation. This predicts that in the absence of GalT I, capacitation would occur at an accelerated rate and may be independent of regulatory cofactors in the female reproductive tract (or in vitro). We tested this prediction by measuring the rate of capacitation in wild-type and GalT I-null sperm incubated in the absence of the obligatory cofactors albumin, Ca^{2+} and HCO_3^-. As expected, wild-type sperm require each of these co-factors to undergo capacitation (Figs 5 and 6). Interestingly, in the absence of Ca^{2+} or HCO_3^-, GalT I-null sperm still display maximal binding to the zona pellucida and sensitivity to ionophore. In addition, GalT I-null sperm require albumin for zona pellucida-binding but not for the A23187-induced acrosome reaction. GalT I-null sperm may display a differential requirement for albumin because the state of a plasma membrane competent to undergo vesicle fusion may not be the same as the state of a membrane competent to undergo ligand binding.

Precocious capacitation of GalT I-null sperm correlates with elevated cAMP levels

As GalT I-null sperm undergo capacitation in the absence of Ca^{2+} and HCO_3^- at the same rate as wild-type sperm undergoing capacitation in complete medium, it is possible that processes regulated by these two co-factors are constitutively active in GalT I-null sperm. We initially focused on a target that is influenced by both Ca^{2+} and HCO_3^-: cAMP produced by the soluble isoform of adenylyl cyclase (Garbers et al., 1982; Litvin et al., 2003). We determined whether this enzyme (or other sources of cAMP) might be constitutively active in GalT I-null sperm by measuring the cAMP content of sperm during capacitation.

cAMP levels correlate closely with the capacitation phenotype of wild-type and GalT I-null sperm (Fig. 7). Basal levels of cAMP are constitutively elevated in GalT I-null sperm consistent with their constitutively higher rates of zona pellucida binding and acrosomal exocytosis. Furthermore, capacitating GalT I-null sperm in the absence of HCO_3^- leads to cAMP levels similar to that in wild-type sperm capacitated in complete medium; conditions in which GalT I-null sperm behave similar to wild-type sperm during capacitation (Figs 5 and 6). This result suggests that the precocious capacitation of GalT I-null sperm could be because of increased levels of cAMP and/or other intracellular targets of Ca^{2+} and HCO_3^-.

The elevated cAMP levels in GalT I-null sperm may also reflect signal transduction pathways in addition to those regulated by Ca^{2+} and HCO_3^-. Adenosine receptors, through the action of fertilization promoting peptide, stimulate cAMP production in a heterotrimeric G protein-dependent manner (Baxendale and Fraser, 2003; Fraser and Adeoya-Osiguwa, 1999). GalT I is functionally linked with a pertussis toxin-sensitive G protein during the acrosome reaction and it may interact with adenosine receptors during capacitation. In addition, cAMP production in mammalian sperm may be stimulated by reactive oxygen species and regulated by intracellular pH (Aitken et al., 1998). pH is regulated by HCO_3^- (Zeng et al., 1996) and could be altered in GalT I-null sperm. In any event, the accelerated capacitation rate of GalT I-null sperm is positively correlated with elevated cAMP levels, and further experiments are required to determine whether this increase is causal or incidental to the accelerated capacitation of GalT I-null sperm.

The precocious capacitation phenotype in GalT I-null sperm is confined to functional aspects of the sperm head

We suspected that the elevated levels of cAMP would lead to increased PKA activity and a consequent increase in PKA-dependent tyrosine phosphorylation. However, neither PKA activity nor tyrosine phosphorylation were significantly different between wild-type and GalT I-null sperm (Fig. 8). The only detectable difference in the profile of tyrosine-phosphorylated proteins was an additional ~200 kDa protein of unknown significance in GalT I-null sperm lysates. This protein may directly or indirectly promote the accelerated capacitation of GalT I-null sperm, but this waits further testing. The kinetics of tyrosine phosphorylation in sperm from these mice occurs much more rapidly than previously reported (Visconti et al., 1995a), which is probably due to differences in mouse strains used or in the composition of the capacitation medium.

It is unclear why the PKA activity in GalT I-null sperm did not correlate with the elevated levels of cAMP. Others have experimentally increased cAMP concentration in sperm using cAMP analogs and demonstrated an effect on PKA activity (Demarco et al., 2002; Visconti et al., 1995b; Visconti et al., 1999). One explanation is that there are multiple steps between cAMP production and tyrosine phosphorylation, and the amount of cAMP in the cell may not directly correlate with every downstream target (Harrison, 2003). Alternatively, the elevated cAMP in GalT I-null sperm may act on a limited number of targets and/or in a localized manner in the sperm head. cAMP may also influence targets in the sperm head other than PKA, such as cyclic nucleotide-gated ion channels and cAMP-activated guanine-nucleotide exchange proteins (Harrison, 2003).

Collectively, these results suggest that capacitation can be segregated into GalT I-dependent events in the head (zona pellucida binding, acrosomal exocytosis) and GalT I-independent events in the tail (protein tyrosine phosphorylation, sperm motility). This conclusion is supported by observations in the literature in addition to results presented here. For example, calmodulin antagonists prevent capacitation as assayed by in vitro fertilization and acrosomal exocytosis, but fail to block protein tyrosine phosphorylation (Si and Olds-Clarke, 2000). Many substrates of protein tyrosine phosphorylation are components of the principle piece and fibrous sheath of the flagellum (Carrera et al., 1996; Jha and Shivaji, 2002; Naaby-Hansen et al., 2002), or associated with microtubule function, such as AKAP3, AKAP4, dynein intermediate chain and PKA (Ficarro et al., 2003). Although there is evidence that some PKA subunits localize to the sperm head (Flesch et al., 2001; Harrison et al., 2000; Visconti et al., 1997), it is believed to act on substrates primarily in the sperm tail. Consistent with this, the sperm-specific catalytic subunit of PKA has been detected in the midpiece, mitochondria and the axoneme of murine and ovine sperm (San Agustin and Witman, 2001), a location where it would not be subject to regulation by GalT I.

Although capacitation was first described over 50 years ago, the identity and mechanism of extracellular and intracellular signaling molecules regulating this process are still unclear. The study of capacitation has been hindered by the lack of defined mutations that interfere with its normal progression. To our knowledge, only one other class of mutations affects the capacitation process in mammalian sperm: the t haplotypes. Several genes affected by the t haplotypes have been identified (Herrmann et al., 1999; Samant et al., 2002); this group of mutations appears to overwhelmingly affect flagellar function (Olds-Clarke and Johnson, 1993). The present study reports the first mutation in a sperm surface receptor that leads to a constitutively activated capacitation phenotype, and which recapitulates wild-type capacitation in the absence of co-factors normally required for capacitation. We expect further study of this mutant to aid understanding of this physiologically complex process.

The authors thank Dr Bill Roudebush of Reproductive Biology Associates (Atlanta, GA) for use of the Hamilton-Thorne CASA instrument. We also thank Dr Patricia Olds-Clarke of Temple University and Dr Robin A. P. Harrison of the Babraham Institute (United Kingdom) for enlightening discussions of these data. All

procedures involving animals conformed to local and national animal welfare protocols and were approved by the Emory IACUC board. This work was supported by grants from the NIH (HD23479 to B.D.S. and T32 GM08367 to C.R.).

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