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GTP-bound Rab3A exhibits consecutive positive and negative roles during human sperm dense-core granule exocytosis

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Exocytosis of mammalian sperm dense-core secretory granule relies on the same fusion molecules as all other secretory cells; one such molecule is the small GTPase Rab3A. Here, we report an in-depth biochemical characterization of the role of Rab3A in secretion by scrutinizing the exocytotic response of streptolysin O-permeabilized human sperm to the acute application of a number of Rab3A-containing constructs and correlating the findings with those gathered with the endogenous protein. Full length, geranylgeranylated, and active Rab3A elicited human sperm exocytosis *per se*. With Rab3A/Rab22A chimeric proteins, we demonstrated that the carboxy-terminal domain of the Rab3A molecule was necessary and sufficient to promote exocytosis, whereas its amino-terminus prevented calcium-triggered secretion. Interestingly, full length Rab3A halted secretion when added after the docking of the acrosome to the plasma membrane. This effect depended on the inability of Rab3A to hydrolyze GTP. We combined modified immunofluorescence and acrosomal staining protocols to detect membrane fusion and the activation status of endogenous Rab3 simultaneously in individual cells, and found that GTP hydrolysis on endogenous Rab3 was mandatory for fusion pores to open. Our findings contribute to establishing that Rab3 modulates regulated exocytosis differently depending on the nucleotide bound and the exocytosis stage under study.

Keywords: Rab3 cycle, GTP hydrolysis, exocytosis, acrosome, calcium, sperm

Introduction

Exocytosis is controlled by a complex and highly conserved protein machinery; isoforms of these proteins carry out similar functions in all cells (Burgoyne and Morgan, 2003; Lang and Jahn, 2008; Malsam et al., 2008; Sudhof and Rothman, 2009; Wang and Thurmond, 2009; Sudhof and Rizo, 2011). At the core of this machinery are SNARE and Rab proteins. Rabs are a family of monomeric GTPases that cycle between active, GTP-bound, and inactive, GDP-bound, states. Rab3 is probably the most studied Rab in exocytotic cells, and the most controversial. Different approaches show both positive and negative roles for various Rab3 family members. Thus, despite a wealth of genetic, overexpression, electrophysiological, and biochemical data regarding Rab3s (A, B, C, and D), it is difficult to formulate a coherent hypothesis about the functions of these proteins in membrane fusion. Rab3A has been claimed to maintain the supply of vesicles into the releasable pool (Coleman and Bykhovskaia, 2009) and to be unnecessary for refilling this pool (Geppert et al., 1997; Leenders et al., 2001); to increase the docking of vesicles to the plasma membrane (Tsuboi and Fukuda, 2006; Coleman et al., 2007; van Weering et al., 2007) and not to be involved in docking but in the recruitment of vesicles (Leenders et al., 2001; Tian et al., 2012); to regulate the number of vesicles that fuse during stimulation (Wang et al., 2008) and the amount of transmitter in a quantum (Geppert et al., 1997; Coleman and Bykhovskaia, 2009); to be unnecessary for fusion itself (Johannes et al., 1994) but to control exocytosis of vesicles through direct interaction with fusion pores of unusual characteristics (Wang et al., 2008); to limit evoked release to a single quantum for each release site during a single impulse (Johannes et al., 1998); to participate in priming (Dulubova et al., 2005; Huang et al., 2011), etc. Perhaps the only notion all studies agree on is that Rab3A modulates exocytosis.

Exocytosis of the sperm single dense-core secretory granule—the acrosome—is a synchronized process that happens only once in the life of the cell and shares the basic fusion molecules with all other secretory cells. Acrosomal exocytosis (termed the

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acrosome reaction, AR) depends on most members of the proteinaceous fusion machinery described to date, including the Rab3-interacting protein RIM (Bello et al., 2012) and several small GTPases such as Rab3A, Rab27 (Bustos et al., 2012), Rho family members (Ducummon and Berger, 2006; Baltierrez-Hoyos et al., 2012), and Rap (Branham et al., 2009). Rab3A is required for the AR triggered by calcium (Belmonte et al., 2005; De Blas et al., 2005), cAMP (Branham et al., 2006), sphingosine 1-phosphate (Suhaiman et al., 2010), and diacylglycerol (Lopez et al., 2012) and for the docking of the acrosome to the plasma membrane (Bello et al., 2012). Moreover, Rab3A is activated (exchanges GDP for GTP) during the exocytosis signaling pathway (Branham et al., 2009; Lopez et al., 2012) through a RabGEF cascade recently described (Bustos et al., 2012).

The acrosomal granule behaves as an internal store of releasable calcium; efflux from this reservoir through inositol 1,4,5-trisphosphate (IP_3)-sensitive channels is required for the AR (De Blas et al., 2002, 2005; Darszon et al., 2005; Herrick et al., 2005; Branham et al., 2006; Lopez et al., 2007, 2012; Costello et al., 2009; Suhaiman et al., 2010). When the AR is initiated with inducers in the presence of intra-acrosomal calcium chelators or IP_3-channel blockers, exocytosis is halted at the stage that acrosomal calcium should be released. Morphologically, this stage correlates with abundant close contacts (shorter than 8 nm) between the outer acrosomal and plasma membranes (Zanetti and Mayorga, 2009). Molecularly, it corresponds to a state where partially assembled (sensitive to botulinum toxins but resistant to tetanus toxin) *trans* SNARE complexes bridge these two membranes together in preparation for fusion (De Blas et al., 2005; Rodriguez et al., 2011). We define this stage as the docking step of the exocytotic cascade. When *O*-nitrophenyl EGTA acetoxymethyl ester (NP-EGTA-AM), a photolabile calcium chelator widely used in the field of exocytosis, is introduced into sperm whose plasma membrane has been permeabilized with streptolysin O (SLO), NP-EGTA-AM crosses the plasma and outer acrosomal membranes, accumulates inside the acrosome, and halts the AR triggered by all inducers by sequestering intra-acrosomal calcium for as long as the system is kept in the dark. UV photolysis of NP-EGTA-AM rapidly replenishes the acrosomal calcium pool, resuming exocytosis (De Blas et al., 2002; Ackermann et al., 2008; Hu et al., 2010). By combining this tool with AR blockers, we have been able to classify a dozen or so components of the signaling cascade that drives acrosomal exocytosis into early- or late-acting factors based on whether they are required before, at, or after intra-acrosomal calcium release. Rab3A is one of the fusion-related proteins and plays its role in the AR before the release of calcium from the intracellular store (De Blas et al., 2005).

In addition to blocking antibodies and protein cassettes that target the endogenous protein (Branham et al., 2009; Bustos et al., 2012), acute (15 min) delivery of recombinant Rab3A into human sperm is our method of choice to investigate the role of this protein in regulation of exocytosis. Because sperm neither transcribe nor translate, overexpression and silencing RNA technologies are not applicable to these cells. Recombinant, full length Rab3A, geranylgeranylated and loaded with GTP (Belmonte et al., 2005) or GTP-γ-S (Yunes et al., 2000), stimulates the AR in SLO-permeabilized human sperm; a permeant version of this protein elicits exocytosis in non-permeabilized cells (Lopez et al., 2007). In many aspects, secretion elicited by exogenous Rab3A resembles the physiological AR. For instance, recombinant Rab3A mobilizes calcium from the acrosome (De Blas et al., 2002) and disassembles the toxin-resistant *cis* SNARE complexes (the predominant form in resting sperm) to allow subsequent engagement *in trans* (De Blas et al., 2005).

In summary, multiple pieces of evidence support the role of Rab3A as a positive regulator of acrosomal exocytosis. Here, we describe an experimental strategy showing that Rab3A also behaves as a negative regulator of sperm secretion. Persistently activated Rab3A halts exocytosis when added after the docking of the acrosome to the plasma membrane. Thus, Rab3A is a molecule with dual properties evidenced at different times and within different molecular contexts during the secretory cascade. The positive and negative attributes of Rab3A on exocytosis are segregated to different domains of the protein. More importantly, we describe that endogenous Rab3A undergoes transient activation following the challenge of sperm with an AR inducer. In other words, as much as a GEF activity is essential during the early stages, GTP hydrolysis is mandatory to accomplish the late steps of secretion.

Results

The carboxy-terminal domain of Rab3A is necessary and sufficient to elicit acrosomal exocytosis

To characterize the behavior of Rab3A in dense-core granule exocytosis, we expressed the protein fused to GST in *Escherichia coli*, geranylgeranylated it (p) or not (np) *in vitro*, and loaded with GDP-β-S or GTP-γ-S. When introduced into SLO-permeabilized human sperm, GDP-β-S-loaded Rab3A did not affect exocytosis *per se* but prevented the calcium-triggered AR regardless of its lipid modification state (Figure 1A). Geranylgeranylated, GTP-γ-S-loaded Rab3A alone induced the AR to the same extent accomplished by calcium. Treatment with Rab3A plus calcium did not augment further the percentage of reacting cells, suggesting that their effects are neither additive nor synergistic (Figure 1B). To rule out that these effects might be due to guanine nucleotides leaking from Rab3A and acting on endogenous G proteins, we repeated the experiments with the Rab3A point mutant Q81L, whose $k_{off\ (GTP)}$ is almost 9-fold lower than that of wild-type (Brondyk et al., 1993). As observed with wild-type Rab3A, when GDP-β-S-loaded and geranylgeranylated Rab3AQ81L was introduced into SLO-permeabilized human sperm, it did not affect the AR *per se* but prevented the calcium-triggered exocytosis (Supplementary Figure S1A). Geranylgeranylated Rab3AQ81L-GTP-γ-S accomplished the AR in as many cells as calcium did. Once again, the effect of these two exocytosis inducers was not additive or synergistic (Supplementary Figure S1B).

To address which portion of the Rab3A molecule is responsible for its exocytosis-inducing activity, we designed two chimeric constructs. In the Rab3A-22A chimera, the amino-terminus (amino acids 1–63) of Rab22A, a non-secretory Rab, was replaced by the corresponding region of Rab3A (amino acids 1–80). In the Rab22A-3A chimera, the first 63 amino acids of Rab22A were fused to the carboxy-terminus (amino acids 81–221) of Rab3A (Figure 1C). We used the I-TASSER internet server (Zhang, 2008; Roy et al., 2010, 2011) to model the chimeras *in silico* and

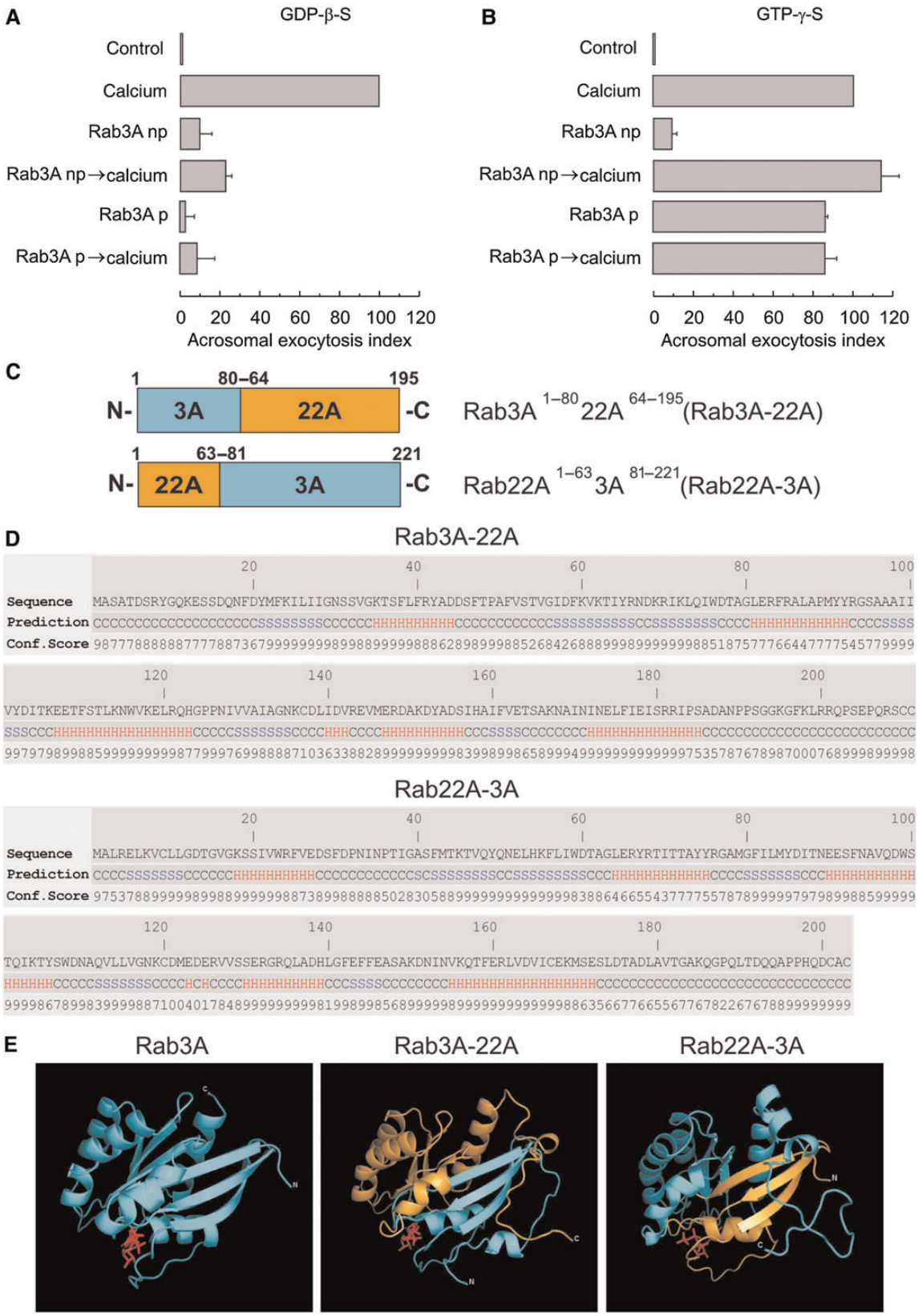

Figure 1 Rab3A-22A and Rab22A-3A chimeric proteins are structurally similar to Rab3A. (**A** and **B**) 300 nM GST-Rab3A loaded with GDP-β-S (**A**) or GTP-γ-S (**B**) and geranylgeranylated (p) or not (np) were introduced into SLO-permeabilized human sperm and incubated for 15 min at 37°C. Cells underwent no further treatment or were challenged with 0.5 mM $CaCl_2$ for 15 min at 37°C as indicated. Exocytosis was evaluated by post-fixation

predict their secondary (Figure 1D) and tertiary (Figure 1E) structures. The predicted conformations contain a six-stranded β sheet with five parallel and one anti-parallel strands flanked by five α helices; these structures are a signature of Rab proteins. We used TM-score, an algorithm that compares topologies between two proteins (Zhang and Skolnick, 2004), to determine the structural similarity between the predicted models and the native structure of full length Rab proteins. The TM-score was 0.71 ± 0.11 for Rab3A-22A and 0.78 ± 0.1 for Rab22A-3A. Because a TM-score >0.5 suggests that a model exhibits the correct topology, we were confident that the chimeric Rab proteins that we had designed would adopt a tridimensional conformation similar to that of Rab proteins. I-TASSER identified five enzyme analogs for each chimera, four of them fitting the small monomeric GTPase classification in the ExPaSy enzyme database. We completed our analysis of the predicted 3D models generated by I-TASSER using the server to identify potential functional analogs. The structure of ligand-binding sites in the chimeric proteins predicts their interaction with GDP, GTP, Mg^{2+}, rabenosyn-5, GDI, and Rabex-5. In short, our *in silico* analysis suggests that Rab3A-22A and Rab22A-3A would adopt the tridimensional structure expected of a Rab protein and interact with known Rab3A and Rab22A ligands.

Both chimeras expressed well in *Escherichia coli*, which we took as an indicator that they folded correctly. The gel filtration profiles of the chimeric proteins were similar to that of wild-type Rab3A, which confirms that they are properly folded and monomeric (data not shown). They were subsequently geranylgeranylated (p) or not (np) *in vitro* and loaded with GTP-γ-S. As observed with Rab3A, geranylgeranylated Rab22A-3A, but not the unmodified form, elicited the AR by itself. Also like full length Rab3A, this chimera did not influence the level of exocytosis accomplished by calcium (Figure 2A). In contrast, Rab3A-22A did not induce the AR even when geranylgeranylated and loaded with GTP-γ-S (Figure 2B). These data indicate that the exocytosis-inducing activity of Rab3A resides in its carboxy-terminal portion.

Would differential binding to interacting proteins explain the opposite behaviors of the chimeras on the AR? The only Rab3 effector described to date in sperm is RIM (Bello et al., 2012). Thus, we conducted far-western blots to assess the binding of all Rab3A-containing proteins to RIM. The selected monoclonal antibody to Rab3A recognizes its carboxy-terminal portion (amino acids 191–207) (Baumert et al., 1993; Schluter et al., 2002). This antibody detected Rab3A and Rab22A-3A, but not Rab3A-22A, in dot blot assays. Conversely, the anti-Rab22A antibody, raised against a peptide encompassing amino acids 143–192 of human Rab22A, recognized the Rab3A-22A chimera but not full length Rab3A or Rab22A-3A (Figure 2C). Rab3A, Rab3AQ81L and Rab22A-3A bound immobilized RIM in far-western experiments but Rab3A-22A did not (Figure 2C). These findings allow us to speculate that in order to elicit the AR, Rab3A-containing proteins must be able to bind effectors (e.g. RIM).

Rab3A-22A inhibits the AR after the fusion machinery has reached the docking stage during the exocytotic cascade

The Rab3A-22A hybrid protein not only failed to elicit the AR but also prevented calcium-triggered exocytosis (Figure 2B). Next, we set out to determine the temporal characterization of this inhibition. We took advantage of an assay that allows us to assess whether a given factor can perturb exocytosis when added after an AR inducer has caused the system to accomplish the docking of the acrosome to the plasma membrane. This assay rests on the knowledge that docking precedes intracellular calcium mobilization. Briefly, when AR inducers are added to cells previously loaded with NP-EGTA-AM (De Blas et al., 2005), they put the AR signaling cascades into motion. Nevertheless, exocytosis cannot proceed beyond the stage that requires localized calcium release from the intracellular store targeted by NP-EGTA-AM as long as the tubes are protected from light. This is a relatively late stage that happens after the activation of endogenous Rab3A (Branham et al., 2009) and correlates biochemically with sperm SNAREs engaged in partially assembled *trans* complexes (De Blas et al., 2005) and morphologically with docked acrosomes (Zanetti and Mayorga, 2009). UV photolysis of NP-EGTA-AM rapidly replenishes the intracellular calcium pool, accomplishing exocytosis. We first conducted control experiments using full length Rab22A to make sure that the effects elicited by the chimeras were attributed to the Rab3A portion of the molecules. Addition of recombinant, isoprenylated, and activated Rab22A did not prevent calcium-triggered exocytosis either in the standard assay or in cells pretreated with NP-EGTA-AM (Supplementary Figure S1C). When we challenged NP-EGTA-AM-loaded sperm with extracellular calcium, which allowed exocytosis to proceed to the stage requiring intracellular calcium mobilization, and then added recombinant Rab3A-22A, there was no exocytosis even after illuminating the tubes (Figure 2D, black bar). These data

staining with FITC-PSA and the data were normalized as described under Supplementary Materials and methods. Plotted results represent the mean ± SEM of three independent experiments. (**C**) Schematic representation of chimeric Rab proteins used in this study. The numbers above each bar indicate the residue at which each Rab was fused to generate the hybrid protein. Rab22A is shown in bright orange and Rab3A in cyan. (**D** and **E**) Prediction of secondary and tertiary structures for chimeras Rab3A-22A and Rab22A-3A performed with the I-TASSER server (Zhang, 2008; Roy et al., 2010). (**D**) Predicted secondary structures are typical for Rab proteins containing β strands (blue S letters), α helices (red H letters), and coils (black C letters). The confidence score prediction for each residue is shown with values ranging between 0 and 9 (a higher score indicates a prediction with higher confidence). (**E**) Best predicted 3D structures selected based on the highest C-scores calculated by I-TASSER, which were 0.01 for chimera Rab3A-22A and 0.5 for Rab22A-3A. C-score is a confidence score for estimating the quality of predicted models; it is typically in the range of $[-5, 2]$. In general, models with C-score > -1.5 have a correct fold. The tridimensional structure of Rab3A is shown for comparison. Color coding is the same as in **C**; the guanosine nucleotide GNP (guanosine 5′-[β,γ-imido]triphosphate, a non-hydrolyzable analog of GTP that binds and irreversibly activates G proteins) is shown in red.

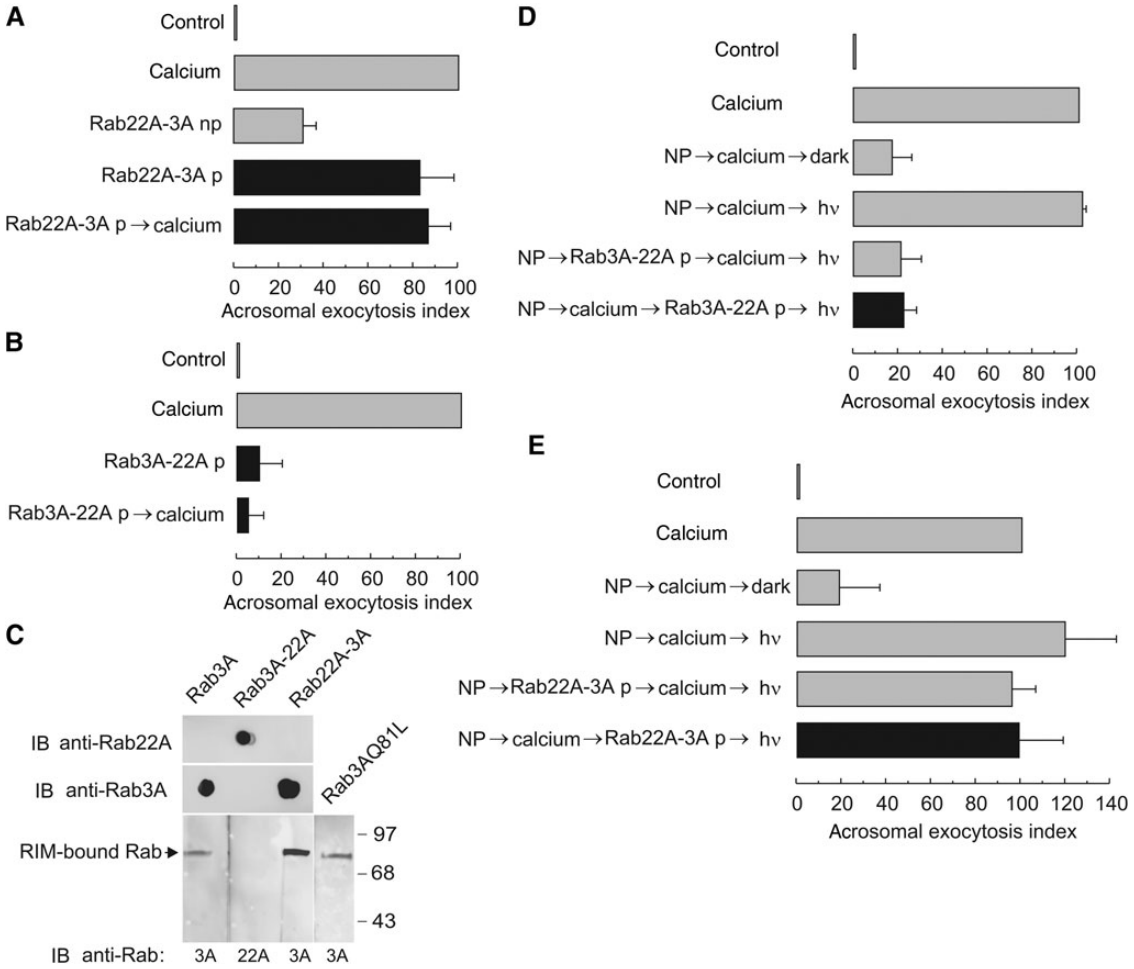

Figure 2 Rab22A-3A triggers and Rab3A-22A inhibits the AR. (**A** and **B**) 300 nM of non-modified (np) or geranylgeranylated (p) Rab22A-3A-GTP-γ-S (**A**) or Rab3A-22A-GTP-γ-S (**B**) were introduced into SLO-permeabilized sperm and incubated for 15 min at 37°C. Cells underwent no further treatment or were challenged with 0.5 mM $CaCl_2$ for 15 min at 37°C. Exocytosis was evaluated and the data were normalized as described under Supplementary Materials and methods. Plotted results represent the mean ± SEM of three independent experiments. (**C**) Top: 500 ng of GST-Rab3A, GST-Rab3A-22A, or GST-Rab22A-3A were spotted on nitrocellulose and probed with anti-Rab22A and anti-Rab3A antibodies. Bottom: 100 ng of GST-RIM-RBD were electrophoresed in 10% Tris-glycine gels and transferred to nitrocellulose. Blots were overlaid with 1 μg/ml of full length GST-Rab3A (wild-type or Q81L mutant), GST-Rab3A-22A, or GST-Rab22A-3A loaded with GTP-γ-S and probed with anti-Rab22A antibody (Rab3A-22A) or the monoclonal anti-Rab3A antibody (the rest). Mr standards ($\times 10^3$) are indicated on the right. The arrow points to Rabs bound to electrophoresed RIM. Shown is an experiment representative of two repetitions. (**D** and **E**) Permeabilized spermatozoa were loaded with 10 μM NP-EGTA-AM (NP) for 10 min at 37°C to chelate intra-acrosomal calcium. The AR was subsequently initiated by adding 0.5 mM $CaCl_2$. After 15 min incubation at 37°C to allow exocytosis to proceed up to the intra-acrosomal calcium-sensitive step, sperm were treated for 15 min at 37°C with 300 nM of geranylgeranylated and GTP-γ-S-loaded Rab3A-22A (**D**) or Rab22A-3A (**E**). All these procedures were carried out in the dark. UV flash photolysis of the chelator was induced at the end of the incubation period (hν), and the samples were incubated for 5 min at 37°C (NP→ calcium→ RabX→ hν, black bars). Several controls were run (gray bars): background AR in the absence of any stimulation (control); AR stimulated by 0.5 mM $CaCl_2$ (calcium); inhibitory effect of NP-EGTA-AM in the dark (NP→ calcium→ dark); the recovery upon illumination (NP→ calcium→ hν); and the effect of the chimeras when present throughout the experiment (NP→ RabX→ calcium→ hν). Acrosomal exocytosis was evaluated as described in **A** and **B**. Plotted results represent the mean ± SEM of three independent experiments.

suggest that the amino-terminal portion of Rab3A bears an activity capable of inhibiting relatively late stages of sperm secretion. We tested this premiss by using the converse chimeric protein that lacks this domain. When we substituted Rab3A-22A by the stimulatory Rab22A-3A chimera, we did not observe any inhibition on the AR in cells previously treated with NP-EGTA-AM (Figure 2E, black bar). These results indicate that the inhibitory activity of Rab3A resides in its amino-terminal portion, a domain that is missing in the Rab22A-3A hybrid protein. Equally important was the observation that the Rab3A-22A chimera was able to halt exocytosis when added after the endogenous machinery had catalyzed the docking of the acrosome to the plasma membrane. We were surprised to discover that the step inhibited by Rab3A-22A occurred later than the one catalyzed by endogenous Rab3A.

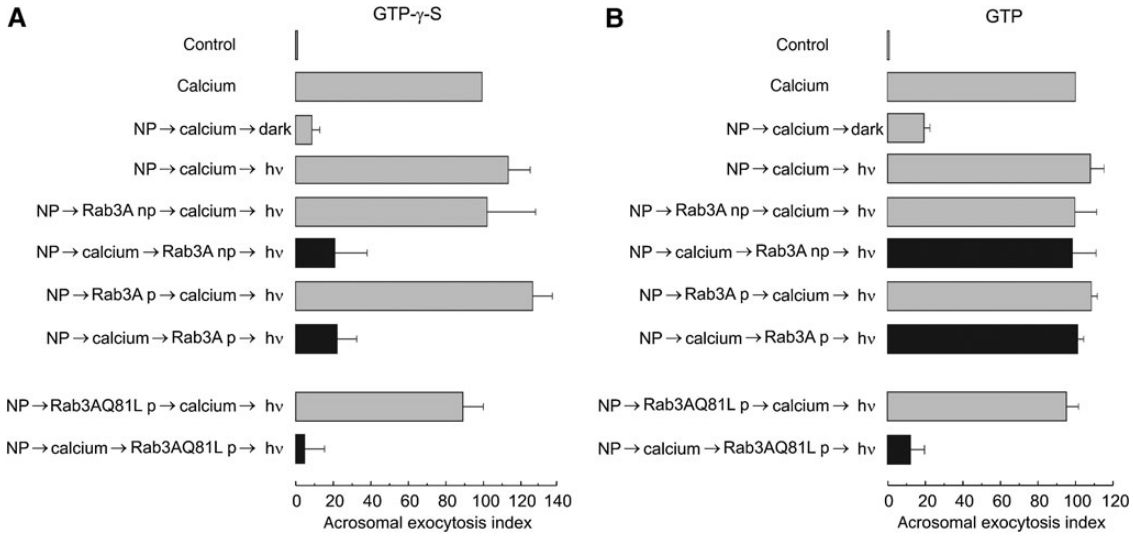

Figure 3 Rab3A halts the AR at a relatively late stage as long as it cannot hydrolyze GTP. Permeabilized spermatozoa were loaded with 10 μM NP-EGTA-AM (NP) for 10 min at 37°C. The AR was subsequently initiated by adding 0.5 mM $CaCl_2$. After 15 min incubation at 37°C, sperm were treated for 15 min at 37°C with 300 nM of non-modified (np) or geranylgeranylated (p) and GTP-γ-S- (**A**) or GTP- (**B**) loaded wild-type (top) or Q81L (bottom) Rab3A. All these procedures were carried out in the dark. UV flash photolysis of the chelator was induced at the end of the incubation period (hν), and the samples were incubated for 5 min at 37°C (NP→ calcium→ Rab3A/Rab3AQ81L→ hν, black bars). Controls were as described in Figure 2D and E. Exocytosis was evaluated and the data were normalized as described under Supplementary Materials and methods. Plotted results represent the mean ± SEM of three independent experiments.

Persistently active Rab3A can inhibit exocytosis if added after the system has reached the docking stage

Full length wild-type Rab3A contains the amino-terminal portion present in the inhibitory Rab3A-22A chimera. Therefore, we hypothesized that the amino-terminal portion of Rab3A bears an inhibitory activity that is normally masked in the whole molecule by the stimulatory activity of the carboxy-terminal domain. In order to unveil this putative inhibitory capacity, we tested the effect of Rab3A in cells previously loaded with NP-EGTA-AM. Full length, persistently activated Rab3A did not measurably affect the AR when added before calcium stimulation (Figure 3A). To our surprise, adding Rab3A after calcium abolished exocytosis completely; geranylgeranylated (p) Rab3A halted exocytosis as did the unprenylated (np) protein (Figure 3A, top, black bars). Thus, lipid modification grants Rab3A the ability to elicit the AR when added alone to SLO-permeabilized sperm (Figure 1B) but does not govern its inhibitory properties (Figure 3A). It is worth stressing that geranylgeranylated Rab3A is an AR inducer; hence, its ability to halt exocytosis when added after calcium in NP-EGTA-AM-loaded sperm was totally unexpected. By replacing Rab3A with dibutyryl cAMP, which is an AR inducer when added alone as well as in the context of NP-EGTA-AM and did not show any post-docking inhibitory effect (Supplementary Figure S1D), we ruled out that this behavior was simply a consequence of adding a second inducer after initiating the AR with exogenous calcium.

Similar experiments with substitution of the non-hydrolyzable GTP-γ-S by the naturally occurring guanosine nucleotide GTP showed that, in contrast to the persistently activated form, GTP-loaded Rab3A was unable to halt the AR (Figure 3B, top, black bars). The results were the same regardless of the isoprenylation status of the molecule. The Rab3AQ81L mutant has an undetectably low basal GTPase activity and is considered as not able to hydrolyze bound GTP (Brondyk et al., 1993). When added to SLO-permeabilized sperm loaded with NP-EGTA-AM and calcium, Rab3AQ81L inhibited the AR when or whether bound to GTP-γ-S (Figure 3A, bottom, black bar) or GTP (Figure 3B, bottom, black bar). These results indicate that as long as GTP were hydrolyzed, recombinant Rab3A would not act as an inhibitor.

In short, the amino-terminal portion of Rab3A inhibited the AR at a late stage through a mechanism independent of the lipid modifications that occurred at the carboxy-terminus end of the molecule. More strikingly, an excess active Rab3A was detrimental to the progress of the exocytotic cascade when added after SNAREs were assembled in partial *trans* complexes and the acrosome was docked to the plasma membrane. We summarized the Rab3A-related changes during the AR in a sequence (1) where a single arrow indicates one step between the terms connected, and double arrows indicate unknown number of steps between the connected terms:

$$\text{Rab3A - GDP} \rightarrow \text{Rab3A - GTP} \rightarrow\rightarrow \text{intra - acrosomal calcium release} \rightarrow\rightarrow \text{Rab3A - GTP} \rightarrow \text{Rab3A - GDP} \rightarrow\rightarrow \text{exocytosis} \quad (1)$$

Endogenous Rab3 undergoes transient activation during the AR: (i) the activation step

Next, we used several approaches to investigate whether the endogenous protein undergoes changes similar to those summarized in (1) while the exocytotic cascade progresses. We previously showed an activation (exchange of GDP for GTP) of Rab3 in human

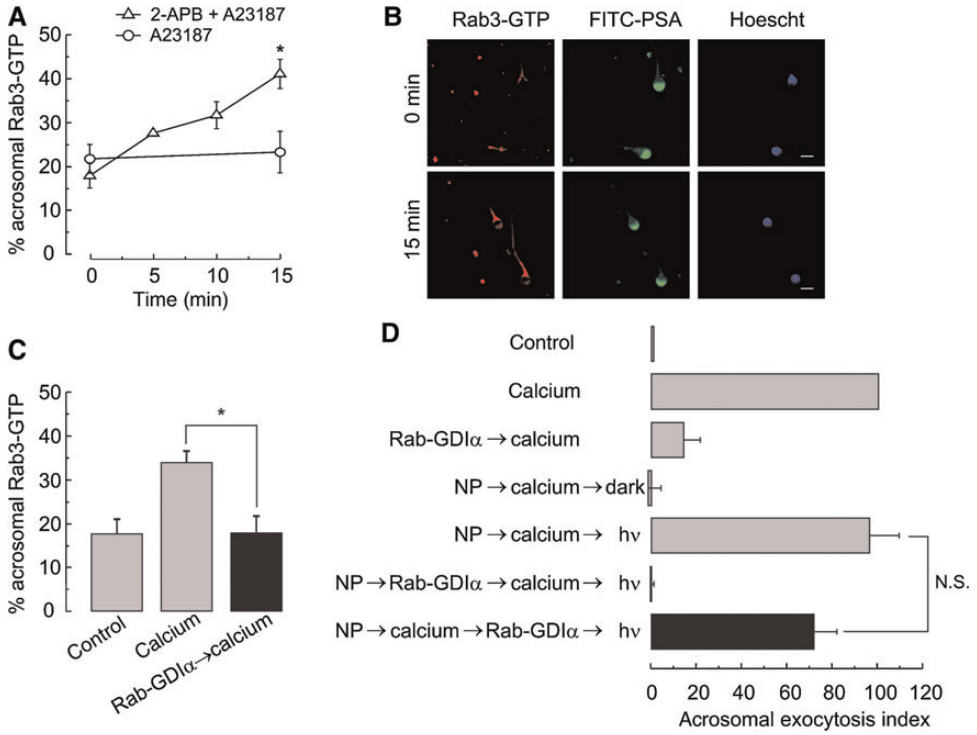

Figure 4 Endogenous Rab3 is activated before intracellular calcium mobilization during the AR. (**A** and **B**) Capacitated sperm incubated with 10 μM A23187 for the indicated times were fixed in 2% paraformaldehyde, attached to poly-L-lysine-coated coverslips, and overlain with 140 nM GST-RIM-RBD in blocking solution. Cells were triple stained with an anti-GST antibody to detect endogenous active Rab3 (left), FITC-PSA to assess acrosomal status (middle), and Hoechst 33342 to visualize all cells (right). (**A**) Percentage of cells immunodecorated in the acrosomal region with the anti-GST antibody at 0, 5, 10, and 15 min after treating with A23187 and 100 μM 2-APB (open triangles) or 0 and 15 min after treating with A23187 alone (open circles). The data represent the mean ± SEM of five independent experiments. *$P < 0.05$ comparing plus/minus 2-APB, 15 min. (**B**) Fields photographed before (top, 0 min) and after (bottom, 15 min) the addition of A23187 to cells treated with 2-APB. Scale bar, 5 μm. (**C**) SLO-permeabilized sperm were treated with 100 μM 2-APB and with or without 400 nM Rab-GDIα. The AR was initiated with 0.5 mM $CaCl_2$. Incubations were for 15 min at 37°C after each addition (black bar). Samples were processed for Rab3-GTP immunodetection as in **A** and **B**. The data represent the mean ± SEM of four independent experiments. *$P < 0.05$. (**D**) Permeabilized spermatozoa were loaded with 10 μM NP-EGTA-AM (NP) for 10 min at 37°C. The AR was subsequently initiated by adding 0.5 mM $CaCl_2$ and 15 min incubation at 37°C. Sperm were treated for 15 min at 37°C with 400 nM recombinant Rab-GDIα. All these procedures were carried out in the dark. UV flash photolysis of the chelator was induced at the end of the incubation period (hν), and the samples were incubated for 5 min at 37°C (NP→ calcium→ Rab-GDIα→ hν, black bar). Controls were as described in Figure 2D and E. Exocytosis was evaluated and the data were normalized as described under Supplementary Materials and methods. Plotted results represent the mean ± SEM of three independent experiments. N.S. indicates statistically non-significant difference between two groups ($P > 0.05$).

sperm during the initial phases (15 min from the time of addition of inducers) of the AR (Branham et al., 2009; Bustos et al., 2012; Lopez et al., 2012). Similar results were obtained from a recently developed fluorescence microscopy-based protocol (refer to as far-immunofluorescence) that determines simultaneously the localization and activation status of endogenous small G proteins (Bustos et al., 2012). By applying this technology, we detected Rab3-GTP in the acrosomal region in 18% of resting cells. The percentage increased with time upon incubation with the calcium ionophore A23187 until it reached a maximum of 41% at 15 min (Figure 4B, anti-GST, red, and 4A, open triangles). It is worth pointing out that we carried out these experiments in sperm preloaded with 2-APB, an IP_3-sensitive calcium channel blocker that halts exocytosis at the docking stage (Supplementary Figure S3C). The rationale for this strategy is that the subcellular compartments that bear Rab3 are shed upon exocytosis (Supplementary Figure S4C and D). This feature makes it impossible to detect Rab3 inactivation (precisely the hypothesis to test) when the acrosome is lost. When we conducted the experiments in sperm treated with A23187 but not with 2-APB, the percentage of cells exhibiting acrosomal labeling for Rab3-GTP did not increase (Figure 4A, open circles). These results are consistent with the notion that when intra-acrosomal calcium release is allowed, sperm that exocytose lose Rab3-GTP staining. On the contrary, when calcium efflux from the acrosomal store is prevented by 2-APB, cells stained for Rab3-GTP accumulate.

In systems with repetitive trafficking rounds, a Rab-GDP is maintained in a cytosolic pool bound to Rab-GDI until a GEF activity exchanges GDP for GTP on Rab. Once this active Rab has exerted its actions, it hydrolyzes GTP, GDI removes the newly formed GDP-bound Rab from membranes and maintains it in a reserve

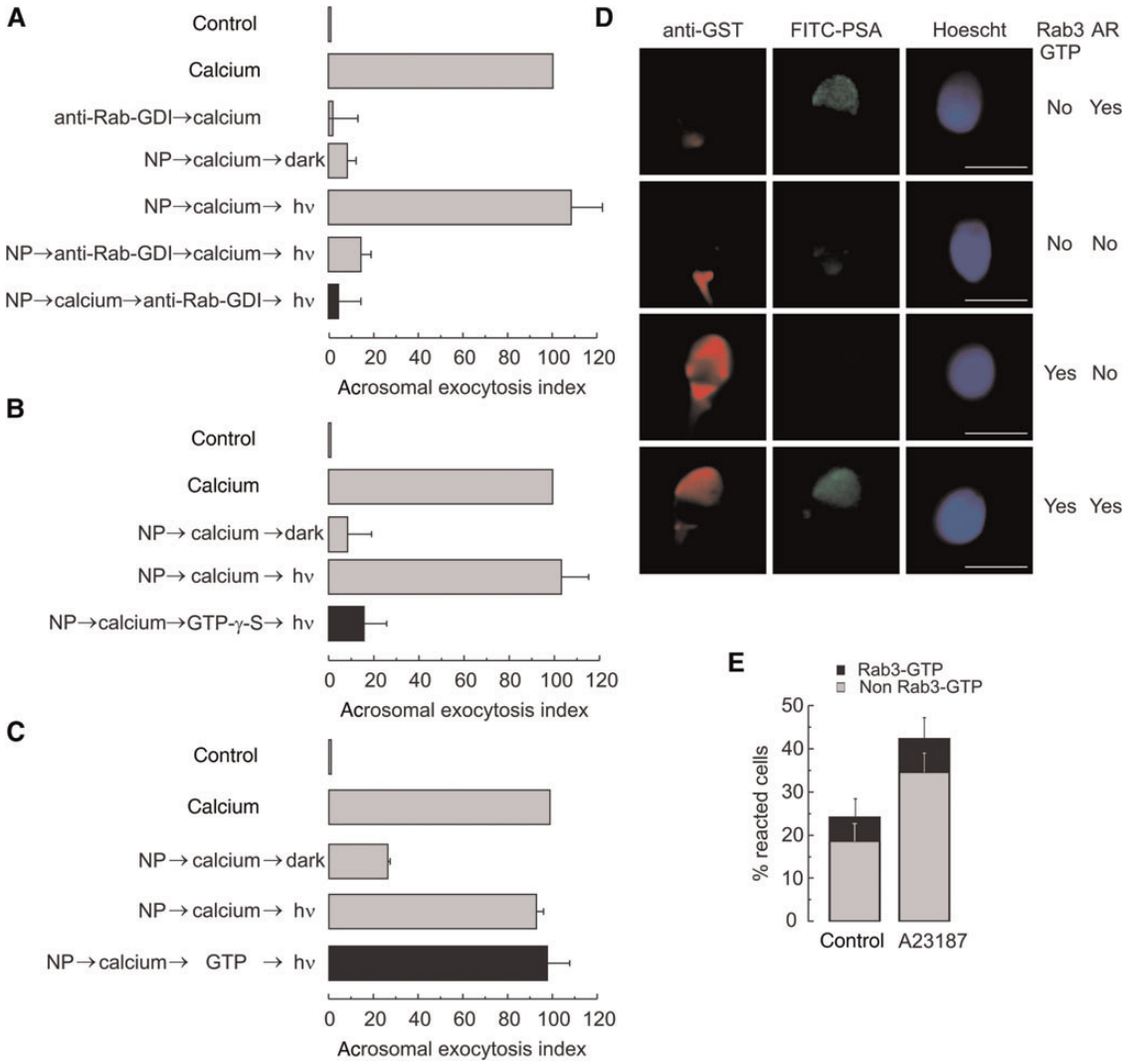

Figure 5 Endogenous Rab3 must hydrolyze GTP for fusion pore opening. (**A**–**C**) Permeabilized spermatozoa were loaded with 10 μM NP-EGTA-AM (NP) for 10 min at 37°C. The AR was subsequently initiated by adding 0.5 mM $CaCl_2$ and 15 min incubation at 37°C. Next, sperm were treated for 15 min at 37°C with 7 nM anti-Rab-GDI antibodies (**A**). Alternatively, after an incubation with EDTA/Mg^{2+} to increase the off rate of bound nucleotides to endogenous G proteins, and 40 μM GTP-γ-S (**B**) or GTP (**C**) for 5 min, bound nucleotides were stabilized with a further 10 min incubation with 15 mM $MgCl_2$. All these procedures were carried out in the dark. UV flash photolysis of the chelator was induced at the end of the incubation period (hν), and the samples were incubated for 5 min at 37°C (NP→ calcium→ anti-Rab-GDI/GTP-γ-S/GTP→ hν, black bar). Controls were as described in Figure 2D and E. Exocytosis was evaluated and the data were normalized as described under Supplementary Materials and methods. Plotted results represent the mean ± SEM of three (**A**), mean ± range of two (**B**), and mean ± SEM of four (**C**) independent experiments. (**D** and **E**) Capacitated sperm were incubated with 10 μg/ml FITC-PSA and 10 μM A23187 for 15 min, fixed in 2% paraformaldehyde, attached to poly-L-lysine-coated coverslips, and overlain with 140 nM GST-RIM-RBD in blocking solution. Cells were triple stained with an anti-GST antibody to detect endogenous Rab3-GTP (left), *in vivo* FITC-PSA for fusion pore opening (middle), and Hoechst 33342 to visualize all cells in the field (right). (**D**) Shown are examples of the four fluorescent staining patterns. Scale bars, 5 μm. (**E**) Quantification shows the percentage of reacted cells that exhibited (black bars corresponding to bottom rows in **D**) or not (gray bars corresponding to top rows in **D**) active Rab3. Plotted results represent the mean ± SEM of four independent experiments.

pool in the cytosol until it is next needed; through this cycle, a Rab molecule can be used multiple times. We then determined the presence of Rab-GDI in human sperm by probing detergent extracts prepared from mouse testis and human sperm with an anti-Rab-GDI antibody that recognizes isoforms α and β on western blots. Data summarized in Supplementary Figure S2A show a single ~55 kDa protein band that comigrates with the positive control, Rab-GDIα from mouse brain cytosol. Rab-GDI partitioned to the aqueous phase following treatment with Triton X-114 (Supplementary Figure S2A and C) and fractionated entirely into the cytosol after subcellular fractionation (Supplementary Figure S2B and C). Thus, a Rab-GDI, likely the α isoform, is present in human sperm and behaves biochemically like that from somatic cells. The anti-Rab-GDI antibodies prevented the calcium-triggered

AR in a dose-response manner (Supplementary Figure S2D), indicating that Rab-GDI is required for exocytosis. We reasoned that an excess of recombinant Rab-GDIα introduced into sperm would bind endogenous Rab3-GDP and impede its activation by AR inducers. We tested this premiss by far-immunofluorescence assay that monitors active Rab3. The percentage of SLO-permeabilized sperm exhibiting Rab3-GTP in the acrosomal region increased ~16% over the basal when cells were challenged with calcium to undergo the AR. When we preloaded the cells with 400 nM Rab-GDIα, stimulation with the same amount of calcium for the same period of time was unable to activate Rab3 (Figure 4C). Accordingly, Rab-GDIα prevented calcium-triggered AR both in the standard assay and when added at the beginning of the incubation to NP-EGTA-AM-loaded sperm (Figure 4D). In contrast, when we inverted the sequence of additions and offered Rab-GDIα after the system had completed the activation of endogenous Rab3, there was a non-statistically significant inhibition of the AR (Figure 4D, black bar). These results provide independent proof to the previous findings that Rab3 must be activated for exocytosis to take place. This activation takes place prior to calcium efflux from the acrosome during the exocytotic cascade, as summarized in sequence (1).

Endogenous Rab3 undergoes transient activation during the AR: (ii) the inactivation step

The inability to hydrolyze GTP is detrimental when recombinant proteins containing the amino-terminal portion of Rab3A are added after calcium challenging during the exocytotic cascade. If GTP hydrolysis was also required at this stage on the endogenous Rab3, it would be expected that a Rab-GDI would play its necessary role through binding to the newly formed Rab3-GDP. We studied the temporal requirement of endogenous Rab-GDI during the AR by using specific antibodies that sequester the protein in cells preloaded with NP-EGTA-AM. The anti-Rab-GDI antibodies blocked the AR when added after the inducer (Figure 5A, black bar). These data suggest that Rab-GDI is required during the AR with a timeframe identical to that when inability to hydrolyze GTP turns recombinant Rab3A into an AR blocker. We interpret these results as follows: before challenging with AR inducers, endogenous Rab3 is inactive and therefore recombinant Rab-GDIα blocks exocytosis because it sequesters Rab3 and maintains it in a GDP-bound state. Fifteen minutes after adding exogenous calcium (while preventing intracellular calcium efflux), endogenous Rab3 has exchanged GDP for GTP and therefore exogenous Rab-GDIα is no longer able to bind it or to inhibit exocytosis. The experiments conducted with the anti-Rab-GDI antibodies suggest that a Rab-GDI continues to be required after incubation with the AR inducer and photodestruction of NP-EGTA-AM with UV light. The most straightforward explanation is that to accomplish the late stages, endogenous Rab3 would need to hydrolyze GTP and be removed from membranes by Rab-GDI.

To test the prediction that GTP hydrolysis is necessary for exocytosis, we compared the response of SLO-permeabilized cells to GTP and the non-hydrolyzable analog GTP-γ-S. We first loaded sperm with NP-EGTA-AM and incubated for 10 min in the dark. Next, we initiated the AR with calcium and after 15 min incubation at 37°C, we added EDTA/Mg^{2+} to increase the off rate of nucleotides bound to endogenous G proteins (Burstein and Macara, 1992) and 40 μM GTP-γ-S (Figure 5B) or GTP (Figure 5C). After incubating for 5 min, we stabilized bound nucleotides with a further 10 min incubation with 15 mM $MgCl_2$, and finally illuminated the tubes. Interestingly, GTP-γ-S blocked calcium-triggered exocytosis (Figure 5B, black bar) but GTP did not (Figure 5C, black bar). These results show that pharmacological intervention to prevent GTP hydrolysis correlates with the inhibition of exocytosis. In other words, we are showing for the first time that a member of the endogenous G protein family must hydrolyze GTP to accomplish the late stages of sperm exocytosis.

Last, we investigated whether such a hydrolysis is required on Rab3A and if so, how does it relate to membrane fusion. GTP hydrolysis is virtually impossible to correlate with exocytosis by static methods in fixed samples, because the acrosomal matrix detected with fluorescent FITC-coupled *Pisum sativum* agglutinin (FITC-PSA) is shed together with the compartment containing Rab3 in cells that undergo exocytosis (Supplementary Figure S4C and D). Thus, the acrosomes of cells that have not undergone exocytosis are bright green (see central panels in Figure 4B and Supplementary Figure S4C, 'non-reacted') whereas cells that have lost their acrosomes do not display fluorescent staining (Supplementary Figure S4C, 'reacted'). Shedding can be prevented with 2-APB, but this treatment affects the read out for Rab3 activation/deactivation (Figure 4A and Supplementary Figure S3B vs. C) and is therefore not suitable to answer the question we posed above. To overcome this limitation, we combined an alternative acrosomal staining method (Zoppino, et al., 2012) with the far-immunofluorescence protocol. In the modified version, live sperm are bathed in FITC-PSA during the incubation with the AR inducer. In consequence, cells that undergo exocytosis during this incubation time stain their acrosomes green because the lectin penetrates into the acrosome through the fusion pores generated when the outer acrosomal membrane fuses with the plasma membrane; all other cells remain unstained (see schematics in Supplementary Figure S3D and E). Normally, after fusion pore opening, plasma and outer acrosomal membranes vesiculate and subsequently vesicles and acrosomal contents disperse into the medium (hence, reacted sperm are not stained with PSA in the standard, indirect protocol). Luckily, in the alternative (direct) staining method, PSA traps vesicles with some acrosomal contents attached to them in an insoluble matrix tethered to reacting sperm. In consequence, the fluorescent label of cells undergoing exocytosis is not transient but permanent and can be readily detected. Because of the topology of the AR, cytosolic or membrane-bound Rab3 would be trapped inside the vesicles that form upon membrane fusion. This is an additional fortunate feature of the direct detection system because we can be sure that lack of Rab3-GTP staining is due to GTP hydrolysis and not to Rab3 loss. We nevertheless ascertained this by indirect immunofluorescence using anti-Rab3 antibodies, which do not distinguish between active and inactive Rab3A, as probe. We scored sperm stained for Rab3A in the acrosomal region of resting cells as well as sperm challenged with A23187 and stained with FITC-PSA before fixing. Both

unreacted and reacted sperm displayed high percentages of stained cells (Supplementary Figure S4C and D). These results confirm that Rab3A is not lost upon exocytosis when the AR is assessed with lectin in the direct AR assay. As expected, reacted cells did not exhibit acrosomal Rab3A staining when FITC-PSA was added to fixed and permeabilized sperm after exocytosis took place in the indirect AR assay (Supplementary Figure S4A and B). There were four different fluorescence patterns in cells subjected to the direct FITC-PSA staining protocol combined with Rab3-GTP far-immunofluorescence (Figure 5D). The vast majority of the cells that underwent exocytosis did not exhibit Rab3-GTP (Figure 5E, gray bars). These data suggest that Rab3 must hydrolyze bound GTP to accomplish the late stages of the exocytotic cascade and allow fusion pores to open between the outer acrosomal and plasma membranes. In this last series of experiments, we did not capture Rab3 during the window of time when it was transiently active due to the absence of 2-APB. Taken together, our data indicate that Rab3 undergoes transient activation during the AR and therefore fits the sequence delineated in (1). The activation phase is sensitive to an excess Rab-GDI; deactivation correlates temporarily with the need for GTP hydrolysis and the requirement for Rab-GDI. Put into perspective, the results obtained with recombinant Rab3A helped us to unveil a key mechanism driven by its native counterpart to achieve fusion.

Discussion

In spite of many years of intense investigation, the role of the Rab3 family in regulating exocytosis still remains controversial. Studies conducted with tissues/cells isolated from Rab3A null mice (Geppert et al., 1994) do not always agree on their interpretation of what is the precise role of this small GTPase in secretion. For instance, it is clear that Rab3A is necessary for insulin secretion because exocytosis is impaired in Rab3A null islets of Langerhans (Yaekura et al., 2003; Merrins and Stuenkel, 2008). Likewise, calcium-triggered exocytosis of α-melanocyte stimulating hormone is selectively diminished in melanotrophs from Rab3A null mice compared with that in wild-type cells (Sedej et al., 2013). Because Rab3A is highly expressed in the brain and associates with synaptic vesicles, much effort has been devoted to identify the role of this protein in synaptic release. Unfortunately, the specific effects of Rab3A deletion on synaptic transmission appear to vary in not only the type of synapse analyzed but also the experimental preparations and therefore determining whether Rab3A exhibits a positive or a negative role in the nervous system remains controversial (Geppert et al., 1994, 1997; Leenders et al., 2001; Sons and Plomp, 2006; Coleman et al, 2007, Coleman and Bykhovskaia, 2009).

Another way to study the role of endogenous Rab3A is to counteract its expression with antisense probes to Rab3A transcripts, which leaves the cells less time for compensatory mechanisms than whole animal knockout approaches. Microinjection of antisense oligonucleotides directed against Rab3A mRNA into adult rat melanotrophs (Rupnik et al., 2007), bovine adrenal chromaffin cells (Johannes et al., 1994, 1998), or PC12 cells interferes with secretion, pointing once again to the requirement of this small GTPase for exocytosis.

Overexpression studies have suggested that Rab3A is a negative modulator of exocytosis; however, not all studies support this view. Depending on the system under study or even the level of expression, transfection with Rab3A enhances, does not modify, or inhibits exocytosis (see Supplementary Table S1 for a non-comprehensive summary).

A strategy more closely related to the one we applied in this study, and designed to circumvent the potential artifacts caused by transfection and overexpression, is to introduce proteins into cells and record secretory activity within hours. In rat melanotrophs, microinjection of wild-type Rab3A does not affect calcium-dependent secretory responses but Rab3AQ81L augments it 3-fold (Rupnik et al., 2007). Microinjected recombinant wild-type Rab3A does not impair secretion induced by a depolarizing voltage step in bovine chromaffin cells, but the Q81L mutant does (Johannes et al., 1994). Dialysis of this mutant into photoreceptors via whole patch clamp blocks synaptic release in an activity-dependent manner (Tian et al., 2012). This study proposes that the role of GTP-bound Rab3A would be to escort a vesicle to the zone of release during the initial attachment. Next, Rab3A would hydrolyze GTP, dissociate from the vesicle, and return to the cytoplasm where it would encounter and escort another vesicle (Tian et al., 2012). This apparently straightforward model is in agreement with work conducted with intracellular trafficking Rabs. Three very important corollaries are derived from this model: (i) active Rab3 participates in the docking of a vesicle to the fusion site, (ii) Rab3 must inactivate and detach, and (iii) these two events happen in a sequence, whereby 'active Rab'-mediated docking precedes the inactivation of Rab, which might be important for fusion pore opening or expansion. How does this translate to experimental preparations? First, we need to bear in mind that most cells house functionally different pools of secretory vesicles. If the experiments zoom in a pool of vesicles that need to reach and attach to the plasma membrane, it is likely that the results would suggest a requirement for active Rab3 translated into a positive role for this GTPase. If, on the contrary, the experiments focus on a pool of already docked vesicles (for instance the readily releasable pool), applying a Rab3 unable to inactivate would be detrimental and therefore the outcome would suggest a negative role for this GTPase. Perhaps there is such an enormous controversy in the field, because it is a serious challenge to design experiments to investigate Rab3 function in cells where many vesicular pools coexist and the overexpression or depletion of Rab3 takes place in very long time scales compared with the life cycle of a vesicle.

We would like to report here that we have dissected this pathway taking advantage of a biological system that lacks these shortcomings because: (i) each sperm contains a single secretory granule rather than a heterogeneous pool, (ii) before applying an exocytotic stimulus, all granules (in all cells) are undocked, (iii) the AR can be halted at different stages (e.g. before docking or after docking but prior to fusion), and (iv) the whole experiment takes place in a short time scale (minutes) in cells with preformed granules and without biogenesis of new ones. The short incubations used throughout our study represent a technical advantage because they do not allow time for compensatory mechanisms or deep perturbations

of the endogenous fusion machinery; thus, the results are basically a straightforward read out for the role of the introduced proteins in exocytosis.

Our findings suggest that Rab3A is a protein with positive and negative attributes segregated to different domains of the molecule. Its positive and negative functions are evidenced at different times and within different molecular contexts during the secretory cascade. Thus, persistently active wild-type Rab3A triggered exocytosis when introduced into SLO-permeabilized cells at the beginning of the incubation (when all granules are undocked, Figure 1B). Unexpectedly, the same protein blocked the AR when added after docking of the acrosome to the plasma membrane (Figure 3A). By means of chimeric proteins, we were able to determine that the carboxy-terminal half of the Rab3A molecule bears the stimulatory activity (Figure 2A) whereas the amino-terminal portion carries the inhibitory activity (Figure 2B and D). The molecular mechanisms through which recombinant Rab3s elicit exocytosis appear to depend on their ability to bind Rab3 effectors, given that full length wild-type Rab3A, Rab3AQ81L, and Rab22A-3A, which bind RIM, are AR inducers whereas Rab3A-22A, which does not bind RIM, is not (Figure 2C). To the best of our knowledge, there is only one published study reporting the behavior of chimeric proteins containing portions of Rab3 isoforms in secretion. In that work conducted in the β-cell line HIT-T15, Rab3AQ81L exhibits a potent inhibitory effect on secretion whereas Rab3CQ81L does not. Interestingly, a Rab3A–Rab3C chimera, in which the carboxy-terminal domain of Rab3AQ81L was replaced with the corresponding region of Rab3C, behaves as Rab3A. In contrast, a Rab3C–Rab3A chimera containing the carboxy-terminal domain of Rab3AQ81L and the amino-terminal portion of Rab3C behaves as Rab3C (Iezzi et al., 1999). Therefore, the two studies agree that chimeric proteins containing the amino-terminal region of Rab3A inhibit secretion whereas those containing the carboxy-terminal portion do not.

Results obtained with recombinant Rab-GDIα and RIM-RBD, anti-Rab-GDIα/β antibodies, and the guanine nucleotides GTP and GTP-γ-S (Figures 4 and 5A–C) indicate that, as hypothesized in (1), endogenous Rab3 is activated and inactivated in a chronological succession to accomplish sperm exocytosis. A new assay that combines far-immunofluorescence with a direct acrosomal staining protocol (Figure 5D and E and Supplementary Figure S4C and D) allows us to monitor simultaneously fusion pore opening and the activation status of Rab3 in individual cells, thus providing direct evidence that Rab3 hydrolyzes GTP in sperm undergoing membrane fusion. The population of human sperm containing active Rab3 in the acrosomal region increases >2-fold at 15 min after challenging 2-APB-pretreated cells with a calcium ionophore. Rab3 activation must be transient, because such an accumulation is not detected in the absence of 2-APB (Figure 4A). These data agree with a study showing that global Rab3A-GTP hydrolysis correlates with massive exocytosis of synaptic vesicles in rat brain synaptosomes (Stahl et al., 1994). We previously published data pointing to the activation of a Rab3-GEF during the first 15 min of treatment with AR inducers (Michaut et al., 2000; Branham et al., 2009; Bustos et al., 2012). Here, we show that recombinant Rab-GDIα sequesters endogenous Rab3-GDP, impedes the exchange of GDP for GTP (Figure 4C), and blocks the AR (Figure 4D) when applied during this window of time. These results are in agreement with those summarized in Figure 1A, which show that recombinant Rab3A loaded with GDP-β-S inhibits the calcium-triggered AR; a possible explanation is that this protein sequesters endogenous Rab3-GEF/s. We are currently trying to identify Rab3-GEF and Rab3-GAP activities relevant for sperm exocytosis; our first candidates are Rab3il1 and Rab3gap1-2, the only GEF and GAP described in mouse epididymis and testis proteomes, respectively (Guo et al., 2010; Chauvin et al., 2012). In short, sequence (1) delineates our working model based on studies conducted with recombinant proteins. The model proposes that during the early stages of the AR, Rab3 exchanges GDP for GTP to accomplish docking and later hydrolyzes GTP to complete fusion pores opening. We found that the behavior of endogenous Rab3 fits in this model.

Materials and methods

Ethics statement

We are cognizant of the Argentinean (ANMAT 5330/97) and international (Declaration of Helsinki) principles and bioethical codes, and guarantee that all procedures carried out in conducting the research reported here were in compliance with both. Human subjects were involved in this project for the purpose of semen donation. The subject population consisted of healthy male donors 21 years of age or over. All donors signed a written Informed Consent form at the time of their enrollment. The Bioethical Committee of the Medical School (Comité de Bioética de la Facultad de Ciencias Médicas de la Universidad Nacional de Cuyo) approved our protocol for the collection and manipulation of human sperm samples. All laboratory procedures followed the safety regulations of the Medical School.

Assessment of acrosomal status by post-fixation (indirect) staining with FITC-PSA

Sperm capacitated as described in Supplementary Materials and methods were spotted on teflon-printed slides, air dried, and fixed/permeabilized in ice-cold methanol for 20 sec. Acrosomal status was evaluated by staining with FITC-PSA (25 μg/ml in PBS) for 40 min at room temperature followed by a 20-min wash in water (Mendoza et al., 1992). We scored at least 200 cells per condition using an upright Nikon Optiphot II microscope equipped with epifluorescence optics. Basal ('control', no stimulation) and positive ('calcium', 0.5 mM $CaCl_2$ corresponding to 10 μM free calcium estimated by MAXCHELATOR, a series of program(s) for determining the free metal concentration in the presence of chelators; available on the World Wide Web at http://www.stanford.edu/~cpatton/maxc.html, Chris Patton, Stanford University, Stanford, CA, USA) controls were included in all experiments. Acrosomal exocytosis indices were calculated by subtracting the number of spontaneously reacted spermatozoa (basal control without stimulation, ranged 5%–20% before normalization) from all values and expressing the results as a percentage of the AR observed in the positive control (ranged 15%–35% before normalization; assigned 100%

of responsive cells for normalization). We only included in our analysis results derived from experiments that produced similar responses and where the difference between basal and calcium-stimulated conditions was of at least eight percentage points. Samples with a level of spontaneously reacted sperm higher than 20% were excluded from our analysis. Data were evaluated using the Tukey–Kramer *post hoc* test for pairwise comparisons. Differences were considered significant at the $P < 0.05$ level.

Double staining procedure for the assessment of nucleotide-binding status of endogenous Rab3 by far immunofluorescence and the acrosomal status by post-fixation (indirect) or in vivo (direct) staining with FITC-PSA

Experiments were conducted with capacitated sperm, permeabilized (Figure 4C) or not (Figures 4A, B and 5D, E) with SLO and incubated with (Figure 4A, open triangles) or without (Figures 4A, open circles, and 5D, E) 100 μM 2-APB. The AR was induced in non-permeabilized cells with 10 μM A23187 and in SLO-permeabilized cells with 0.5 mM $CaCl_2$, incubating for 15 min at 37°C. For experiments where acrosomal status was assessed with the *in vivo* (direct) staining method (Figure 5D and E), sperm suspensions were bathed in 10 μg/ml FITC-PSA for all the incubation time. In all cases, aliquots were withdrawn and fixed in 2% paraformaldehyde, neutralized with 100 mM glycine, attached to poly-L-lysine (stock 0.1% *w*/*v* (Sigma) diluted 1:20 in water) coated, 12 mm round coverslips by incubating for 30 min at room temperature, and stored overnight at 4°C in a moisturized chamber. Sperm membranes were permeabilized in 0.1% Triton X-100 in PBS for 10 min at room temperature, cells were washed three times (6 min each) with PBS/0.1% PVP (PBS/PVP), and non-specific reactivity was blocked in 5% BSA in PBS/PVP for 1 h at 37°C. Slides were overlain with 140 nM GST-RIM-RBD in 3% BSA in PBS/PVP for 1 h at 37°C. After washing (three times, 6 min each, PBS/PVP), anti-GST antibodies were diluted at 31.5 μg/ml (210 nM) in 3% BSA in PBS/PVP, added to the coverslips, and incubated for 1 h at 37°C in a moisturized chamber. After washing twice for 10 min with PBS, we added Cy3-conjugated goat anti-rabbit IgG (1.67 μg/ml in 1% BSA in PBS/PVP) and incubated for 1 h at room temperature protected from light. Coverslips were washed six times for 6 min each with PBS/PVP. In experiments where acrosomal status was assessed with the post-fixation (indirect) staining method (Figure 4A–C), cells were subsequently stained for acrosomal contents as described under 'Assessment of acrosomal status by post-fixation (indirect) staining with FITC-PSA' but without air drying. All samples were mounted with 1% propyl-gallate/50% glycerol in PBS containing 2 μM Hoechst 33342 and stored at −20°C in the dark until examination with an Eclipse TE2000 Nikon microscope equipped with a Plan Apo 40×/1.40 oil objective and a Hamamatsu digital C4742-95 camera operated with MetaMorph 6.1 software (Universal Imaging Corp.). We scored the presence of red and/or green staining in the acrosomal region by manually counting between 100 and 200 cells either directly at the fluorescence microscope or in digital images from at least 10 fields. Data were evaluated using the Tukey–Kramer *post hoc* test for pairwise comparisons. Background was subtracted and brightness/contrast were adjusted to render an all-or-nothing labeling pattern using Image J (freeware from N.I.H.).

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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Conflict of interest: none declared.

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