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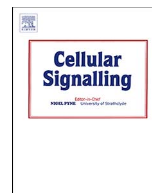
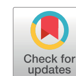


Membrane-permeable Rab27A is a regulator of the acrosome reaction: Role of geranylgeranylation and guanine nucleotides



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The acrosome reaction is the regulated exocytosis of mammalian sperm's single secretory granule, essential for fertilization. It relies on small GTPases, the cAMP binding protein Epac, and the SNARE complex, among other components. Here, we describe a novel tool to investigate Rab27-related signaling pathways: a hybrid recombinant protein consisting of human Rab27A fused to TAT, a cell penetrating peptide. With this tool, we aimed to unravel the connection between Rab3, Rab27 and Rap1 in sperm exocytosis and to deepen our understanding about how isoprenylation and guanine nucleotides influence the behaviour of Rab27 in exocytosis. Our results show that TAT-Rab27A-GTP-γ-S permeated into live sperm and triggered acrosomal exocytosis per se when geraylgeranylated but inhibited it when not lipid-modified. Likewise, an impermeant version of Rab27A elicited exocytosis in streptolysin O-permeabilized — but not in non-permeabilized — cells when geranylgeranylated and active. When GDP-β-S substituted for GTP-γ-S, isoprenylated TAT-Rab27A inhibited the acrosome reaction triggered by progesterone and an Epac-selective cAMP analogue, whereas the non-isoprenylated protein did not. Geranylgeranylated TAT-Rab27A-GTP-γ-S promoted the exchange of GDP for GTP on Rab3 and Rap1 detected by far-immunofluorescence with Rab3-GTP and Rap1-GTP binding cassettes. In contrast, TAT-Rab27A lacking isoprenylation or loaded with GDP-β-S prevented the activation of Rab3 and Rap1 elicited by progesterone. Challenging streptolysin O-permeabilized human sperm with calcium increased the population of sperm with Rap1-GTP, Rab3-GTP and Rab27-GTP in the acrosomal region; pretreatment with anti-Rab27 antibodies prevented the activation of all three. The novel findings reported here include: the description of membrane permeant TAT-Rab27A as a trustworthy tool to unveil the regulation of the human sperm acrosome reaction by Rab27 under physiological conditions; that the activation of endogenous Rab27 is required for that of Rab3 and Rap1; and the connection between Epac and Rab27 and between Rab27 and the configuration of the SNARE complex. Moreover, we present direct evidence that Rab27A's lipid modification, and activation/inactivation status correlate with its stimulatory or inhibitory roles in exocytosis.

1. Introduction

Exocytosis is a highly regulated process whereby the membrane surrounding secretory vesicles and granules fuses with the plasma membrane, so that the organelles release their contents. Rab GTPases are key players in exocytic and endocytic membrane trafficking. During membrane fusion, Rab proteins direct the recognition and physical attachments of the compartments that are going to fuse [2,49]. Newly synthesized Rab GTPases undergo posttranslational addition of geranylgeranyl (20-carbon) isoprenoids onto two cysteines at or near their carboxyl terminus via thioether linkages. Geranylgeranylation is necessary for proteins to reversibly associate with cellular membranes in order to execute their intracellular functions [32]. After prenylation, the inactive, GDP-bound Rab protein can be delivered to a target membrane, where a guanine nucleotide exchange factor (GEF) facilitates GDP release. GTP binds immediately to the guanine nucleotide-free Rab because of its high cytosolic concentration (ten-fold more abundant than GDP). In this active state, Rab-GTP interacts with

Abbreviations: 2-APB, 2-aminoethoxydiphenylborate; AR, acrosome reaction; BoNT/B, botulinum toxin B; Epac, guanine nucleotide exchange factor activated by cAMP; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; GST, glutathione-S-transferase; HTF, human tubal fluid; IPTG, isopropyl-β-D-thio-galactoside; NSF, N-ethylmaleimide-sensitive factor; PBS, phosphate buffer saline; 8-pCPT-2'-O-Me-cAMP, 8-(p-chlorophenylthio)-2 prime-O-methyladenosine-3′,5′-cyclic monophosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Slac2-b, synaptotagmin-like protein homology domain; SLO, streptolysin O; α-SNAP, α-soluble NSF-attachment protein; SNARE, soluble NSF-attachment protein receptor; TPEN, N,N,N′,N′-tetrakis (2-pyridymethyl) ethylenediamine

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effectors to carry out its biological functions. Finally, a GTPase activating protein stimulates the Rab protein's intrinsic GTPase activity, thereby returning it to its inactive, GDP-bound form. Because these cycles of activation and deactivation are repetitive, Rabs are viewed as molecular switches. Oscillations between active GTP-bound and inactive GDP-and GDI-bound conformations may be coupled to subcellular partitioning into membrane-bound and cytosolic states. Rab proteins that cannot undergo geranylgeranylation remain cytosolic and are inactive [6,49,59].

Rab27 is one of the main Rab subfamilies directly implicated in regulated exocytosis. Functional studies carried out with tissues from Rab27-deficient mice or cultured cells overexpressing Rab27 mutants (T23N, with lower affinity for GTP than for GDP, dominant-negative; N133I, with low affinity for GDP and GTP, dominant-negative; Q78L, with reduced GTPase activity, constitutively active; and C219A/C221A, non-geranylgeranylatable and cytosolic) have established that this small GTPase is a key regulator of intracellular organelle movement and secretion in various cell types (reviewed in [12,21,58]). Activation and/or inactivation of Rab27 isoforms have been described in relation to dense-core granules secretion in several systems, for example in salival amylase-secreting parotid acinar cells [25], insulin secreting MIN6 cells [30] and platelets [31].

The exocytosis of mammalian sperm's acrosome (acrosome reaction, AR) is an essential secretory process that must occur at the appropriate time and location for a productive sperm-egg interaction during fertilization. The AR relies on the same highly conserved molecules that drive intracellular membrane fusion and exocytosis in all other cells, save a few particularities (see [4,50] for recent reviews). Sperm Rab27 does not cycle between membranes and cytosol but it is preferentially membrane-bound [9]. Its activation state is coupled to exocytotic stimuli. Thus, a low percentage of resting sperm contains Rab27 in its GTP-bound, active conformation. This percentage increases in response to exocytosis inducers [9,35,45]. Another singularity of the AR is the regulation of the activity of NSF, the ATPase that disentangles *cis* SNARE complexes. In resting sperm, NSF is repressed by tyrosine phosphorylation and SNAREs are predominantly engaged in neurotoxin-resistant, fusion-incompetent *cis* complexes. Upon initiation of the AR, protein tyrosine phosphatase 1B (PTP1B) dephosphorylates NSF, de-repressing its activity. Hence, exocytosis inducers cause *cis* complexes to dissociate into toxin-sensitive, monomeric SNAREs that subsequently engage in a fusion-productive *trans* configuration [17,61].

The biggest obstacle when trying to apply classic cell biology and biochemistry to the study of sperm exocytosis resides on this cell's inability to synthesize proteins. Two techniques have been developed to overcome this limitation: controlled plasma membrane permeabilization with pore-forming toxins and delivery of permeable proteins (reviewed in [4]). We used both to attain the results reported in this study. The contribution of this paper to the sperm biology field is to expand the small repertoire of permeable proteins that have been engineered to apply protein transduction to mammalian sperm. TAT-Rab27A allowed us to scrutinize in detail the influence of geranylgeranylation and guanine nucleotide status of Rab27 on exocytosis under physiological conditions. The ability to unveil Rab27-driven signaling pathways in live cells in short time frames confers an advantage over genetic methods because cells, their machinery, maturation and properties are preserved. Other novel results reported here are the unsuspected connection between Epac and Rab27 and a link between Rab27 and the disassembly of the SNARE complex. We hope our findings might be extended and contribute to the exocytosis field in general in the future.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant SLO was obtained from Dr. Bhakdi (University of Mainz, Mainz, Germany). The rabbit polyclonals anti-Rab27 (affinity purified with the immunogen), anti-NSF (whole serum) and anti-α-tubulin (affinity purified with the immunogen) antibodies were from Synaptic Systems (Göttingen, Germany). The rabbit polyclonal anti-GST antibody (purified IgG) was from EMD Millipore Corporation (Billerica, MA). The mouse monoclonal anti-His_6 antibody (purified IgG 2a) was from GE Healthcare (Buenos Aires, Argentina). Horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) was from Kierkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Horseradish peroxidase- and Cy™3-conjugated goat anti-rabbit IgGs (H + L) were from Jackson ImmunoResearch (West Grove, PA). 8-(p-chlorophenylthio)-2 prime-O-methyladenosine-3′,5′-cyclic monophosphate (8-pCPT-2’-O-Me-cAMP) was from Axxora, LLC (San Diego, CA). N,N,N′,N′-tetrakis (2-pyridymethyl) ethylenediamine (TPEN) from Molecular Probes was purchased from Invitrogen (Buenos Aires, Argentina). Prestained molecular weight markers were from Boston BioProducts Inc. (Worcester, MA). 2-aminoethoxydiphenylborate (2-APB) from Calbiochem was purchased from Merck Química Argentina S.A.I.C. (Buenos Aires, Argentina). Glutathione-Sepharose and Ni-NTA-agarose were from GE Healthcare. All other chemicals were purchased from Sigma-Aldrich™ Argentina S.A., Genbiotech, or Tecnolab (Buenos Aires, Argentina).

2.2. Expression and purification of recombinant proteins

A pQE9 (Qiagen GmbH, Hilden, Germany) construct encoding full length, wild type α-SNAP was a kind gift from Dr. S. Whiteheart (University of Kentucky, Lexington, KY). A plasmid encoding NSF in pET28a (Stratagene, La Jolla, CA) was generously provided by Dr. D. Fasshauer (University of Lausanne, Lausanne, Switzerland). The cDNA encoding the light chain of botulinum toxin B (BoNT/B) fused to His_6 (pQE3, Qiagen) was generously provided by Dr. T. Binz (Medizinische Hochschule Hannover, Hannover, Germany). A pGEX-6p (GE Healthcare) construct encoding Rab27A was a kind gift from Dr. D. Munafó (The Scripps Research Institute, La Jolla, CA). GST fusion protein with the Rab3-GTP-binding domain of rat RIM (amino acids 11–398, RIM-RBD) [14] in a pGEX2p vector was generously provided by Dr. R. Regazzi (University of Lausanne, Lausanne, Switzerland). The Rap-GTP binding cassette Ral-GDS-RBD fused to GST [54] was a kind gift from Dr. O. Coso (Universidad de Buenos Aires, Buenos Aires, Argentina). DNA encoding the Rab27-GTP binding domain of Slac2-b (synaptotagmin-like protein homology domain-2; amino acids 1–79) in pGEX-2T [31] was a kind gift from Dr. R. Shirakawa (Kyoto University, Kyoto, Japan).

The plasmid encoding NSF was transformed in *Escherichia coli* BL21(DE3) $T1^R$ (New England Biolabs, Ipswich, MA). cDNA encoding α-SNAP was transformed into *Escherichia coli* XL-1Blue (Stratagene) and that encoding BoNT/B was transformed into *Escherichia coli* M15pRep4 (Qiagen). Protein expression was induced with 0.6 mM isopropyl-β-D-thio-galactoside (IPTG) (3 h at 37 °C, α-SNAP) or 1 mM IPTG (3 h at 37 °C, NSF; 4 h at 30 °C, BoNT/B). Plasmids encoding all GST-fused proteins were transformed in *Escherichia coli* BL21(DE3) $T1^R$ and protein expression was induced with IPTG as follows: GST-Slac2-b and GST-Rab3A, 0.5 mM IPTG for 3 h at 37 °C; GST-Ral-GDS-RBD 0.1 mM IPTG, overnight at 22 °C; GST-RIM-RBD 0.5 mM IPTG, overnight at 22 °C; GST-Rab27A 1 mM IPTG, 3 h at 37 °C. GST-fused recombinant proteins were purified on glutathione-Sepharose beads following standard procedures. Purification of His_6-tagged recombinant proteins was carried out under native conditions according to Qiagen's instructions except that the purification buffers contained 20 mM TrisHCl, pH 7.4 and 200 mM NaCl; lysis buffer contained 10 mM imidazole, washing buffer contained 50 mM imidazole; and elution buffer contained 250 mM imidazole and that all solutions involved in the purification of NSF contained 5 mM ATP, 5 mM $MgCl_2$, and 2 mM β-mercaptoethanol plus NaCl (500 mM for lysis and 200 mM for washing and elution buffers). Recombinant protein concentrations were determined by the Bradford method (Bio-Rad) using bovine serum albumin as a standard in 96-well microplates and quantified on a BioRad 3550 Microplate

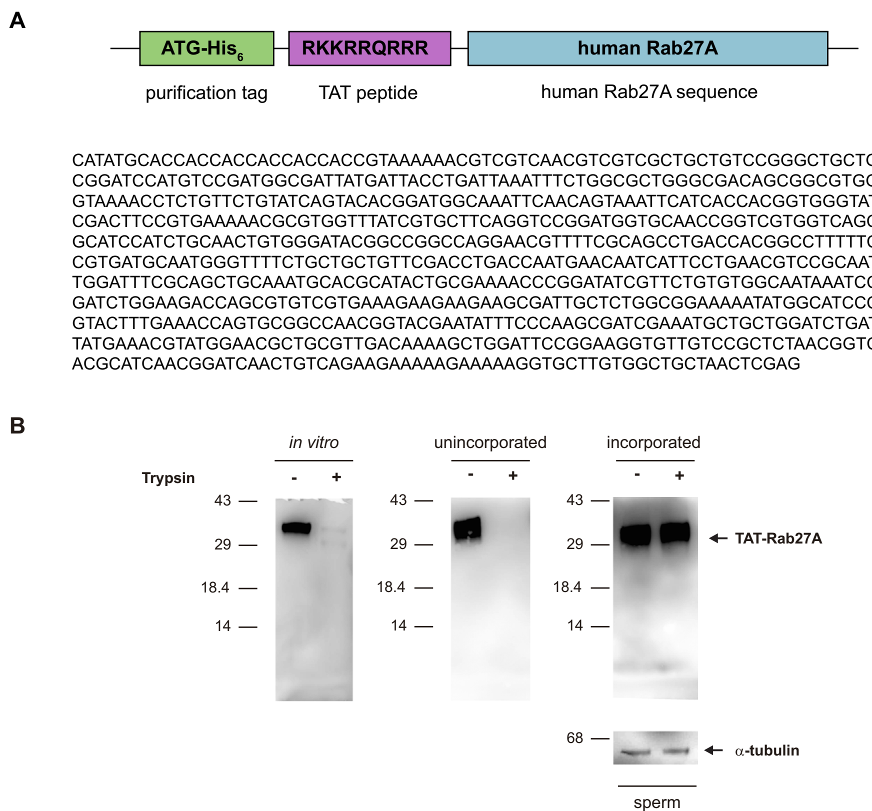


Fig. 1. TAT-Rab27A transduces into human sperm. A, Schematic representation of the TAT-Rab27A protein. The sequence encoding human Rab27A (blue) was fused at its N-terminus to the TAT peptide from HIV virus (violet) for protein transduction and a His_6 tag (green) for affinity purification and immunodetection. The DNA sequence encoding pemeable Rab27A optimized for expression in *Escherichia coli* by Genscript OptimumGene™ algorithm is shown at the bottom. B, TAT-Rab27A was delivered to the intracellular compartment of human sperm because it resisted degradation by trypsin. Left, "In vitro" panel shows trypsin-sensitivity of TAT-Rab27A in solution: 65 ng of the protein were incubated with (+) or without (−) 0.5 μg/ml trypsin for 20 min at 37 °C. Central and right panels: human sperm (35×10^6 cells/ml) were incubated with 65 ng (125 nM) TAT-Rab27A for 20 min at 37 °C. Subsequently, cells were treated with (+) or without (−) 0.5 μg/ml trypsin. After 20 min at 37 °C, samples were centrifuged and proteins from both the sperm pellet (right, "incorporated") and the extracellular supernatant (centre, "unincorporated") were processed for anti-His_6 Western blot. Anti-α-tubulin was used to show equal loads. Mr. standards ($\times 10^3$) are indicated on the left. Shown is an experiment representative of two repetitions.

Reader or from the intensities of the bands in Coomassie Blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis (SDS-PAGE) gels.

2.3. Construction and expression of the TAT-Rab27A

The cDNA encoding a permeable version of human Rab27A optimized for bacterial expression was synthesized by Genscript (Piscataway, NJ). The insert was subcloned in frame with the TAT sequence of HIV virus (RKKRRQRRR) for protein transduction into the HindIII-BamHI cloning site of pET28a(+) vector (Novagen, now Merck-Millipore). The cDNA encoding His_6-TAT-Rab27A (Fig. 1A) was transformed into *Escherichia coli* BL21(DE3) $T1^R$ and expression of recombinant protein was accomplished by incubation with 0.5 mM IPTG for 3 h at 37 °C. Purification of the TAT-Rab27A was carried out following standard procedures. When indicated, TAT-Rab27A was geranylgeranylated and loaded with guanine nucleotides as described [9].

2.4. Human sperm sample preparation procedures

After at least two days of abstinence, semen samples were provided by masturbation from healthy volunteer donors (age range 21–45) who were free from sexually transmitted diseases. Only samples that met the quality parameters established by the World Health Organization (WHO) were included in the analysis. Semen was allowed to liquefy for 30–60 min at 37 °C. Following a swim-up protocol to isolate highly motile cells, sperm concentrations were adjusted to 10^7/ml before incubating for at least 2 h under capacitating conditions (Human Tubal Fluid media as formulated by Irvine Scientific (Santa Ana, CA) supplemented with 0.5% bovine serum albumin, HTF medium), 37 °C, 5% CO_2/95% air). Samples were processed for AR assays, Western blot and far-immunofluorescence [35].

2.5. Trypsin protection assay for the TAT-Rab27A transduced into human sperm

Sperm incubated under capacitating conditions were washed and resuspended in HTF without BSA (7×10^6 cells in 200 μl) and incubated for 10 min at 37 °C with 100 μM 2-APB for 10 min at 37 °C to prevent acrosomal loss due to exocytosis [8,16] followed by 125 nM His_6-TAT-Rab27A for 30 min at 37 °C. After washing once with PBS, sperm were exposed to 0.5 μg/ml trypsin (Sigma) for 20 min at 37 °C. Cells were washed once with PBS, boiled for 3 min at 95 °C in 60 μl of sample buffer and analyzed by Western blot with anti-His_6 antibodies as probe to detect the internalized recombinant protein. Proteins from cell-free supernatants were precipitated with CCl_3H–CH_3OH–H_2O. Precipitated proteins were dissolved in sample buffer by heating once at 60 °C for 10 min and once at 95 °C for 3 min and processed for Western blot.

3. Results

3.1. TAT-Rab27A transduces into human sperm

Sperm lack the machinery to synthesize proteins, which seriously restricts the biochemistry that can be performed with them. Two methods have been developed to overcome this limitation: a SLO-plasma membrane permeabilization protocol and the delivery of permeable proteins into living cells. Here, we describe a permeable version of Rab27A in which the entire coding sequence of the human Rab27A gene was synthesized and inserted in a vector for bacterial expression of permeable proteins (Fig. 1A) [35]. Rab27A coupled to a TAT peptide (that confers membrane permeability) and a His_6-tag (for purification and detection) was expressed in *Escherichia coli,* purified by affinity chromatography on Ni-NTA-agarose and applied to suspensions of living human sperm. We analyzed the incorporation — reported by resistance to trypsin digestion — of TAT-Rab27A into human sperm by Western blot with anti-His_6 antibodies. We first established the minimal

concentration of trypsin capable of digesting the amount of recombinant TAT-Rab27A to be used subsequently in incorporation assays (65 ng, corresponding to 125 nM in 200 µl final volume). As shown in the left panel of Fig. 1B, 0.5 µg/ml trypsin erased the anti-His_6 signal, which reflects proteolytic cleavage of TAT-Rab27A. Next, we incubated sperm with 125 nM TAT-Rab27A for 30 min at 37 °C, divided the sample, added 0.5 µg/ml trypsin to one tube and incubated for another 20 min at 37 °C. We recovered both cells and supernatants and processed them for anti-His_6 Western blot. A significant proportion of TAT-Rab27A was protected from trypsin digestion (Fig. 1B, right panel). As a control, 0.5 µg/ml trypsin removed the His_6 signal of all unincorporated TAT-Rab27A, which indicates that the enzyme was active and its concentration sufficient (Fig. 1B, center panel). These results indicate that TAT-Rab27A transduced into live human sperm.

3.2. Permeable Rab27A serves as an inducer or blocker of sperm exocytosis depending on its geranylgeranylation and guanine nucleotide status

When added to human sperm, recombinant, lipid modified and active small GTPases Rab3A [11,33,60] and ARF6 [39] trigger exocytosis. To investigate whether Rab27A induces the AR, we introduced geranylgeranylated TAT-Rab27A into human sperm and assessed exocytosis by lectin-binding at the fluorescence microscope. TAT-Rab27A, geranylgeranylated and activated with GTP-γ-S *in vitro*, induced the AR in a dose–response fashion (Fig. 2A, closed circles). In contrast, a non-permeant version of recombinant Rab27A, isoprenylated and loaded with GTP-γ-S did not elicit an exocytotic response (Fig. 2B). These results suggest that the effects of TAT-Rab27A on the AR are due to its transduction into cells.

Both progesterone and the Epac-selective cAMP analogue 8-pCPT-2'-O-Me-cAMP induce the AR in human sperm. Three hundred nM TAT-Rab27A-GTP-γ-S triggered the AR to the same extent accomplished by both of them (Fig. 2C). Geranygeranylated TAT-Rab27A loaded with GDP did not affect the AR per se at any of the concentrations tested (Fig. 2A, open circles). However, TAT-Rab27A-GDP-β-S prevented the AR triggered by progesterone and 8-pCPT-2′-O-Me-cAMP (Fig. 2C). In short, geranylgeranylated Rab27A behaves as a human sperm exocytosis inducer when bound to GTP and as an inhibitor when bound to GDP. Both these activities were lost when the protein was added to sperm without prior geranylgeranylation: neither TAT-Rab27A-GTP-γ-S induced nor TAT-Rab27A-GDP-β-S inhibited the AR (Fig. 2D). On the contrary, TAT-Rab27A-GTP-γ-S prevented progesterone and 8-pCPT-2′-O-Me-cAMP from triggering exocytosis (Fig. 2D). Taken together, these results indicate that geranylgeranylation and guanine nucleotides govern the actions of Rab27A on the AR.

3.3. Epac stimulation by 8-pCPT-2′-O-Me-cAMP induces the exchange of GDP for GTP on Rab27

Antibodies against Rab27 introduced into SLO-permeabilized human sperm inhibit the AR elicited by calcium [9,45]. We have previously shown that anti-Rap1 and anti-Rab3 antibodies halt the exocytotic cascade because they impair the activation of endogenous small promoted by exocytosis triggers [45]. Here, we investigated whether

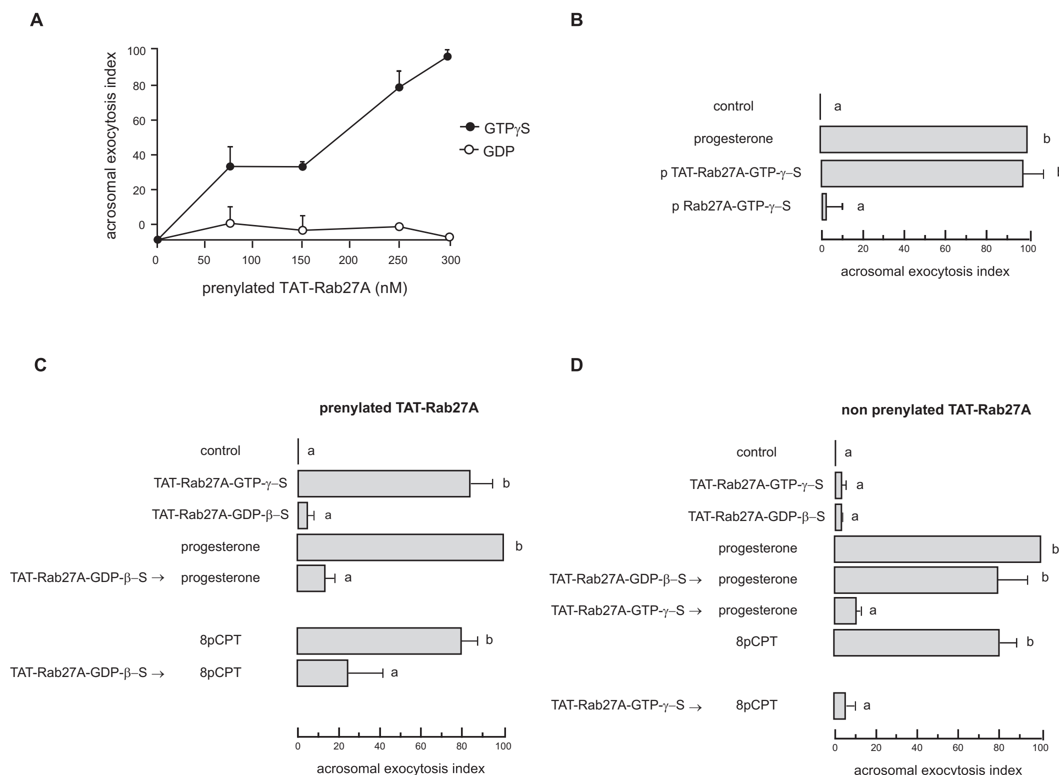


Fig. 2. Permeable Rab27A influences sperm exocytosis differently depending on its geranylgeranylation and guanine nucleotide status. A, Geranylgeranylated TAT-Rab27A loaded with GDP (○) or GTP-γ-S (●) was introduced into human sperm and incubated for 15 min at 37 °C. Sperm were fixed and the AR was measured by FITC-PSA binding. Values were normalized to the AR induced by 15 µM progesterone and assigned 100%. Plotted results represent the mean ± SEM of at least three independent experiments. B, Sperm were incubated for 15 min at 37 °C with 15 µM progesterone, 300 nM geranylgeranylated TAT-Rab27A loaded with GTP-γ-S, or 300 nM geranylgeranylated Rab27A (non-permeant) loaded with GTP-γ-S. Samples were processed and AR scored as explained in A. Plotted results represent the mean ± SEM of at least three independent experiments. Tukey–Kramer post hoc test was used for pairwise comparisons. Different letters indicate statistical significance ($P < .001$). C, D Sperm were incubated for 15 min at 37 °C with 300 nM TAT-Rab27A geranylgeranylated (C) or not (D) and loaded with GDP-β-S or GTP-γ-S. When indicated, cells were further incubated with 15 µM progesterone or 50 µM 8-pCPT-2′-O-Me-cAMP (8pCPT). Controls included background AR in the absence of any stimulation (control) and AR stimulated by 15 µM progesterone or 50 µM 8-pCPT-2′-O-Me-cAMP. Samples were processed and AR scored as explained in **A**. Plotted results represent the mean ± SEM of at least three independent experiments. Tukey–Kramer post hoc test was used for pairwise comparisons. Different letters indicate statistical significance (C: $P < .05$, D: $P < .001$).

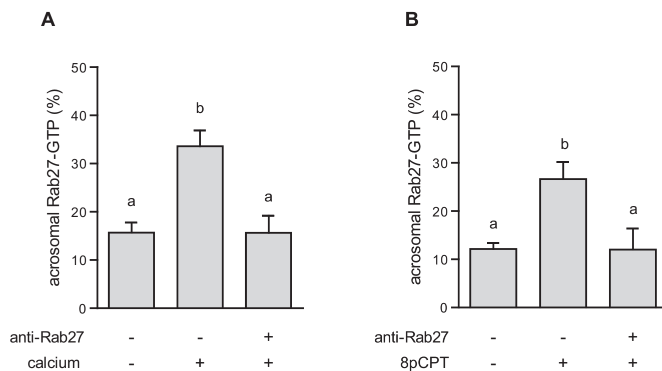


Fig. 3. The Epac selective cAMP analogue 8-pCPT-2′-O-Me-cAMP activates Rab27 in the acrosomal region. Human sperm were permeabilized with SLO, incubated with 100 μM 2-APB and, when indicated, with 7 nM anti-Rab27 antibodies, followed by 0.5 mM $CaCl_2$ (A) or 50 μM 8-pCPT-2′-O-Me-cAMP (B) for 15 min at 37 °C. Cells were processed for far-immunofluorescence, overlain with GST-Slac2-b-SHD to detect active Rab27 and stained with the anti-GST antibody to visualize the activity probe. Shown are quantifications of the percentage of cells with active Rab27 in the acrosomal region. The data represent the mean ± SEM of three independent experiments. Tukey–Kramer post hoc test was used for pairwise comparisons. Different letters indicate statistical significance (A: $P < .01$, B: $P < .05$).

the anti-Rab27 antibodies inhibit the AR through a similar mechanism. We have developed a far-immunofluorescence protocol to quantify the population of cells exhibiting endogenous Rab27-GTP in the acrosomal domain [9,10]. As shown before, Rab27 was active in a small population of resting sperm. Challenging with AR inducers augmented the percentage of cells with acrosomal Rab27-GTP (Fig. 3 and [9,35]). The proportion of cells with active Rab27 after sequential incubations with anti-Rab27 antibodies and calcium was similar to that in the basal conditions (Fig. 3A). These results suggest that anti-Rab27 antibodies impaired the activation of endogenous Rab27 elicited by calcium.

Progesterone promotes the activation of sperm Rab27 via a cAMP-dependent pathway [35]. The cAMP target relevant for exocytosis in human sperm is Epac [8]. The Epac selective cAMP analogue 8-pCPT-2′-O-Me-cAMP increases the amount of active Rab3 and Rap1 pulled down from human sperm compared to untreated controls [7,9] as well as the number of cells exhibiting Rab3-GTP [45] and Rap1-GTP [35] in the acrosomal domain. We conducted the next experiments to elucidate whether there is a connection between Epac and Rab27. 8-pCPT-2′-O-Me-cAMP was as effective as calcium in promoting the exchange of GDP for GTP on endogenous Rab27. As expected, activation did not take place in sperm preincubated with anti-Rab27 antibodies (Fig. 3B). These results indicate that the stimulation of Epac by cAMP leads to the activation of Rab27.

3.4. Geranylgeranylation and guanine nucleotides status govern how Rab27 influences the activation of Rab3 and Rap1

Because of the central roles played by these small GTPases in the exocytotic cascade, we considered essential to gather additional data to investigate the connection between the three. All AR inducers tested to date elicit the activation of Rab3 in the acrosomal domain of human sperm [9,35,39,45]. Likewise, Fig. 4A shows that the percentage of cells with active Rab3 in the acrosomal domain increases from 20% to 37% upon treatment with calcium. When we introduced anti-Rab27 antibodies into SLO-permeabilized human sperm prior to challenging with calcium, the percentage of cells with active Rab3 remained low (Fig. 4A). These findings show that endogenous Rab27 is required for calcium to activate sperm Rab3.

We reasoned that to become a valuable reporter of physiological events, TAT-Rab27A should modulate the same exocytotic stage as endogenous Rab27. When live human sperm were incubated with geranylgeranylated (p) TAT-Rab27A-GTP-γ-S (III), the percentage of cells displaying Rab3-GTP in the acrosomal region was higher than in the untreated controls (I). Similar proportions of cells exchanged GDP for GTP on Rab3 in response to Rab27A (III) and to progesterone (II), the positive control (Fig. 4B). These results indicate that lipid-modified TAT-Rab27A-GTP-γ-S is a bona fide AR inducer. Geranylgeranylated TAT-Rab27A-GDP-β-S (IV) as well as non-isoprenylated (np) TAT-Rab27A-GTP-γ-S (V) prevented progesterone from activating Rab3 (Fig. 4B).

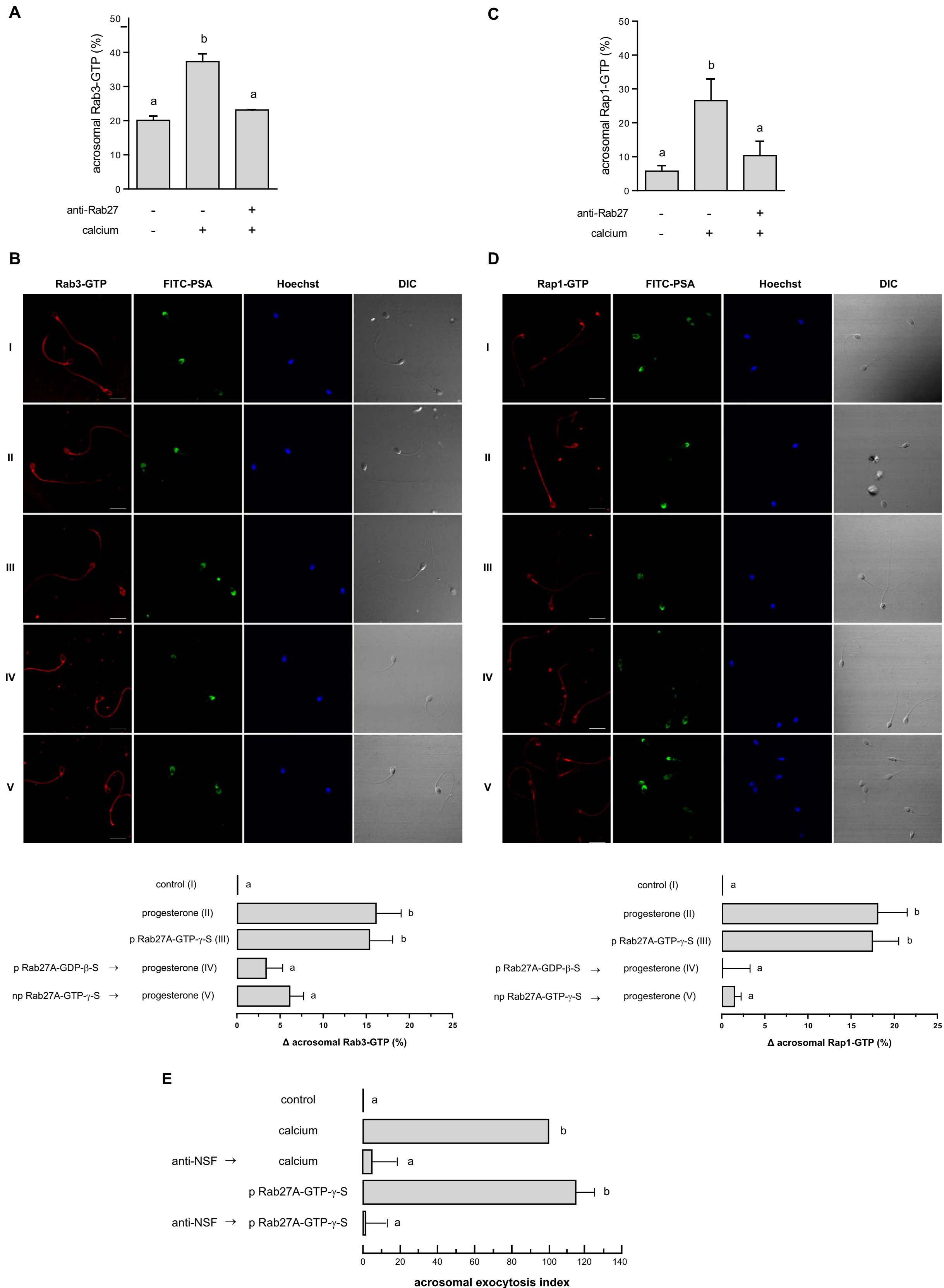
Next, we asked whether calcium requires endogenous Rab27 to activate sperm Rap1. We run far-immunofluorescence experiments similar to those described in Fig. 4A-B but replacing the RIM-RBD cassette by a Ral-GDS-RBD activity probe that detects Rap1-GTP. This small G protein was activated in the acrosomal region in response to calcium. Preincubation with anti-Rab27 antibodies abolished the stimulatory effect of calcium on the activation of Rap1 (Fig. 4C). As shown before for Rab3, geranylgeranylated (p) TAT-Rab27A-GTP-γ-S (III) promoted the exchange of GDP for GTP in the acrosomal region; the magnitude of the response was comparable to that of progesterone (II) (Fig. 4D). Geranylgeranylated TAT-Rab27A-GDP-β-S (IV) as well as non-isoprenylated (np) TAT-Rab27A-GTP-γ-S (V) prevented progesterone from activating Rap1 (Fig. 4D).

3.5. Rab27 is required for the AR upstream cis SNARE complex disassembly

The ATPase NSF, its co-factor α-SNAP, their target the SNARE complex and accessory proteins drive exocytosis in all cells. α-SNAP/NSF unpair *cis* (in one membrane) SNARE complexes, making monomeric SNAREs available for subsequent *trans* (between the two membranes that are going to fuse) pairing and fusion [46,62]. α-SNAP, NSF and members of the SNARE complex are essential components of the protein machinery that governs the late stages of the AR (reviewed in [4,50]). If geranylgeranylated TAT-Rab27A-GTP-γ-S were a genuine AR inducer, exocytosis triggered by it should rely on the standard fusion machinery. Unlike the case in non-permeabilized cells (Fig. 2B), 300 nM geranylgeranylated (p) Rab27A-GTP-γ-S elicited exocytosis at a level comparable to that accomplished by calcium in human sperm with their plasma membrane permeabilized with SLO (Fig. 4E). Anti-NSF antibodies prevented calcium-triggered exocytosis (Fig. 4E and [8,45,51]). Sequestration of sperm NSF also impaired the AR elicited by TAT-Rab27A-GTP-γ-S (Fig. 4E). These results suggest that permeable Rab27A requires NSF to accomplish the AR.

We carried out complementary experiments employing anti-Rab27 antibodies to interfere with the endogenous protein and ask for its connection with the fusion machinery. We applied two experimental strategies to reinforce these findings. In the first one, we used a reversible pair. These tools consist of stage-specific blockers and their rescuers (see [45] for a detailed explanation on how they work) and a number of them has been used to investigate signaling pathways during the AR (see for example [9,35,44]). Despite the fact that the endogenous protein is essential for membrane fusion, high concentrations of recombinant α-SNAP block the AR in permeabilized human sperm. This is because the protein binds free syntaxin and impedes its entering the fusogenic SNARE complex; NSF rescues this inhibition [43,51]. Thus, α-SNAP/NSF constitutes a reversible pair. We anticipated that Rab27 would no longer be required once the signaling cascade initiated by calcium arrived at the stage blocked by recombinant α-SNAP. We tested this premise and found that the anti-Rab27 antibodies had no effect on the AR when added after recombinant α-SNAP and calcium (Supp. Fig. S1A, black bar). These results confirm that the step catalyzed by Rab27 is upstream that affected by α-SNAP (Fig. 5). In the second strategy, we combined the reversible pair anti-Rab27/recombinant Rab27A [9,45] with BoNT/B. Clostridial toxins are zinc-dependent proteases that cleave neuronal SNARE isoforms, impair their assembly into ternary complexes and therefore prevent exocytosis (reviewed in [1,38]). SNAREs are resistant to toxin-cleavage when engaged in *cis* complexes, which is their configuration in resting sperm.

Complexes disassemble in response to AR inducers, in consequence, SNAREs acquire sensitivity to neurotoxins. Thus, BoNT/B is a reliable tool to assess SNAREs configuration because it cleaves sperm monomeric synaptobrevin-2 and inhibits the AR; the zinc chelator TPEN antagonizes its effects [17]. When permeabilized human sperm were challenged with calcium after introducing anti-Rab27 antibodies,



(caption on next page)

Fig. 4. Rab27, Rab3A and Rap1 exhibit sequential roles during exocytosis. A, C, SLO-permeabilized sperm were treated with 100 μM 2-APB and 7 nM anti-Rab27 antibodies followed by 0.5 mM $CaCl_2$. B, D, Sperm treated with 100 μM 2-APB were incubated with 300 nM TAT-Rab27A geranylgeranylated (p) or not (np) and loaded with GDP-β-S (IV) or GTP-γ-S (III, V). When indicated, cells were incubated with 15 μM progesterone (II, IV, V). Incubations were for 15 min at 37 °C after each addition. Samples were processed for far-immunofluorescence, overlain with GST-RIM-RBD to detect active Rab3 (A, B) or GST-Ral-GDS-RBD to detect active Rap1 (C, D) followed by anti-GST antibodies. B, D, Shown are epifluorescence micrographs of cells triple stained with the anti-GST antibody (to visualize the activity probes; red, left panels), FITC-PSA (to confirm that the AR was effectively prevented by 2-APB; green, central panels), and Hoechst 33,342 (to visualize all cells in each field; blue, right panels). Differential interference contrast (DIC) microscopy images are shown at the far right. Bars = 10 μm. Quantifications of the percentage of cells with active GTPases in the acrosomal region (flagellar staining is variable from sample to sample and from batch to batch of activity probes and it is considered non-specific) are shown at the bottom of each group of micrographs. The percentage of cells with acrosomal Rab3-GTP (B) and Rap1-GTP (D) in the control condition (untreated cells) was subtracted from all values. Plotted is the mean ± SEM of at least three independent experiments. Tukey–Kramer post hoc test was used for pairwise comparisons. Different letters indicate statistical significance (A, B and C: $P < .05$; D: $P < .01$). E, SLO-permeabilized sperm were treated with anti-NSF antibodies (whole rabbit serum diluted 1:300). The AR was initiated with 0.5 mM $CaCl_2$ or 300 nM geranylgeranylated (p) Rab27A-GTP-γ-S. Incubations were for 15 min at 37 °C after each addition. Controls included background AR in the absence of any stimulation (control) and AR stimulated by 10 μM free calcium or 300 nM geranylgeranylated (p) Rab27A-GTP-γ-S. Samples were processed and AR scored as explained in the legend to Fig. 2, except that values are normalized to the AR induced by 0.5 mM $CaCl_2$ and assigned 100%. Plotted results represent the mean ± SEM of at least three independent experiments. Tukey–Kramer post hoc test was used for pairwise comparisons. Different letters indicate statistical significance ($P < .001$).

the AR took place normally in the presence of BoNT/B (Supp. Fig. S1B, black bar). That synaptobrevin-2 was protected from toxin cleavage suggests that it was engaged in *cis* SNARE complexes. Taken together, our findings suggest that the end point of the Rab27-linked signaling pathway is the dissociation of the SNARE complex (Fig. 5).

4. Discussion

The small GTP-binding protein Rab27 has been implicated in the regulation of different types of membrane trafficking, including regulated exocytosis in a wide variety of secretory cells [12,21,56,58].

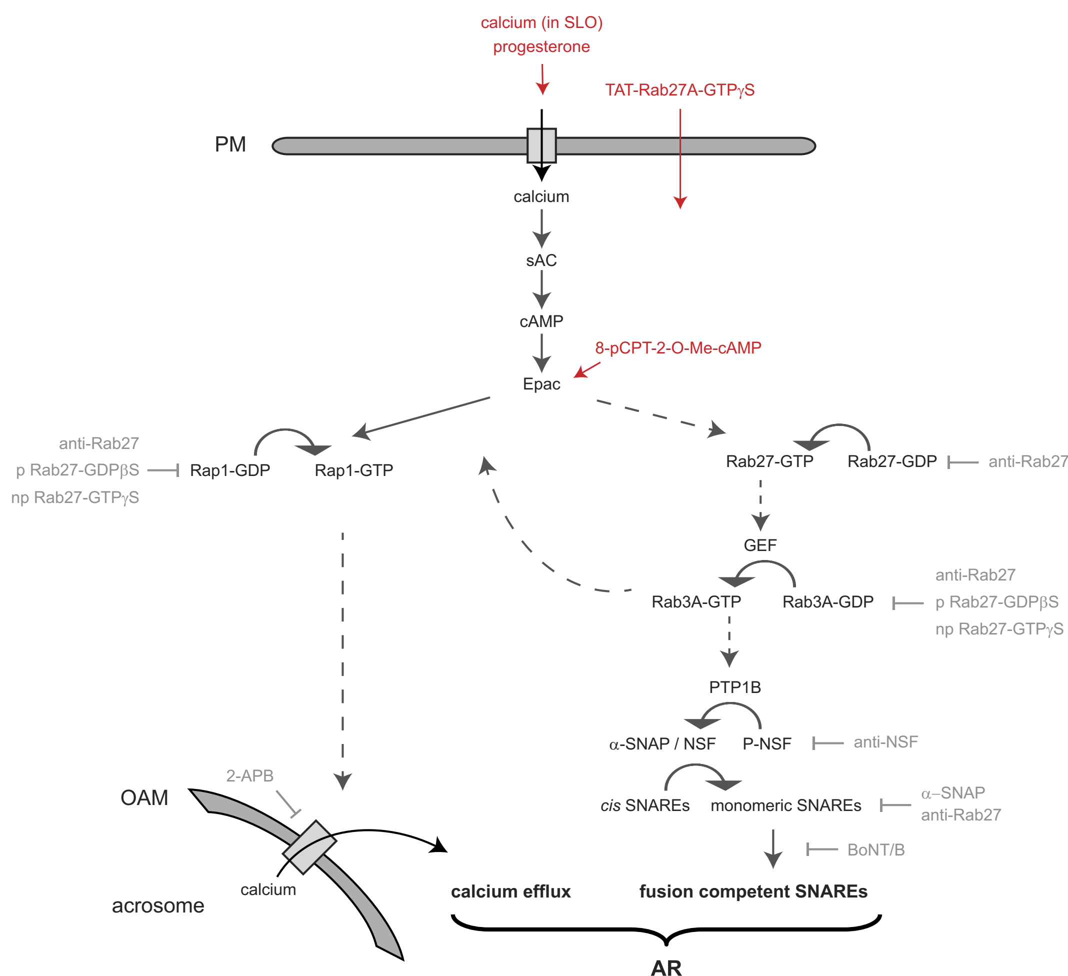


Fig. 5. Updated working model for the AR. Calcium enters the cell from the extracellular milieu through the SLO-generated pores or through calcium channels opened in response to progesterone. It is beyond the scope of this manuscript to define the identity of these calcium channels (but see for instance [5,15]). Downstream this calcium influx, sAC converts ATP into cAMP, which activates Epac as does 8-pCPT-2′-O-Me-cAMP, and here the signaling pathway splits into two limbs. In one of them, Epac catalyzes the exchange of GDP for GTP on Rap1; Rap1-GTP heads a pathway that leads to acrosomal calcium mobilization ("calcium efflux" in the Figure). In the other, Epac-cAMP indirectly activates secretory Rabs 27 and 3. Rab27-GTP heads a pathway that leads to the correct assembly of the fusion machinery ("fusion competent SNAREs" in the Figure). Somewhere downstream of Rab3-GTP, there is a unidirectional (Rab3 limb → Rap1 limb) connection between both arms of the pathway. After this point, PTP1B is activated and/or recruited to the sites where it dephosphorylates NSF, derepressing its activity. Next, active, dephospho-NSF, in a complex with α-SNAP, renders SNARE proteins fusion competent. The step driven by active SNAREs converges with the local increase in calcium coming from the acrosome downstream of Rap1-GTP to accomplish the final steps of membrane fusion ("AR" in the Figure). Blockers used in this manuscript are indicated in light gray. Anti-Rab27 and recombinant TAT-Rab27As are shown next to all reactions scrutinized in data Figures rather than pointing at the first inhibited reaction (which presumably affects all those downstream of it). AR inducers calcium, progesterone, TAT-Rab27A-GTP-γ-S and 8-pCPT-2′-O-Me-cAMP are shown in red. PM, plasma membrane; OAM, outer acrosomal membrane. Solid arrows mean there is one step between the terms connected, dashed arrows mean that the number of steps is either unknown or not depicted for simplicity. Modified from [35,45].

Studies on the levels and/or subcellular localization of members of the Rab family have been reported in relationship to capacitation, a maturation process mammalian sperm must go through before fertilizing eggs (for instance Rab2 [42] and Rab3 [3,55]). Rab27, Rab3 and Rap1 are required for the exocytosis of the acrosome in human sperm (Fig. 5). The three small G proteins are activated in response to exocytosis inducers [7,9,11,34,35,39,45]. On the other hand, recombinant Rab27A-GTP induces the exchange of GDP for GTP on sperm Rab3 [9]. Here, we show that endogenous Rab27 acts in concert with, and plays its role upstream of, the other small G proteins because anti-Rab27 antibodies and inhibitory versions of TAT-Rab27A, which precluded the activation of sperm Rab27, prevented the exchange of GDP for GTP on Rab3 (Fig. 4A-B) and Rap1 (Fig. 4C-D) — and therefore inhibited the AR — in permeabilized and intact sperm respectively. We are putting forth the first pieces of direct evidence that the signaling cascades initiated by AR triggers rely on the activation of endogenous Rab27 to exchange GDP for GTP on the small G proteins Rab3 and Rap1. We are only beginning to understand how these GTPases coordinate their actions, for instance Rab27 and Rab3 are organized in a Rab-GEF cascade [9]. A functional link has been inferred for between Rab3 and Rap1, which act in separate branches of the signaling pathways that govern the AR [7,45]. The observations that reagents that interfere with endogenous Rab27 hinder the activation of Rap1 (Fig. 4C-D) provide further evidence about the existence of the link between Rab27 (and Rab3) in one, and Rap1 in the other, arms of the pathway (Fig. 5). Functions for Rap1 [53], Rab3A, Rab3D and Rab27A [63] have been described for the exocytosis of Weibel-Palade bodies in endothelial cells. Likewise, Rap1 [47], Rab27A [24], Rab27B [13,23] and Rab3D play regulatory roles in pancreatic acinar exocytosis. The small GTPases Rab27B [26], Rab3D [41] and Rap1 [57] regulate amylase release from rat parotid acinar cells. Our findings are unique in the sense that all three GTPases have been scrutinized in the same study.

The experiments summarized in Supporting Fig. S1 indicate that the activation of Rab27, precluded by the antibody, is necessary for the disassembly of the *cis* SNARE complex into monomeric proteins. Despite the fact that a connection between Rab27 and members of the SNARE complex or accessory proteins has been described in several systems, there are no reports on the influence of Rab27 on SNAREs configuration. Protein complexes consisting of Rab27-effector-monomeric Q-SNAREs have been involved in the tethering/docking of secretory granules to the plasma membrane. For example Munc13-4 and doc2 interact with Rab27A on secretory lysosomes and syntaxin 11 on the plasma membrane of cytotoxic lymphocytes (reviewed in [18,22]); granulophilin binds Rab27A on insulin granules and Munc18-1 and/or syntaxin1a on the plasma membrane in pancreatic β cells (reviewed in [28]); synaptotagmin-like protein-4 and rabphilin bind Rab27A on secretory granules and Munc18–1/syntaxin1a, Munc18-2 or SNAP-25 on the plasma membrane in PC12 and parotid acinar cells (reviewed in [20,52]); etc. In all these cases, Rab27 and a member of the Munc18 or SNARE families are bridged by a Rab27 effector; in sperm, in contrast, there are several players (rather than a single one) between Rab27 and the SNARE complex (Fig. 5).

Human sperm Rab27 partitions into the Triton-X114 detergent phase and localizes to membranes after subcellular fractionation [9]. A permeable version of recombinant Rab27A was designed in the laboratory and characterized in this study. Geranylgeranylated TAT-Rab27A-GTP-γ-S triggered, and TAT-Rab27A-GDP-β-S inhibited, the AR (Figs. 2A-C and 5E). Similarly, the active protein promoted, and the inactive inhibited, the exchange of GDP for GTP on Rab3 (Fig. 4B) and Rap1 (Fig. 4D). The requirement for GTP points to the interaction of the recombinant protein with effectors. Geranylgeranylation of TAT-Rab27A was crucial because when lacking, the active protein blocked the AR whereas the inactive one did not influence it (Fig. 2D). The dependence on geranylgeranylation for function suggests that TAT-Rab27A targets to sperm membranes and thus shares biochemical properties with the endogenous protein; presumably, non prenylated TAT-Rab27A did not localize to membranes. In this context, it is licit to speculate that, when loaded with GTP-γ-S, non-geranylgeranylated TAT-Rab27A inhibited the AR because it bound effectors, retained them in the wrong compartment and made them inaccessible to membrane-bound endogenous Rab27. These findings agree with those reported with the GTPase-deficient Rab27A-Q78L and non-lipid modifiable Rab27A-C219A/C221A mutants in melanocytes [19,27,36] and platelets [48]. Last, the inhibitory effect of isoprenylated TAT-Rab27A-GDP-β-S might be due to sequestration of sperm's Rab27 GEFs based on the mechanism proposed to explain the negative phenotype of the Rab27A-T23N mutant (which preferentially binds GDP [36]).

In summary, this manuscript contains direct evidence that Rab27 has a positive role during sperm exocytosis downstream the activation of Epac and upstream that of Rab3, Rap1 and the disassembly of the SNARE complex. Some of the evidence was generated thanks to a permeable version of recombinant Rab27A described and characterized here. This protein must be geranylgeranylated to exert its function, likely reflecting its dependence on membrane targeting. The technology of protein transduction through cell-penetrating peptides has recently begun to be applied to transport biologically active molecules into living sperm [29,33,35,37,40]. Cell-penetrating peptides may be attached to their cargo proteins after or during peptide synthesis. Alternatively, the DNA sequences encoding such peptides can be inserted in plasmids for bacterial expression as in [35] for a cAMP sponge and in this paper for Rab27A. It would be interesting to assess the effects of TAT-Rab27A in other exocytotic models.

5. Conclusions

We have designed a membrane-permeant version of human Rab27A. This protein, expressed in *Escherichia coli* and purified by affinity chromatography, transduced into human sperm and behaved as an AR trigger as long as it was geranylgeranylated and bound to GTP, and therefore, active. TAT-Rab27A-GTP-γ-S also promoted the exchange of GDP for GTP on Rab3 and Rap1. All other combinations of prenylation status and bound guanine nucleotides either did not have a measurable effect or inhibited the AR and the activation of the two small GTPases that act downstream of Rab27. In short, TAT-Rab27A is a trustworthy tool to unveil the regulation of the human sperm AR by Rab27 in intact cells, something impossible to achieve pharmacologically. By means of two complementary approaches, we showed for the first time that endogenous Rab27 must be activated to allow the exchange of GDP for GTP on Rab3 and Rap1 and the disassembly of the SNARE complex by AR inducers. An additional important finding derived from this study is that an Epac-selective cAMP analogue activated Rab27 in the acrosomal region of human sperm. This is the first time a connection between Epac and Rab27 is suggested. In short, the contribution of this paper to the sperm biology field is the description and characterization of a novel tool: a membrane-permeant version of a protein whose endogenous counterpart is relevant for the AR. The contribution of this paper to the exocytosis field is the correlation of dense-core granule secretion with the activation and geranylgeranylation status of Rab27A.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.01.010.

Author contributions

MAB, OL, and CNT designed experiments; MAB, OL, and MCR performed experiments; CNT wrote the manuscript. All authors reviewed and approved the final manuscript.

Conflict of interest

No competing interests declared.

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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.

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