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REVIEW ARTICLE

The proteins of exocytosis: lessons from the sperm model

Claudia Nora Tomes*[1]

*Institute of Histology and Embryology (IHEM-CONICET), School of Medicine, Cuyo National University, 5500 Mendoza, Argentina

Exocytosis is a highly regulated process that consists of multiple functionally, kinetically and/or morphologically definable stages such as recruitment, targeting, tethering and docking of secretory vesicles with the plasma membrane, priming of the fusion machinery and calcium-triggered membrane fusion. After fusion, the membrane around the secretory vesicle is incorporated into the plasma membrane and the granule releases its contents. The proteins involved in these processes belong to several highly conserved families: Rab GTPases, SNAREs (soluble NSF-attachment protein receptors), α-SNAP (α-NSF attachment protein), NSF (N-ethylmaleimide-sensitive factor), Munc13 and -18, complexins and synaptotagmins. In the present article, the molecules of exocytosis are reviewed, using human sperm as a model system. Sperm exocytosis is driven by isoforms of the same proteinaceous fusion machinery mentioned above, with their functions orchestrated in a hierarchically organized and unidirectional signalling cascade. In addition to the universal exocytosis regulator calcium, this cascade includes other second messengers such as diacylglycerol, inositol 1,4,5-trisphosphate and cAMP, as well as the enzymes that synthesize them and their target proteins. Of special interest is the cAMP-binding protein Epac (exchange protein directly activated by cAMP) due in part to its enzymatic activity towards Rap. The activation of Epac and Rap leads to a highly localized calcium signal which, together with assembly of the SNARE complex, governs the final stages of exocytosis. The source of this releasable calcium is the secretory granule itself.

Key words: acrosome reaction, calcium, exocytosis, membrane fusion, Rab, SNARE, SNARE-interacting molecule, sperm.

INTRODUCTION

Sexual reproduction for the perpetuation of species occurs through fertilization, a process in which the haploid sperm and haploid egg merge to allow entry of the sperm head, and subsequently the delivery of the male chromatin, into the egg cytoplasm. As a result, a diploid zygote is formed that will eventually produce an individual genetically distinct from his or her progenitors. All mammalian sperm contain a single, dense-core secretory granule (the acrosome) which releases its contents at fertilization through a regulated exocytosis known as the acrosome reaction (AR). As sperm must travel long distances and encounter many obstacles, within both the male and the female reproductive tracts, the initiation of the AR is tightly co-ordinated with the availability of eggs. Despite decades of intense investigation, reproductive biologists have not yet reached a consensus about crucial issues including where (on/within which egg layer) the AR takes place, what its purpose is and what its physiological inducers are. Fortunately, recent discoveries have advanced the field rapidly, so many answers to these questions might soon be available. These and other important issues have been thoroughly reviewed over the last few years by leading experts in the sperm biology field, so they are not revisited in the present review, but interested readers are referred to the literature [1–8]. Rather, the present review focuses on some of the membrane fusion molecular mechanisms occurring during exocytosis in the latest stages of the AR, which have been clarified after dissection of these events over the past decade.

ARE SPERM A GOOD MODEL FOR EXOCYTOSIS?

This section is devoted to the argument that sperm represent a good model of exocytosis and the AR is worthy of attention. The realization that the central components of the exocytotic machinery have counterparts in most types of intracellular membrane trafficking led to the general belief that the mechanisms of membrane fusion are universal. Indeed, we and others have shown that the AR relies on the same highly conserved molecules and goes through the same stages of exocytosis that occur in neuronal, endocrine and all other cells studied to date (reviewed in the literature [9–11], and to such an extent that some authors [12] refer to the anterior region of the acrosomal cap as the ‘acrosomal synapse’.

Exocytosis is a highly regulated process that consists of multiple functionally, kinetically and/or morphologically definable stages, such as recruitment, targeting, tethering and docking of secretory vesicles with the plasma membrane, priming of the fusion machinery and calcium-triggered membrane fusion, after which the membrane around the secretory vesicle is incorporated into the plasma membrane. A discrete number of molecules involved in this complex cascade of events have been identified (for recent reviews see the literature [13–20]).

Abbreviations: 8-pCPT-2-*O*-Me-cAMP, 8-(4-chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate acetoxymethyl ester; AR, acrosome reaction; BoNT, botulinum neurotoxin; Epac, exchange protein directly activated by cAMP; GDI, GDP dissociation inhibitor; GEF, guanine nucleotide exchange factor; IP_3, inositol 1,4,5-trisphosphate; NSF, N-ethylmaleimide-sensitive factor; PKA, cAMP-dependent protein kinase; PLC, phospholipase C; PTP1B, protein tyrosine phosphatase 1B; sAC, soluble adenylate cyclase; SM, Sec1/Munc18; SNAP, *N*-ethylmaleimide-sensitive factor attachment protein; SNARE, soluble NSF attachment protein receptor; SLO, streptolysin O; TeTx, tetanus toxin.

[1] To whom correspondence should be addressed (ctomes@fcm.uncu.edu.ar).

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Exocytosis of the acrosomal vesicle is somewhat unique. Each sperm contains a single, very large and electron-dense acrosome that is shed entirely, together with the portion of plasma membrane that overlays it, in response to exocytosis inducers. Owing to the size and morphology of the granule (see schematic diagrams in Figure 2B), as well as the nature of the acrosomal contents, this exocytosis is a slow event. Despite these singular morphological and functional features, all of the fusion-related molecules involved in the AR were initially found in somatic cells and implicated in various membrane-fusion events. As such, characterization of the molecular machinery that drives sperm exocytosis may appear to be a simple confirmation, in yet another model, of already known mechanisms.

However, after establishing that sperm share their basic fusion molecules and regulatory components with all other eukaryotic cells, additional mechanisms were unveiled that might be universal but are difficult to analyse in more complex exocytosis scenarios because of the following. (i) Most exocytotic cells house functionally different pools of secretory vesicles. It is a serious challenge to design experiments to investigate the function of a given fusion protein in a specific fusion stage when there is coexistence of vesicular pools undergoing different processes affected by that protein. In contrast, each sperm contains a single secretory granule (rather than a heterogeneous pool) with exocytosis that is driven by unidirectional cascades, thus making the AR more straightforward to dissect compared with other secretory processes. (ii) In some cell types (typically neurons), the same substances (and often more than one substance contained in different vesicle pools and released in response to a single stimulus) are secreted again and again, requiring multiple rounds of fusion such that both membranes and fusion machinery recycle several times. Each sperm contains one preformed granule, with no biogenesis of new ones. Sperm exocytosis is thus a singular event in which the granule membrane is not incorporated into the plasma membrane. Instead, both membranes form vesicles and are shed, together with the acrosomal contents and the scarce cytoplasm around the acrosome during exocytosis (see schematic diagrams in Figure 2B). Thus there is no post-exocytosis membrane remodelling and recycling, and/or endocytosis in these cells. As such, all components of the fusion machinery can exhibit only pre-fusion roles. (iii) In many exocytotic cells, a pool of granules is situated very close to or in direct contact with the plasma membrane even before the application of a stimulus; these granules are defined as morphologically docked. It has been suggested that granules in this pool release their contents faster than others on calcium influx. In contrast, all acrosomes (in all sperm cells) are undocked before the application of an exocytosis stimulus. In other words, docking does not pre-exist but is secondary to the challenge of the cells (but see Tsai et al. [21]). The AR can be reversibly halted at different stages (e.g. before docking or after docking but before fusion), which allows for the detailed analysis of the molecular mechanisms involved. (iv) Overexpression and ablation of genes are two widely used technologies in the exocytosis field that were instrumental in the identification of proteinaceous fusion machinery. Both take place over very long timescales compared with the life cycle of a vesicle. As sperm cannot synthesize new proteins, only pre-existing proteins can be delivered to the intracellular compartments through artificial pores or coupled to cell-permeable peptides. Consequently, experiments can take place in a short timescale (minutes) in sperm. The short incubations used in the sperm model 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 reflection of the role of the introduced factors in exocytosis.

INTERMEDIATE EXOCYTOTIC STAGES DURING THE AR

To release their contents, secretory vesicles must travel from a cytosolic depot pool towards the plasma membrane. Once they identify the compartment with which they are going to fuse, tethering (the initial contact event that bridges the space between the two approaching membranes) ensues [22–25]. Only recently have vesicles loosely connected over a relatively long distance (tethered) to the plasma membrane in mammalian models been captured and visualized. The methods employed to achieve this task include evanescent wave microscopy [26], cryoelectron tomography [27] and TEM [28] (reviewed in Hallermann and Silver [29]). As a result of its size and shape, the acrosome cannot travel to contact the plasma membrane. In capacitated human sperm (capacitation is a maturation process that sperm must go through after leaving the male tract to acquire fertilizing capacity; for review see Salicioni et al. [30]), the acrosome is evenly spaced (18 nm) from the cell membrane in all cells. Only after challenge with AR inducers do the acrosomal contents swell and the acrosomal membrane stretch towards the cell membrane. Unfortunately, no method for visual distinction between tethered and non-tethered acrosomes is available yet. Nevertheless, as these tethering molecules include Rab GTPases and effector protein complexes, and sperm exocytosis depends on isoforms of these proteins, it is likely that the acrosome is tethered to the sperm plasma membrane at some point during the AR.

Docking is a tighter interaction of the two bilayers engaged in fusion achieved by pairing across soluble *N*-ethylmaleimide-sensitive factor (NSF)-attachment protein receptors (*trans*-SNARE complexes). SNARE complexes consist of four intertwined, parallel helices, each supplied by a different SNARE motif. When all cognate SNAREs are located on the same membrane, they spontaneously assemble in stable *cis* complexes, which are functionally inactive. Disentangling these complexes to regenerate monomeric SNAREs that will subsequently be available to engage in productive *trans* complexes requires metabolic energy provided by the NSF via the hydrolysis of ATP. It is believed that the activity of NSF is constitutive in most cells to ensure that *cis* complexes are disassembled under normal steady-state conditions. In sperm, however, the picture is different, perhaps owing to their need to co-ordinate the AR carefully with the exact moment when they encounter the egg. Thus, under resting conditions sperm SNAREs do not cycle but are engaged in *cis* complexes on both plasma and outer acrosomal membranes [31], because the dissociating activity of NSF is repressed by tyrosine phosphorylation [32]. On sperm activation, protein tyrosine phosphatase 1B (PTP1B) dephosphorylates NSF, derepressing its activity. Free SNAREs are subsequently able to reassemble in *trans* formation, a process that is facilitated by complexin [33,34] and Munc18 [35] (Figure 1). At this stage, sperm exhibit swollen acrosomes with numerous tight appositions (distances between 0 and 8 nm) between the outer acrosomal and plasma membranes [36]. These distances are comparable to those reported in the literature for morphologically docked secretory vesicles [37,38] and are a hallmark of docked acrosomes. In contrast to our findings in human sperm, docked acrosomes have been detected in capacitated boar sperm that have not been stimulated to undergo exocytosis [21]. We believe that *trans*-SNARE complexes form molecular bridges that stabilize the tight appositions between the acrosome and the plasma membrane during the docking stage of the AR.

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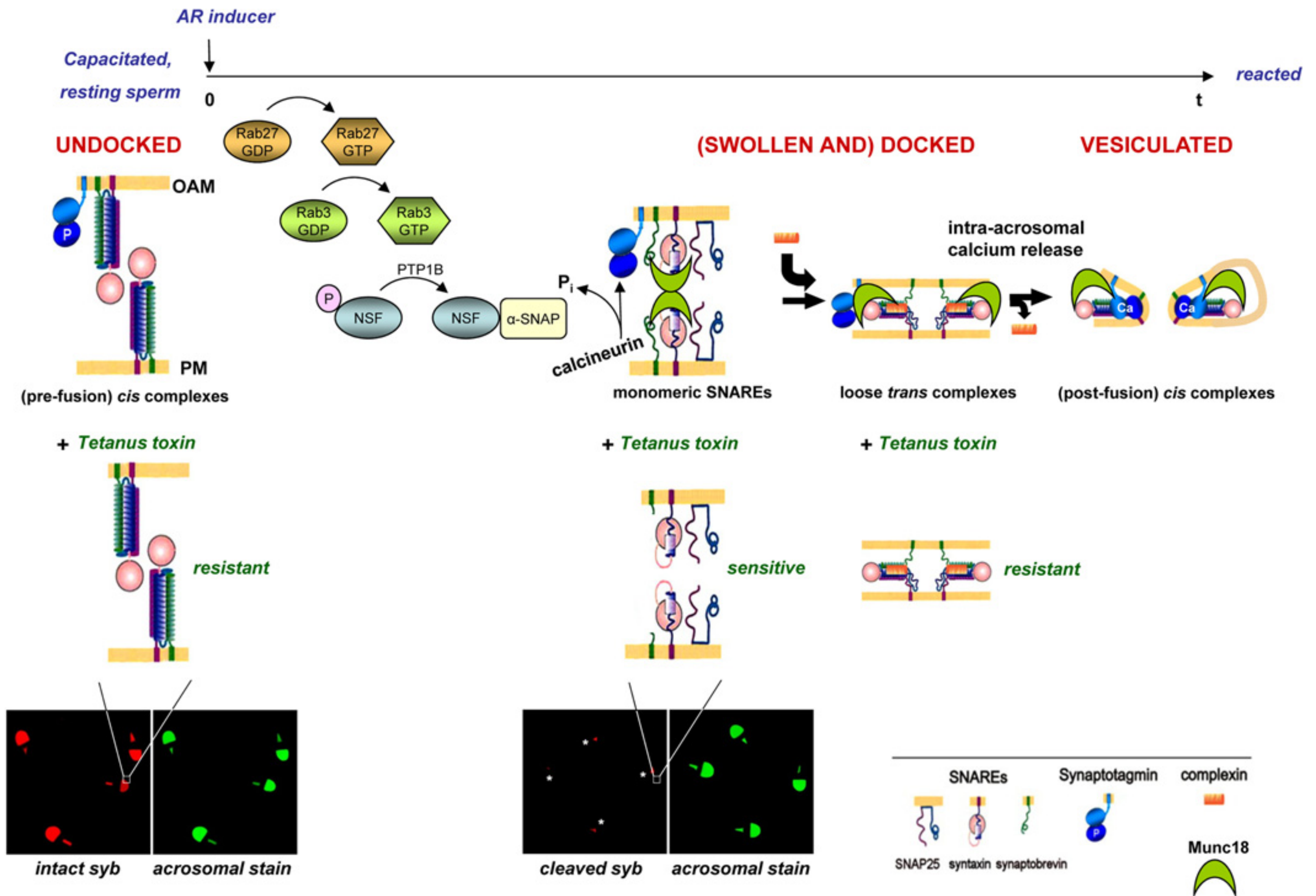

Figure 1 Schematic diagram for the activation/disassembly/assembly of the fusion molecules that drive the AR

Top: in resting sperm, the acrosome is flat, its contents are not swollen and its membrane is not docked to the plasma membrane. SNAREs, synaptotagmin and NSF are inactive, with the former engaged in *cis* complexes and the latter phosphorylated on threonine and tyrosine respectively. One of the earliest events triggered by exposure to AR inducers is the swelling of the acrosome. Also, in response to inducers, Rab27 exchanges GDP for GTP; subsequently, Rab27-GTP promotes the activation of Rab3. Next, two phosphatases dephosphorylate their substrates: calcineurin on synaptotagmin and PTP1B on NSF. Once dephosphorylated, NSF, together with α-SNAP, disassembles *cis*-SNARE complexes. Munc18 binds monomeric syntaxin, keeping it temporarily in a closed configuration. Munc18 and complexin help SNAREs to reassemble in *trans* complexes. At this stage, the swollen acrosome is docked to the plasma membrane. A local increase in calcium coming from the acrosome through IP_3-sensitive channels binds synaptotagmin. Afterwards, a series of sub-reactions that include displacement of complexin lead to the opening of fusion pores and vesiculation of the fusing membranes. Post-fusion *cis*-SNARE complexes remain trapped inside the vesicles. Middle/bottom: in resting sperm, synaptobrevin engaged in *cis*-SNARE complexes is resistant to cleavage by TeTx and therefore an antibody can detect the intact protein in the acrosomal region of the cells (red staining). Binding of a specific lectin demonstrates the integrity of the acrosome (green staining). After the initiation of the AR, monomeric synaptobrevin is susceptible to cleavage by TeTx; the antibody does not detect proteolysed synaptobrevin (left), even though all four cells in the shown field (asterisks) have intact acrosomes (green). OAM, outer acrosomal membrane; PM, plasma membrane. The model was modified from Roggero et al. [33] and the drawings were modified from Söllner [156].

In most cells, a single fusion pore opens between the fusing membranes; eventually the pore widens and the membrane around the secretory vesicle is incorporated into the plasma membrane as the granule contents discharge. In sperm, however, the pores that open at the docking sites widen, but, as the outer acrosomal membrane is as large as the area of plasma membrane with which it is fusing, the result of pore widening is fenestration of the fusion membranes and joining of pores to produce hybrid plasma membrane/outer acrosomal membrane vesicles and tubules. The AR is completed when vesicles, tubules and acrosomal contents are shed [39–41] (see schematic diagrams in Figure 2B). Intermediate stages are hard to capture in sperm undergoing the AR because they are transient. Nevertheless, swelling, docking and vesiculation can be easily visualized by TEM and quantified after simple pharmacological interventions [35,36,42–44].

MEMBERS OF THE STANDARD PROTEINACEOUS FUSION MACHINERY DRIVE THE AR

Secretory Rabs: Rab27 and Rab3

Rab3 (A, B, C and D) and Rab27 (A and B) constitute the two main Rab subfamilies directly implicated in regulated exocytosis. These ‘secretory Rabs’ localize to vesicles and secretory granules in a variety of secretory cell types [13,45–48]. They control the recruitment and attachment of secretory vesicles to the plasma membrane through interaction with effectors [49].

Rab3A is present in the acrosomal region of human [50], rat and mouse sperm [51]; in the latter, it is predominantly membrane bound and sheds during the AR [52]. In human sperm, capacitation [53] and challenging sperm with AR inducers [54] lead to an enhanced association of Rab3A with membranes. Rab3A is required for the AR triggered by calcium [31,53,55], cAMP [56],

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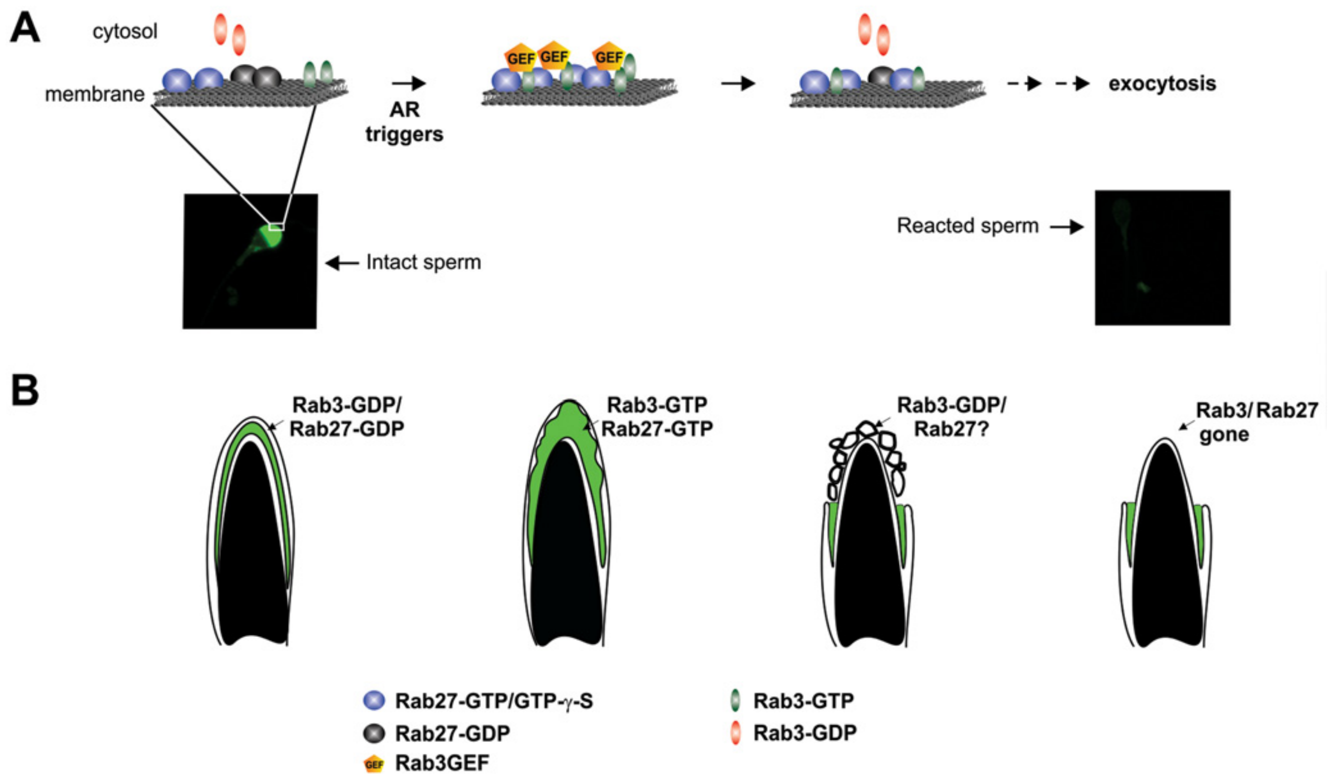

Figure 2 Schematic representation of Rab3 and 27 activation status and localization: morphological changes undergone by sperm during the AR

(**A**) In resting sperm, Rab27 is inactive and membrane bound, whereas Rab3 is also inactive but distributed between the cytosol and the particulate fraction. The acrosome is intact and therefore stained with a green lectin (photograph). On initiation of the AR, Rab27 exchanges GDP for GTP, without appreciably modifying its subcellular localization. Rab27-GTP promotes the activation of Rab3 through the recruitment of a Rab3-GEF activity (orange pentagons); GTP-bound Rab3 is targeted to the particulate fraction. Later, Rab3 hydrolyses GTP, which allows vesiculation of the acrosomal and plasma membranes. We do not have data on Rab27′s activation status at this stage. Finally, the apical portion of the cell, which contains Rab proteins, is shed and the AR completed. Note that when the acrosome is lost, the green lectin cannot stain the cell (photograph). Modified from Bustos et al. [54]. (**B**) The drawings summarize my interpretation of the activation status of Rab proteins combined with the schematic diagrams of the morphological changes undergone by sperm during the AR. Modified from Bustos et al. [61].

sphingosine 1-phosphate [57] and diacylglycerol [58], as well as for the docking of the acrosome to the plasma membrane [42]. The Rab3 effector, Rab3-interacting protein RIM, is present in the acrosomal region of mouse [59] and human [42] sperm, and is required for docking, and therefore the AR, in the latter. Rab3A is activated (exchanges GDP for GTP) in response to exocytosis inducers early during the exocytotic cascade [54,58,60–62] and must be inactivated (e.g. hydrolyse GTP) to accomplish the later phases [61] (Figure 2A). The accessory protein Rab-GDI (GDP dissociation inhibitor) binds Rab-GDP and extracts it from membranes. Rab-GDI is present in the acrosomal region of human sperm and is required during the AR with a timeframe identical to that in which Rab3 must hydrolyse GTP [61].

Despite the fact that Rab3A is absolutely essential for the human sperm AR measured *in vitro*, Rab3A$^{-/-}$ animals are fertile [63]. It is worth noting that the AR has not been characterized in these animals, although other Rabs or even Rab3 isoforms could function in the place of Rab3A *in vivo*. Candidates might be a protein similar to Rab37, which was classified as a secretory Rab by Fukuda [46] and has been predicted to be present in a bull sperm proteome [64]. Meanwhile, transcripts for isoforms of Rab3 and Rab27 have been detected in whole mouse testes [65] and mouse type A spermatogonia, pachytene spermatocytes and round spermatid libraries [66]. Rab3 and Rab27 have also been detected in a human sperm proteome [66a]. Rab3A deficiency can be rescued by calcium elevation at certain synapses [67,68]. My colleagues and I hypothesize that a similar situation might operate during fertilization *in vivo*. In this scenario, sufficient calcium concentrations in the female tract could overcome the Rab3A deficit in sperm from Rab3A$^{-/-}$ males and explain the lack of an infertility phenotype. Interestingly, the human sperm AR is resistant to Rab3A blockers at elevated calcium concentrations (M.A. Bustos and C.N. Tomes, unpublished work).

Rab27 is present in the acrosomal region of human sperm and is also required for the AR. Unlike the Rab3 isoform, Rab27 membrane localization is not coupled to the initiation of the AR, but the levels of acrosomal GTP-bound Rab27 increase on initiation of exocytosis [54] (Figures 1 and 2A). The average litter size in Rab27A knock-out mice (Rab27A$^{ash/ash}$) is reduced compared with the wild-type background strain, which suggests lower fecundity rates [45]. I would like to point out, however, that overt reproductive phenotypes are rarely observed in mating experiments, even when egg- or sperm-specific proteins with well-established roles in fertility are knocked out. The fertility of the null mice might simply attest to Nature's drive to guarantee species perpetuation. In contrast, problems are readily detected when gametes of deficient animals are tested in experiments designed to assess their fertility directly, such as AR assays and *in vitro* fertilization (something that has not been done for Rab3- or Rab27-null animals).

The coexistence of Rab3 and Rab27 on the same membrane raises the question of functional redundancy, and whether and how their roles are co-ordinated to regulate exocytosis. From the sperm model we now know that Rab27A-GTP recruits a Rab3

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guanine nucleotide exchange factor (GEF) activity. The AR's Rab27/Rab3A constitutes the first Rab GEF cascade described in dense-core vesicle exocytosis [54] (Figures 1 and 2A).

Chaperones: α-SNAP and NSF

α-SNAP (α-*N*-ethylmaleimide-sensitive factor attachment protein) and NSF are essential for all fusion events. α-SNAP stimulates the ATPase activity of NSF to disassemble *cis*-SNARE complexes. NSF has been detected in the acrosomal region of horse [69], bull, mouse and rhesus macaque sperm [70,71]. The AR is sensitive to dominant negative mutants of NSF that cannot bind or hydrolyse ATP [72], to NSF-sequestering antibodies [56,62,73] and to reagents that prevent NSF tyrosine dephosphorylation [32]. NSF, and presumably also α-SNAP, exhibits its role in sperm exocytosis downstream of the step catalysed by Rab3-GTP [62] (Figure 1).

α-SNAP has been found on the acrosome of mouse round spermatids [74] and in the acrosomal region of mouse [75] and human [73] sperm. It is essential for sperm exocytosis [56,73]. The only mammalian organism known to date that carries a genetically modified form of α-SNAP is the *hyh* mouse strain, which bears a M105I point mutation. Males from this strain are subfertile due to defective sperm exocytosis [75]. Meanwhile, NSF is present in rat, mouse and human sperm, and localizes to the acrosomal region in the latter [72].

Despite its positive role in membrane fusion, addition of recombinant α-SNAP to native membranes inhibits fusion. This effect has been observed in permeabilized human sperm [43,73], membrane sheets prepared from PC12 cells [76] and vacuoles from *Saccharomyces cerevisiae* [77,78]; NSF, and its yeast homologue Sec18p, reverses this effect (not tested in Schwartz and Merz [78]). Recombinant α-SNAP also inhibits the fusion *in vitro* between liposomes containing bacterially expressed versions of the Q-SNAREs (see below) syntaxin1 and SNAP-25, and chromaffin granules (containing the native R-SNARE synaptobrevin); once again, NSF reverses this effect [79]. Results from human sperm and PC12 cells led to the hypothesis that recombinant α-SNAP inhibits exocytosis because it binds monomeric syntaxin and prevents this SNARE from assembling with its cognates *in trans*. Simulations carried out with the COmplex PAthway SImulator (COPASI [80]) program fed with known rate parameters taken from the literature (not restricted to any particular model system) accurately fit the AR bench data [43]. Furthermore, TEM experiments show that sequestration of free syntaxin impedes docking of the acrosome to the plasma membrane in human sperm treated with recombinant full-length α-SNAP; a truncated version unable to bind syntaxin inhibits neither docking nor fusion [43]. In contrast, in chromaffin granules fusing with liposomes, α-SNAP binds partially assembled *trans*-SNARE complexes and arrests zippering midway, therefore preventing fusion, but not docking. No retardation effects on zippering or fusion are observed when synaptic vesicles are used instead of chromaffin granules, which suggests that there is an influence of the membrane environment on the effect of α-SNAP [79]. α-SNAP binds non-fusogenic, stalled (because of a deletion mutation that impairs full zippering), partially assembled *trans*-SNARE complexes on yeast vacuoles and rescues fusion in an NSF-independent manner [78]. In *in vitro* experiments conducted with soluble yeast proteins, Sec17 binds SNARE complexes and promotes selective loading of members of the Sec1/Munc18 (SM) protein families on to cognate complexes. SM proteins impair Sec18-mediated disassembly and therefore stabilize SNARE complexes [81]. This mechanism is different from that described using the (mammalian) synaptic versions of all fusion proteins in liposome-based assays [82].

It is clear then that more research is necessary to determine whether those functions of α-SNAP not related to *cis*-SNARE complex disassembly proceed through conserved mechanisms; at this point it would appear that they do not. It would be equally interesting to determine whether the effects of α-SNAP also take place in native systems with normal levels of endogenous proteins and, if they do, what their purpose is.

SNAREs

Syntaxin1, SNAP-25 and synaptobrevin2 families are the synaptic isoforms of the SNARE superfamily. Based on the identity of highly conserved residues, syntaxins and SNAPs are classified as Q-SNAREs (glutamine-containing SNAREs), whereas synaptobrevins are R-SNAREs (arginine-containing SNAREs) [83]. The Q- and R-SNAREs join into parallel four-helix bundles during all fusion processes; Q-SNAREs and R-SNAREs contribute three and one helices respectively to these complexes. It is hypothesized that SNARE proteins zipper progressively from the N-terminal portion of the molecules towards the membranes, and this zippering provides the force that overcomes the energy barrier for bilayer mixing (see Gao et al. [84] and references therein). In addition to pulling the membranes together, it has been suggested that the transmembrane domains of these proteins line/regulate the fusion pore opening and/or expansion [19,85–87].

The notion that synaptobrevin2, syntaxin1 and SNAP-25 have a direct function in exocytosis has received strong support from the identification of these proteins as the targets of clostridial neurotoxins. Tetanus toxin (TeTx) and seven structurally related botulinum neurotoxin serotypes (BoNT/A, B, C1, D, E, F and G) are potent inhibitors of secretory vesicle release due to their highly specific, zinc-dependent, proteolytic cleavage of SNARE proteins. BoNT/A and E cleave SNAP-25, BoNT/C cleaves syntaxin and, with much lower efficiency, SNAP-25. The remaining BoNTs, as well as TeTx, are specific for synaptobrevin [88]. The synaptic isoforms of all SNAREs are sensitive to cleavage by neurotoxins only when not packed in tight heterotrimeric complexes [89]. Whatever the steady-state configuration of SNAREs in neuroendocrine cells might be, exocytosis is blocked by neurotoxins, suggesting that SNAREs go through toxin-sensitive stages [90,91].

Published data on the presence of members of the SNARE complex in sperm comprise all three protein homologues in sea urchins [92–94] and mammals [21,31,41,69,95–99]. Treatment with BoNT/A, E, F, B and C and TeTx resulted in inhibition of acrosomal release, indicating a need for toxin-sensitive members of all three SNARE families and their productive assembly in *trans* complexes in the AR, regardless of the stimulus applied [31–33,35,43,56,58,97,100]. As intact SNAREs are required for the docking of the acrosome to the plasma membrane, cleavage of syntaxin with BoNT/C or of synaptobrevin with TeTx prevents docking [36].

Understanding the dynamics of SNARE assembly and disassembly during membrane recognition and fusion is central to unravelling the mechanisms that underlie regulated exocytosis. We have developed a protocol that detects syntaxin and synaptobrevin sensitivity to neurotoxins by indirect immunofluorescence as a reporter for SNARE protein configuration in human sperm. Both R- and Q-SNAREs are stably protected from toxin cleavage in resting cells. Acquisition of toxin sensitivity is coupled to the initiation of exocytosis [31] or

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treatment with recombinant PTP1B [32] or NSF [31]. Therefore, we hypothesize that sperm SNAREs are engaged in *cis* complexes until the cells are challenged to undergo exocytosis (Figure 1).

Differential sensitivity to BoNT/B and TeTx is a powerful approach to distinguish between monomeric synaptobrevin and that which is engaged in partial *trans* complexes. These toxins cleave the same peptide bond, which is exposed in both configurations [101]. However, TeTx binds the N-terminal whereas BoNT/B binds the C-terminal portion of synaptobrevin's coil domain. As SNARE complex assembly starts at the N-terminus, the TeTx-recognition site is hidden in partially assembled SNARE complexes whereas the BoNT/B recognition site is exposed. In other words, TeTx cleaves only monomeric synaptobrevin whereas BoNT/B also targets synaptobrevin loosely assembled in complexes [31,102,103]. Halting the AR with recombinant α-SNAP or Munc18-1 (see below) freezes syntaxin in a monomeric configuration, preventing its assembly in *trans* complexes and therefore the docking of the acrosome to the plasma membrane [35,43]. In contrast, halting exocytosis with intra-acrosomal calcium chelators [31] or exogenous complexin II [33] captures SNAREs in a loose *trans* configuration (sensitive to BoNT/B but resistant to TeTx) and the acrosome docked to the plasma membrane [35,36,42,43] (Figure 1)

The capacity to gather these findings highlights the strength of the sperm model because assigning molecular correlates to exocytotic stages, as defined by toxin sensitivity in other secretory cells, would have been difficult given the heterogeneous vesicular pools undergoing non-synchronic release.

Munc-13 and -18

In addition to SNAREs, members of the Munc-13 and -18 protein families participate in all types of intracellular membrane fusion (for recent reviews see the literature [15–17,104–106]). These proteins do not act independently but engage in supramolecular complexes [107] and co-operate to achieve fusion [82].

Munc18 is proposed as both a regulator of SNARE function (e.g. it assists in the formation of SNARE complexes) and an intrinsic component of the fusion apparatus. Members of the Munc18 family are present in human (Munc18-1 [35]) and boar (Munc18-2 [41]) sperm. Munc18-1 is essential for the human sperm AR. It plays a key role in the dynamics of *trans*-SNARE complex assembly and/or stabilization, which is necessary for the docking of the outer acrosomal membrane to the plasma membrane [35] (Figure 1).

Members of the Munc13 family have been reported to catalyse the priming of the fusion machinery and to aid in the assembly of *trans*-SNARE complexes by themselves or through interaction with other proteins, e.g. Munc18 or RIM [108]. Munc13-1 is present in the acrosomal region of human sperm and is required for exocytosis. It interacts functionally with Rab3 and RIM in these cells [42]. However, the exact point(s) of the signalling pathways where Munc13-1 is required during the AR remains unclear and as such this protein has been omitted from the models shown in Figures 1 and 3.

Complexin

Complexins are small soluble proteins that regulate exocytosis through their binding to the SNARE complex. They exhibit both activating and inhibitory functions on secretion; these activities have been mapped to different domains of the complexin polypeptides (reviewed in the literature [17,109–111]). Antiparallel binding of the complexin N-terminus to the SNARE complex has been proposed as having a stabilizing effect. The binding of the complexin C-terminal region to phospholipids is also thought to mediate its activating functions. Moreover, it is proposed that complexin acts as a prefusion clamp which arrests SNARE complexes and prevents fusion; this clamp is removed on activation of synaptotagmin by calcium and its binding to the SNARE complex, which causes a rearrangement of the complexin inhibitory helix. These and other concepts have been thoroughly explored in recent publications [112–117].

Complexin exhibits different localizations in non-capacitated and capacitated boar sperm heads, neither of which are predominantly acrosomal [41]. In contrast, complexin is present in the acrosomal region of human [33] and mouse [34,118] sperm. Experiments conducted with streptolysin O (SLO)-permeabilized human [33,55] and complexin I-null [34,118] sperm demonstrated the requirement of complexin in the calcium- and zona pellucida-induced AR respectively. In the former, endogenous complexin facilitates the assembly of *trans*-SNARE complexes, whereas, in the zona pellucida-induced AR, complexin is necessary to penetrate the zona pellucida at fertilization. An excess of recombinant complexin II arrests the AR at the docking stage [36,43] and this inhibition can be relieved by the C2B domain of synaptotagmin VI (see below) [33]. In porcine sperm, the plasma and outer acrosomal membranes are stably docked during *in vitro* capacitation; however, they do not fuse until an AR-inducing signal arrives [21]. This lack of spontaneous fusion has been attributed to stabilization of trimeric *trans*-SNARE complexes by complexin [41]. Thus it would appear that mammalian sperm fit into the model in which complexin acts as a transient fusion clamp that is released by calcium and synaptotagmin (Figure 1).

Synaptotagmin

In the early stages of the secretory pathway, in both constitutive exocytosis and the endocytic pathway, vesicle fusion follows immediately after the two fusing membranes make contact. In regulated exocytosis a trigger is required, so that vesicle fusion starts only when an appropriate signal, most frequently an elevation of the intracellular calcium concentration, is received [14,15,119]. It follows that, notwithstanding its earlier actions, calcium must act at a very late stage on vesicles poised for fusion. Thus synaptotagmins were found to be calcium sensors that act during synchronic release to detect the concentration of calcium by binding it and thereafter signalling to other targets via conformational changes. Synaptotagmins constitute a large family of transmembrane proteins found predominantly on synaptic and secretory vesicles. They contain two calcium-binding C2-domains (C2A and C2B) with distinct apparent calcium affinities. Synaptotagmins also bind the core fusion machinery composed of SNAREs and accessory proteins [15,119].

Several synaptotagmin isoforms have been described in mammalian sperm, e.g. synaptotagmin VI localizes to the acrosomal region in human [120], synaptotagmin VIII in mouse [121], synaptotagmin I in stallion [69], and undefined isoforms in hamster, bull, rhesus monkey, mouse and human [95] sperm; synaptotagmin IV has been reported in porcine sperm [41]. The requirement for synaptotagmin in human and mouse sperm exocytosis has been demonstrated by pre-incubation of SLO-permeabilized cells with various peptides encompassing portions of the cytoplasmic domains of synaptotagmins I, VI and VIII, or antibodies to synaptotagmin VI and VIII [98,120,122].

Binding of synaptotagmin VI to the protein machinery involved in sperm exocytosis can be disturbed by protein kinase C (PKC)-catalysed phosphorylation on Thr^{419} (and by phosphomimetic

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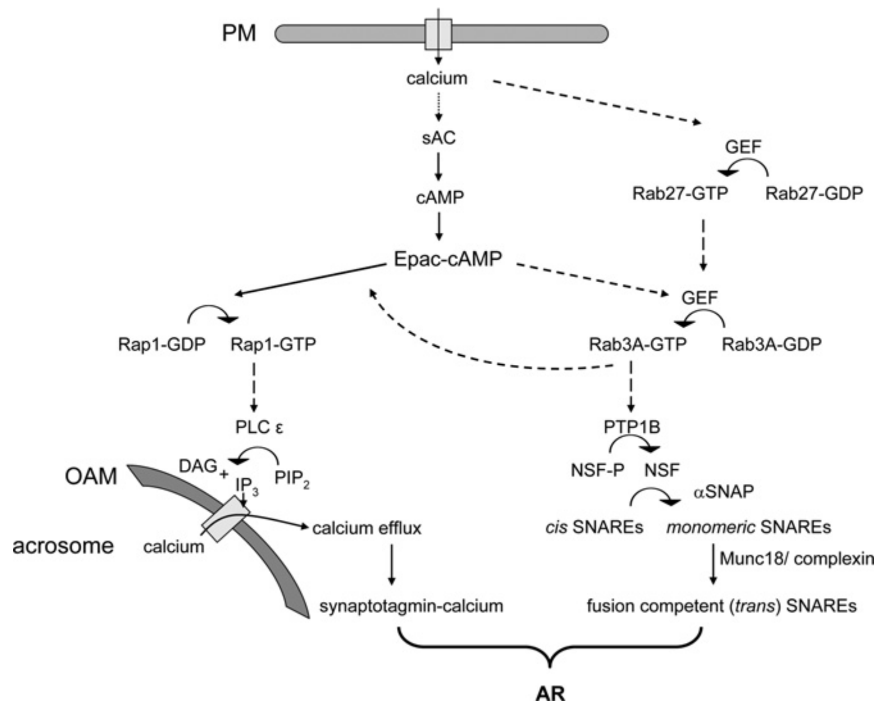

Figure 3 Updated working model for the AR showing a bifurcated signalling pathway downstream of cAMP/Epac

Calcium enters the cell from the extracellular milieu through channels or SLO-generated pores and stimulates, directly or indirectly, the exchange of GDP for GTP on Rab27. Cyclic AMP synthesized by sAC activates Epac and here the signalling pathway splits into two branches. In one, Epac catalyses the exchange of GDP for GTP on Rap; in the other, Epac-cAMP indirectly activates Rab3A. Rap-GTP heads a pathway that leads to acrosomal calcium mobilization (marked as 'calcium efflux'). Downstream of active Rap, a PLCε hydrolyses phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and IP_3, which mobilizes calcium from the acrosomal store. This local increase in calcium activates synaptotagmin. Rab3A-GTP heads a pathway that leads to the correct assembly of the fusion machinery ['fusion competent (*trans*) SNAREs']. Somewhere downstream of Rab3-GTP, there is a unidirectional (Rab3 branch → Rap1 branch) connection between both branches of the pathway. After this point, PTP1B is activated and/or recruited to the sites where it dephosphorylates NSF, derepressing its activity. Next, dephospho-NSF, in a complex with α-SNAP, disassembles *cis*-SNARE complexes. Monomeric SNAREs engage in heterotrimeric *trans* complexes aided by Munc18 and complexin. The steps catalysed by *trans*-SNARE complexes and synaptotagmin–calcium converge to accomplish the final steps of membrane fusion ('AR'). OAM, outer acrosomal membrane; PM, plasma membrane. Unbroken arrows mean that there is one connecting step between the terms whereas dashed arrows indicate that the number of steps is either unknown or not depicted for simplicity. Modified from Ruete et al. [62].

mutants) within the polybasic region of the C2B domain. Phosphorylation in this polybasic region affects its overall charge, which prevents its interaction with effectors and therefore renders the motif inactive. Synaptotagmin is phosphorylated in resting sperm [122]. The initiation of the AR induces calcineurin-dependent dephosphorylation [123] (Figure 1). Dephosphorylated synaptotagmin VI engages in an interplay with complexin during the late stages of the AR [33]. Our model proposes that the calcium to which synaptotagmin binds to achieve fusion comes from the intra-acrosomal store. Synaptotagmin binds to loose *trans*-SNARE complexes and contributes to their full zippering by relieving the complexin clamp (Figure 1). This does not rule out a potentially direct role for synaptotagmin in acrosome-to-plasma membrane fusion as has been suggested in other models [124,125].

OTHER COMPONENTS: cAMP, Epac, Rap AND INTRAVESICULAR CALCIUM

When an AR trigger binds to its receptor, an influx of calcium into the cytosol through plasma membrane channels ensues, with the consequent initiation of complex signalling cascades, which include synthesis of second messengers, protein–protein interactions, post-translational modifications and intracellular calcium mobilization. This productive, sequential and orderly assembly of the fusion protein machinery is crucial for accomplishing the late stages of sperm exocytosis. Calcium is also essential for all regulated exocytosis and therefore for sperm secretion, except that, in this case, the relevant calcium comes from inside the cell. Sperm lack endomembranes that typically behave as calcium reservoirs (e.g. endoplasmic reticulum, Golgi apparatus, lysosomes). Instead, the acrosome itself is the internal store of releasable calcium necessary for sperm exocytosis [62,126–128]. Intracellular calcium is mobilized via inositol 1,4,5-trisphosphate (IP_3)-sensitive channels in human sperm [58,60,62,128]. In the last part of the present review, I focus on the signalling pathway that drives calcium mobilization and present our current integrated molecular model which includes both phenomena: calcium signalling and the assembly of the fusion machinery (Figure 3). The key components of these cascades are not arranged in a linear sequence: a GEF activated by cAMP specific for Rap1 and Rap2 (exchange protein directly activated by cAMP or Epac) sits at a central point of the signalling cascade, after which the exocytotic pathway branches, with one arm assembling the fusion machinery into place and the other eliciting the release of calcium from the acrosome. Both parts of this pathway must act in concert to achieve exocytosis [60,62].

Cyclic AMP/Epac

Cyclic AMP is a central second messenger that controls many vital functions. In certain neurons, and neuroendocrine and exocrine acinar cells, cAMP potentiates calcium-dependent exocytosis whereas, in various non-neuronal cells, cAMP is the

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principal trigger of regulated secretion [13,129,130]. Previously the effects of cAMP in regulated exocytosis were thought to be mediated by cAMP-dependent protein kinase (PKA) through the phosphorylation of relevant substrates. More recently, cAMP was found to modulate exocytosis by co-ordinating both PKA-dependent and PKA-independent mechanisms. The latter are mediated by Epacs (reviewed in the literature [131–133]), although how Epac and PKA cross-talk during cAMP signalling is still unclear.

An elevation of cAMP alone is sufficient to trigger exocytosis in human sperm [56]. Cyclic AMP is produced endogenously by a soluble adenylate cyclase (sAC) that is essential for the AR [60,62,134]. Epac-1 and -2 have been detected using Western blotting in human, stallion and boar [135] sperm, Epac2 in hamster sperm [136] and Epac1 in mouse sperm [137]. Messenger RNAs corresponding to Epac-1 and -2 have been detected by reverse transcriptase PCR in mouse spermatogenic cells [138]. Epac is also present in the acrosomal region in human sperm. In these cells, calcium-induced AR is mediated by cAMP/Epac and is independent of PKA [56], which makes sperm an ideal model for the direct study of the role of Epac in secretion, without the confounding effects that the superimposed actions of PKA may cause in other systems. Rab3A is indirectly activated by cAMP/Epac, which puts into motion the fusion machinery branch of the cascade [54,60,62] (Figure 3).

Rap

Rap proteins (Rap1a and -b, and Rap2a, -b and -c) are small-molecular-mass GTPases of the Ras family, which controls cell growth, differentiation, adhesion-related events and survival. Raps are activated by a number of GEFs, with Epac being one, that connect different inputs to the many Rap functions [131,139–142], one of which is regulated exocytosis. Cyclic AMP/Epac/Rap1 is required for the potentiation of insulin release [143,144], the secretion of pancreatic amylase [145] and the non-amyloidogenic soluble form of the amyloid precursor protein α [146], as well as the exocytosis of Weibel–Palade bodies in endothelial cells [147].

Rap1 has been detected in human [60,135], boar [135] and mouse [136] sperm, and Rap2 is present in hamster sperm [137]. The AR elicited by the Epac-selective cAMP analogue 8-(4-chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate acetoxymethyl ester (8-pCPT-2-*O*-Me-cAMP), recombinant Rab3A-GTP-γ-S, diacylglycerol and calcium requires endogenous active Rap1 [58,60,62]. Calcium used as an AR trigger activates Rap1 in the acrosomal region of human sperm in an Epac-dependent manner [62] (Figure 3). Likewise, the amount of Rap1-GTP pulled down from cells challenged with 8-pCPT-2 -*O*-Me-cAMP is substantially higher than that from untreated controls in human [60], mouse [137] and boar [135] sperm.

Intra-acrosomal calcium mobilization

Cyclic AMP binds both PKA and Epac to regulate intracellular calcium release. In many models, cAMP/PKA and/or cAMP/Epac facilitates the opening of calcium release channels located in intracellular stores, typically the endoplasmic reticulum; such release is important to accomplish biological responses. In pancreatic β cells, perhaps the most-studied secretory model for these pathways (reviewed in the literature [129,148]), additional sources of intracellular calcium, e.g. endosomes, lysosomes and insulin secretory granules themselves, have been described. The activation of Epac by cAMP sensitizes intracellular calcium release channels, which increase cytosolic calcium concentrations and, subsequently, insulin secretion [149,150]. Metabolites, such as IP_3, cADP-ribose (the proposed endogenous ligand for ryanodine receptors) and nicotinic acid adenine dinucleotide phosphate, enhance the ability of cytosolic calcium to activate various calcium release channels located on intracellular organelles [132,151]. Pharmacological blocking of these pathways impairs glucose-induced insulin secretion, pointing to the requirement of intracellular calcium efflux for exocytosis [152].

A large number of effectors that are either recruited or activated by GTP-bound Rap to induce biological responses have been identified (reviewed in Raaijmakers and Bos [142]). The one implicated in Epac-mediated secretory responses is phospholipase C epsilon (PLCε) [144,153]. PLCs catalyse the hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and IP_3. Hence, PLCε might be the link that connects cAMP/Epac/Rap with intracellular calcium mobilization through pathways sensitive to its reaction products. In fact, this enzyme is required downstream of cAMP/Epac2/Rap1 for the potentiation of glucose-induced insulin release and intracellular calcium mobilization [144,153].

A PLC activity is required for the AR induced by calcium, persistently active Rab3A, 8-pCPT-2-*O*-Me-cAMP [60], diacylglycerol and a non-hydrolysable analogue [58]. Furthermore, 8-pCPT-2-*O*-Me-cAMP elicits a calcium signal in population studies that is abrogated by the PLC blocker U73122 [60]. We have recently detected the presence of PLCε1 in the acrosomal region of human sperm and its requirement for the AR (O. Lucchesi, M. C. Ruete, M. F. Quevedo, M. A. Bustos and C. N. Tomes, unpublished work), and it is exciting to have found one more link to complete the pathway (Figure 3). Pharmacological blockers applied in different experimental strategies have demonstrated that the PLC's reaction product diacylglycerol exhibits essential roles during the AR. In addition to its other functions, diacylglycerol engages in a positive feedback loop that contributes to maintain the production of IP_3, the other reaction product of PLC [58]. This second messenger is required for the AR elicited by all inducers [56,58,128,154].

The acrosomal calcium store can be readily visualized with the calcium indicator Fluo3-AM in sperm with plasma membranes permeabilized with SLO (in non-permeabilized cells there is a diffuse cytosolic staining in the whole cell that impairs the visualization of acrosomal calcium; see Tomes [9]). The AR inducers active Rab3A [128], diacylglycerol [58], cytosolic calcium [62] and 8-pCPT-2-*O*-Me-cAMP (O. Lucchesi, M. C. Ruete, M. F. Quevedo, M. A. Bustos and C. N. Tomes, unpublished work) decrease the concentration of calcium inside the acrosome assessed by single-cell fluorescence microscopy. So does adenophostin A, an IP_3 receptor agonist that rescues all AR blockers that interfere with intra-acrosomal calcium mobilization [60,62]. Cyclic AMP and Epac are also necessary for intra-acrosomal calcium efflux [62]. The complete Rap/PLC/intra-acrosomal calcium efflux pathway is summarized in Figure 3.

Both branches (right and left arms in Figure 3) of the exocytotic cascade are joined at or near the stage catalysed by Rab3 in a unidirectional, hierarchical connection, in which the intra-acrosomal calcium mobilization branch is subordinated to the fusion protein branch; somewhere after Rab3, the pathways become independent [60,62,128]. My colleagues and I have proposed the existence of a loop that connects Rab3 with cAMP/Epac, wherein all components of this loop must be available and active to achieve secretion. Once the system has progressed beyond the loop, the calcium mobilization branch will proceed regardless of the status of the fusion machinery branch.

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We speculate that the Rab3 effector RIM might play some part in this loop based on its reported interaction with Epac [155]. Whichever the mechanism, we hypothesize that its purpose is to target Epac, and therefore Rap-GTP and PLCε, to the productive contact sites between the acrosome and the plasma membrane created by active Rab3 and *trans*-SNARE complexes. In this way, intravesicular calcium will be released at or near these points. This hypothetical requirement for a highly localized calcium signal explains the dependence of the AR on intracellular calcium mobilization and why this cannot be overcome with high overall cytosolic concentrations from an extracellular source.

CONCLUDING REMARKS

In addition to the core fusion proteins, sperm contain accessory molecules that tightly regulate the exocytotic cascade. These molecules act as 'gatekeepers' of the fusion reaction, holding components temporarily in an inactive state and coupling their transition to a fusion-competent state before the arrival of the appropriate signal. It is believed that this level of strict regulation avoids the risk of inappropriate activation of the fusion machinery (e.g. when an egg is not readily available for fertilization). The human sperm exocytosis model is privileged in terms of permitting the direct inspection, in great detail and with high resolution, of the dynamics of individual molecular species in an almost *in vivo* configuration. This has allowed the development of a conceptual model that integrates biochemical and morphological data and assigns molecular correlates to each stage of the fusion cascade. It will be exciting to determine whether the cascades covered in the present review are unique to the sperm system or part of a more universal, and still unexplored, regulated exocytosis mechanism.

ACKNOWLEDGEMENTS

I am grateful to Dr Luis Mayorga for revising this manuscript critically for important intellectual content and to Dr Matías Bustos for creating Figure 2.

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Received 16 September 2014; accepted 29 October 2014
Published on the Internet 22 January 2015, doi:10.1042/BJ20141169

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