BOR Papers in Press. Published on January 20, 2016 as DOI:10.1095/biolreprod.115.133231

Acrosomal swelling is triggered by cAMP downstream the opening of store-operated calcium channels during acrosomal exocytosis in human sperm[1]

Running title: Acrosomal swelling during exocytosis

Summary sentence: Acrosomal swelling is a required step of the acrosome reaction in human sperm and is triggered by a signaling pathway that involves calcium and cAMP.

Key words: human sperm, acrosome reaction, acrosomal swelling, regulated exocytosis, calcium, cAMP, secretory granules

Claudia M. Sosa[3], M. Natalia Zanetti[3], Cristian A. Pocognoni, and Luis S. Mayorga[2]
Instituto de Histología y Embriología de Mendoza (IHEM/CONICET-UNCuyo), School of Medicine, National University of Cuyo, Mendoza, Argentina

[1]This work was supported by grants from Universidad Nacional de Cuyo, Argentina (06/J388), CONICET, Argentina (PIP 112-200801-02277), and ANPCYT, Argentina (PICT-2008-1114 and PICT-2011-2310) to L.S.M., and fellowships from CONICET and ANPCYT to C.A.P., M.N.Z., and C.M.S.
[2]Correspondence: Luis S. Mayorga; Instituto de Histología y Embriología de Mendoza (IHEM/CONICET-UNCuyo), School of Medicine, National University of Cuyo, Casilla correo 56, 5500 Mendoza, Argentina. E-mail: lmayorga@fcm.uncu.edu.ar
[3]These authors have contributed equally to this work.

ABSTRACT

Acrosomal exocytosis in mammalian sperm is a regulated secretion with unusual characteristics. One of its most striking features is the post fusion loss of the outer acrosomal membrane and the overlying plasma membrane as hybrid vesicles. We have previously reported in human sperm that, by preventing the release of calcium from the acrosome, the exocytic process can be arrested at a stage where the acrosomes are profusely swollen, with invaginations of the outer acrosomal membrane. In this report, we show by transmission electron microcopy swelling with similar characteristics without arresting the exocytic process. Acrosomal swelling was observed when secretion was promoted by pharmacological and physiological inducers of the acrosome reaction that trigger exocytosis by different mechanisms. We show that progesterone- and thapsigargin-induced swelling depended on a calcium influx from the extracellular medium through store-operated calcium channels. However, calcium was dispensable when sperm were stimulated with cAMP analogs. KH7, an inhibitor of the soluble adenylyl cyclase, blocked progesterone-induced swelling. Our results indicate that swelling is a required process for acrosomal exocytosis triggered by activation of an adenylyl cyclase downstream the opening of store-operated calcium channels.

INTRODUCTION

The acrosome reaction is a special type of regulated exocytosis essential for fertilization [1]. In mammals, the acrosome is a large and flat granule situated between the anterior part of the nucleus and the plasma membrane. The acrosomal membrane underlying the plasma membrane is referred to as the ‘‘outer’’ acrosomal membrane, and that overlying the nucleus is referred to as

Copyright 2016 by The Society for the Study of Reproduction.

the ‘‘inner’’ acrosomal membrane. In human sperm, the outer acrosomal and the plasma membranes are in close proximity; however, there is not obvious interactions between these two structures until the sperm is stimulated in the proximity of the mature oocyte to undergo the acrosome reaction. During acrosomal exocytosis, the membranes fuse at multiple points leading to the release of hybrid vesicles, formed by patches of the outer acrosomal membrane and plasma membrane. Probably the most crucial role of acrosomal exocytosis is the change in the topology and composition of the limiting membrane of the sperm [2]. These changes are fundamental for sperm-egg interaction and fusion.

Progesterone is a physiological stimulus of acrosome reaction in human sperm. The signaling cascade triggered by the hormone is still unclear. One important target for progesterone is the sperm-specific calcium channel CatSper located in the principal piece of the flagellum [3]. These channels can be activated by a large set of small molecules, including prostaglandin E1 and cAMP analogs. Opening of CatSper triggers a complex calcium signaling cascade that includes the release of calcium from intracellular stores [4]. Changes in the calcium content of these stores is sensed by Store-Operated Calcium channels (SOC channels) at the plasma membrane and generate Store-Operated Calcium Entry (SOCE) [5;6]. SOCE may be mediated by different proteins, including TRP (Transient Receptor Potential) channels that are present in sperm and have physiological implications for sperm motility and the acrosome reaction [7]. A different mechanism involves Orai channels that are activated by STIM, a calcium sensor with the correct orientation for detecting changes in the calcium content of intracellular compartments. Orai and STIM have been detected in mouse and human sperm [7;8]. The prevalent mode of activation of Orai by STIM is the physical interaction between these proteins by apposition between the plasma membrane and the intracellular organelle whose calcium content is sensed by STIM. Not all sperm that respond to the hormone with an increase of cytosolic calcium underwent secretion [9]. In fact, there is a time dissociation between the first raise of calcium and the onset of secretion. Hence, it is not clear what signal stimulates the membrane fusion machinery responsible for acrosomal exocytosis.

The molecular mechanism of acrosomal exocytosis has been intensively studied by us and by other laboratories (see references in [10;11]). Fusion between the outer acrosomal and plasma membranes shares a common molecular mechanism with calcium-dependent exocytoses reported in different cell types. According to several pieces of evidence, we have proposed that, in resting sperm, the membrane fusion machinery is inactive. Key factors, such as synatotagmin and NSF, are phosphorylated and SNAREs are assembled in inactive cis complexes (i.e., complexes where all SNAREs are in the same membrane). In permeabilized cells, addition of calcium is sufficient to trigger exocytosis. Secretion is also observed in the presence of cAMP, even at low concentrations of external calcium. Depletion of cAMP prevent calcium-triggered secretion, indicating that this nucleotide is a downstream effector of calcium. When the fusion process is activated, synaptotagmin and NSF are desphosphorylated, Rab3A exchange GDP by GTP, and cis SNARE complex are disassembled. cAMP also activates an EPAC (Exchange Proteins directly Activated by cAMP)-dependent pathway that promotes a Ca^{2+} efflux coming from the acrosome through inositol 1,4,5-trisphosphate (IP_3) sensitive calcium channels [12;13]. The assembly of a functional fusion machinery and the efflux of acrosomal calcium are required for triggering fusion pore opening and acrosomal content release [11;13-15]. Notice that it is not obvious how this complex mechanism of membrane fusion will lead to the formation of hybrid vesicles.

Several studies have characterized acrosomal exocytosis in different mammalian species

[16-20]. A commonly reported observation is the swelling of the acrosome before fusion [16;18;20;21]. We have proposed that acrosomal swelling is an important step in acrosomal exocytosis that actively participates in the formation of hybrid vesicles [22]. In our model, the acrosome swells upon sperm stimulation. The swollen granule undergoes invaginations of the outer acrosomal membrane. The edges of these invaginations contact the plasma membrane and form tight ring-shaped appositions stabilized by the assembly of trans SNARE complexes. We have recently shown that the Endosomal Sorting Complex Required for Transport (ESCRT) is essential for this process [23]. Calcium release from the acrosome in the restricted cytosolic compartment enclosed by the invagination and the plasma membrane triggers the opening and expansion of fusion pores in this ring, leading to the release of hybrid vesicles. The membrane of the invagination would contribute with acrosomal membrane to the vesicle and the cap sealing the invagination, the plasma membrane domain. In a recent publication we measured the kinetics of swelling and observed that it is a slow process and probably the rate limiting-step of acrosomal exocytosis [24].

In this report, we analyzed acrosomal swelling in live, non-permeabilized sperm. Our results indicate that swelling is a required process for acrosomal exocytosis triggered by activation of an adenylyl cyclase downstream the opening of SOC channels.

MATERIALS AND METHODS

Reagents

Progesterone (Pg), 1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole (SKF-96365), NNC 55-0396 dihydrochloride, 4-methy-40-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide (YM-58483), Pisum sativum lectin labeled with fluorescein isothiocyanate (FITC-PSL), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Argentina SA. 1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM), 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) were from Molecular Probes (Invitrogen Argentina). A23187 was from Alomone Labs (Alomone Labs., Jerusalem, Israel). Xestospongin C (XC) and thapsigargin (Tg) were from Calbiochem (MERCK Química Argentina, Buenos Aires, Argentina). N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (db-cAMP) and 8-*p*CPT-2′-*O*-Me-cAMP (8-pCPT) were from Axxora, LLC (San Diego, CA). (±)-2-(1*H*-benzimidazol-2-ylthio)propanoic acid 2-[(5-bromo-2-hydroxyphenyl)methylene]hydrazide (KH7) was from R&D Systems (Minneapolis, MN). All electron microscopy supplies were from Pelco (Ted Pella INC. California, USA). All other chemicals were purchased from Sigma-Aldrich™ Argentina S.A., Genbiotech, or Tecnolab (all from Buenos Aires, Argentina). A plasmid encoding human Rab3A fused to a cell-penetrating peptide and to His_6 was used to produce membrane permeant Rab3A (R-Rab3A) as previously described [25]. R-Rab3A was prenylated *in vitro*, and loaded with GTPγS as described [12].

Sperm preparation

Human semen samples were obtained from six healthy donors (age range: 25-35 years). The signed informed consent and the protocol for semen handling were approved by the Ethic Committee of the Medical School, Universidad Nacional de Cuyo. Samples were provided by masturbation after at least 2 days of abstinence. Semen was allowed to liquefy for 30–60 min at 37ºC. Highly motile sperm were recovered by swim-up separation for 1 h in human tubal fluid

medium (HTF: 5.94 g/l NaCl, 0.35 g/l KCl, 0.05 g/l $MgSO_4.7H_2O$, 0.05 g/l KH_2PO_4, 0.3 g/l $CaCl_2.2H_2O$, 2.1 g/l $NaHCO_3$, 0.51 g/l d-glucose, 0.036 g/l Na pyruvate, 2.39 g/l Na lactate, 0.06 g/l penicillin, 0.05 g/l streptomycin, 0.01 g/l phenol red) supplemented with 5 mg/ml BSA at 37ºC in an atmosphere of 5% CO_2, 95% air. The pH was maintained within the 7.2–7.4 range. After swim-up, sperm suspensions were diluted to 10 x 10^6 sperm/ml with HTF-BSA and incubated 3–4 hours to promote capacitation (37°C; 5% CO_2, 95% air). In experiments with non-capacitated sperm, HEPES modified HTF (HEPES 5.004 g/l, 5.94 g/l NaCl, 0.35 g/l KCl, 0.05 g/l $MgSO_4.7H_2O$, 0.05 g/l KH_2PO_4, 0.3 g/l $CaCl_2.2H_2O$, 0.33 g/l $NaHCO_3$, 0.51 g/l d-glucose, 0.036 g/l Na pyruvate, 2.39 g/l Na lactate, 0.06 g/l penicillin, 0.05 g/l streptomycin, 0.01 g/l phenol red) without BSA was used for both the swim-up and post swim-up incubations (performed at 37ºC in air).

Acrosomal exocytosis
After incubating as indicated in Fig. 4D, spermatozoa were spotted on Teflon-printed slides, air dried, and fixed/permeabilized in ice cold methanol for 1 min. Acrosomal status was evaluated by staining with FITC–PSL according to [26]. At least 200 cells were scored using a Nikon Optiphot II microscope (Nikon, Inc., Melville, NY)

Swelling protocols
Capacitated or non-capacitaded sperm (10 x 10^6 cells/ml) were incubated with inhibitors for 15 min at 37ºC. Afterwards, the stimulants were added to the sperm suspensions, and the incubation proceeded at 37ºC for 0-30 min, depending on the experiment. Samples were immediately fixed in 2.5% v/v glutaraldehyde in PBS, incubated overnight at 4 °C, and prepared for electron microscopy. To account for the natural variability among semen samples, a negative control (not stimulated) and a positive control were included in all experiments. Hence, swelling under the different conditions tested was always compared with controls run in parallel. In some experiments, 5 mM BAPTA was added to HTF; the free calcium concentration under these conditions was in the 40-80 nM range (Fura2 ratiometric assay [27]).

Transmission Electron Microscopy
Fixed sperm samples were centrifuged for 30 seconds at 10,000 rpm to obtain a pellet, which was maintained throughout the procedure. Pellets were washed twice with PBS and post-fixed in 1% osmium tetroxide in PBS pH 7.4 for 2 hours, washed three times in PBS and dehydrated in an increasing concentration series of acetone on ice. Cells were infiltrated in 1:1 acetone/EPON for 2 hours at room temperature and finally embedded in fresh resin. Thin sections (60–80 nm) were cut with a diamond knife (Diatome, Washington D.C., USA) in a Leica ULTRACUT R ultramicrotome and collected on 200-mesh copper grids. The sections were stained with saturated uranyl acetate in methanol and lead citrate. The samples were observed under a Zeiss 900 electron microscope at 80 kV and photographed with a Gatan Orius SC1000 - 832, CCD camera installed in the left side port of the microscope.

RESULTS

Acrosomal swelling is a necessary step for acrosomal exocytosis
In a previous report, we have described the swelling of the acrosomal granule in sperm stimulated with a calcium ionophore in the presence of different inhibitors that block acrosomal exocytosis

[22]. The rationale for the inclusion of inhibitors was that if swelling were a fast process followed by exocytosis, sperm with swollen acrosomes would not be detected unless exocytosis was blocked. However, when we measured the kinetics of swelling we observed that it is a slow process and probably the rate-limiting step of acrosomal exocytosis [24]. Hence, the percentage of sperm with swollen acrosomes should increase upon stimulation even in the absence of inhibitors. To test this possibility, sperm were challenged with the calcium ionophore A23187 in the absence of inhibitors and cells fixed and analyzed by electron microscopy at different times after stimulation. At the initial time of the incubation, sperm showed acrosomes with electron-dense content and a flat outer acrosomal membrane (Fig. 1D to 1G, sperm labeled "i"). After 20 min of incubation, most sperm showed swollen acrosomes with a ruffled outer acrosomal membrane (Fig. 1E to 1G, sperm labeled "s"). Some of them had lost the acrosome (Fig. 1E, sperm labeled "r"). The percentage of sperm with swollen acrosomes increased with time, reaching a plateau after 8 min. After this time, the percentage of sperm with intact acrosomes continued decreasing at the same time that the number of reacted sperm increased (Fig. 1A). These results are consistent with the idea that first the acrosomes undergo swelling and deformation of the outer acrosomal membrane and then the granules are lost as the sperm react. According to these observations, swelling would be an early and normal step during the acrosomal reaction and not an artifact caused by inhibitors of the exocytosis.

If swelling is a necessary step for secretion, it should be induced by all stimulators of the acrosomal exocytosis. Progesterone is a well-known stimulus for human sperm acrosome reaction. To detect swelling during exocytosis induced by progesterone, sperm were incubated with the hormone in the absence of inhibitors and the cells analyzed by electron microscopy. Similar to the effect of A23187, a time-dependent decrease of sperm with undisturbed acrosomes was observed. In contrast, the percentage of sperm with swollen acrosomes or reacted sperm increased (Fig. 1B, 1F and 1G). Only capacitated sperm react when stimulated with progesterone. To test whether acrosomal swelling induced by the hormone was also capacitation-dependent, the swim-up was performed in HEPES-modified HTF medium in the absence of BSA. After 3 hs of incubation in the same medium, cells were challenged with progesterone for 15 min and further processed for electron microscopy. As shown in Fig. 1C, no swelling was observed in non-capacitated cells, consisting with the idea that swelling is part of the normal secretory process.

Acrosomal swelling was also evaluated with a series of other inducers of exocytosis. To compare percentage of swelling, in these experiments exocytosis was blocked by XC, an IP_3-sensitive calcium channel inhibitor that abrogates acrosomal exocytosis [25] but does not affect acrosomal swelling [22]. As expected, both A23187 and progesterone induced swelling in the presence of XC (Fig. 2A). Calcium pumps responsible for maintaining the levels of this cation in intracellular calcium stores are inhibited by thapsigargin [28]. The decrease of calcium in these stores activates SOC channels. Thapsigargin induces acrosomal exocytosis in sperm from several species, including humans [27;29-31]. db-cAMP is a membrane-permeable cAMP analog that activates the secretion process downstream the opening of calcium channels at the cell surface by activating EPAC, a cAMP binding protein required for acrosomal exocytosis [13;32;33]. 8-pCPT is another cAMP analog that specifically activates EPAC but no other cAMP targets such as protein kinase A (PKA). R-Rab3A is a membrane permeant version of Rab3A carrying a cell-penetrating peptide at its N-terminus [25]. All these inducers of the acrosomal exocytosis promoted swelling (Fig. 2A).

Two characteristics of acrosomal swelling are the increase in size and a decrease in the

electron density of the granule. Strikingly, the equatorial region of the acrosome preserved a condensed aspect and a morphology characteristic of non-stimulated spermatozoa even in profusely swollen granules (examples can be seen in a sperm with a swollen acrosome in Fig. 1F, and sperm heads shown in 2D, and 2E). In some sperm, swelling was accompanied by deformations of the outer acrosomal membrane with deep invaginations and the budding of intraacrosomal vesicles. To compare the swelling caused by the different stimuli, the acrosomes were classified as intact (dense acrosomes with parallel outer and inner acrosomal membrane; Fig. 2C), swollen (enlarged acrosomal compartment with undisturbed outer acrosomal membrane; Fig. 2D), waving with vesicles (enlarged acrosomes with invaginations of the outer acrosomal membrane and presence of intraacrosomal vesicles; Fig. 2E) and reacted sperm (loss of the plasma membrane, outer acrosomal membrane and acrosomal content; Fig. 2F). This classification is shown in Fig. 2B for sperm stimulated with A23187, progesterone, or thapsigargin. The results indicate that all three stimuli induced swelling with similar characteristics.

Our results indicate that all stimulants of the acrosomal exocytosis induce swelling, suggesting that deformations of the acrosomal granule is a required step for human sperm acrosome reaction.

Swelling occurs downstream the opening of SOC channels

The role of SOC channels in the signaling pathway leading to acrosomal reaction has been reported by several laboratories [5;6;34]. The prevalent mode of activation of Orai by STIM is the physical interaction between these proteins by apposition between the plasma membrane and intracellular calcium stores. According to our observations, the swelling of the acrosome (a calcium store) promotes the interaction of its membrane with the plasma membrane. One can speculate that this physical interaction may signal the opening of SOC channels. Hence, the opening of SOC channels would be a post-swelling event. In contrast, we have proposed that the opening of SOC channels initiates the exocytic process, which includes acrosomal swelling. Therefore, in our model, SOC channel activation occurs upstream acrosomal swelling. We then designed experiments to discern between these two possibilities: i) opening of SOC channels is induced by membrane apposition promoted by swelling or ii) opening of SOC channels induces a calcium signal that causes swelling (Fig. 3A)

As discussed above, thapsigargin depletes intracellular calcium stores, activates SOC channels and induces exocytosis. Addition of BAPTA -a fast calcium chelator- to the extracellular medium blocks thapsigargin-stimulated exocytosis [27], likely by preventing the entrance of calcium through the opened SOC channels. If swelling is involved in the opening of SOC channels, acrosomes should be swollen under these conditions. In contrast, if swelling is triggered by the entrance of calcium through SOC channels, acrosomes should be undisturbed. We then analyzed by electron microscopy the morphology of the acrosomes in sperm treated with thapsigargin in the presence of BAPTA. Fig. 3B shows that BAPTA blocked swelling promoted by thapsigargin, suggesting that the swelling is triggered by calcium downstream the opening of SOC channels. To test this conclusion using progesterone, a more physiological stimulus, extracellular calcium was not depleted, but SOC channels were blocked by two specific inhibitors. SKF-96365 is an imidazole-derived compound, widely used to block SOCE. YM-58483 is a pyrazole-derived compound with immunosuppressive effects that efficiently inhibits SOCE [35]. The inhibitors blocked progesterone- and also thapsigargin-induced swelling, but failed to prevent swelling triggered by A23187 (Fig. 3B to 3D). These observations indicate that

acrosomal swelling depends on an influx of calcium through SOC channels opened downstream progesterone and thapsigargin stimuli.

cAMP analogs promote acrosomal swelling independently of calcium

In Fig. 2A, we have shown that db-cAMP, 8-pCPT and R-Rab3A promoted swelling. These three stimuli do not require extracellular calcium to induce acrosomal exocytosis because they activate secretion at a stage downstream the cytosolic calcium increase [25;32;33]. They do require a calcium release from the interior of the acrosome at a later stage, downstream acrosomal swelling, to promote fusion pore opening. These observations suggest that calcium may be dispensable for swelling if exocytosis is triggered by inducers that do not require extracellular calcium. We then tested whether db-cAMP and 8-pCPT could induce swelling when calcium was depleted in both the extracellular medium and intracellular stores. As shown in Fig. 4A, these cAMP analogs induced swelling in the presence of 5 mM BAPTA (that chelates extracellular calcium) and 20 μM BAPTA-AM (that at this relatively high concentration, chelates both cytosolic calcium and calcium in intracellular stores). Notice that the combination of BAPTA plus BAPTA-AM does not affect the morphology in the absence of stimulus, and it does not prevent normal swelling when stimulated by db-cAMP (Fig. 4E and 4F).

The fact that calcium triggers acrosomal swelling but is dispensable when cAMP analogs are used, suggests that calcium promotes swelling indirectly by activating an adenylyl cyclase. Another possibility is that calcium and cAMP activate swelling by independent mechanisms. To assess this possibility we tested the effect of KH7, an inhibitor of the soluble adenylyl cyclase isoform present in sperm. The inhibitor blocked swelling and also acrosomal exocytosis (data not shown) when sperm were stimulated with progesterone (Fig. 4B and 4H). Calcium ionophores can trigger exocytosis even in non-capacitated sperm. Since we postulate that cAMP is activating secretion downstream calcium, we wondered if it could induce acrosomal swelling in non-capacitated sperm. As shown in Fig. 4C, both A23187 and 8-pCPT caused acrosomal swelling in sperm incubated under capacitating and non-capacitating conditions. It has been shown that CatSper can be activated by cAMP analogs when added in the extracellular medium [3]. However, we observed that these compounds activate swelling in the absence of extracellular calcium, indicating that the effect was not due to the opening of CatSper channels. The prediction was then that CatSper inhibitors should not affect swelling triggered by 8-pCPT. NNC55-0396 (NNC), a CatSper inhibitor that affect human sperm motility and acrosome reaction [4;36] was tested at different concentrations (data not shown). Under the conditions tested, 1 μM was sufficient to abrogate progesterone-triggered exocytosis without increasing spontaneous secretion (Fig. 4D). At this concentration, as expected, it did not block acrosomal swelling induced by 8-pCPT (Fig. 4C).

In conclusion, the results indicate that swelling is induced by activation of the soluble adenylyl cyclase isoform downstream an increase of cytosolic calcium.

DISCUSSION

Acrosomal granule swelling during the acrosome reaction has been described by several authors [16;20;21]. In this report we show that swelling was induced by A23187 and progesterone during the normal course of the exocytic process, even in the absence of inhibitors. Moreover, swelling with similar characteristics was observed with several activators of the acrosomal exocytosis that trigger secretion by different mechanisms. There was an evident decondensation of the electron dense matrix inside the granule (except at the equatorial region) together with an

increase in the volume of the acrosome. The outer acrosomal membrane became ruffled with invaginations. The acrosomal swelling promoted the apposition between the outer acrosomal and plasma membrane at the edge of these invaginations. Our results indicate that these membrane-membrane interactions do not participate in the opening of SOC channels and that swelling is activated downstream the SOCE induced by progesterone or thapsigargin. SOC channels respond to a decrease on the calcium content of intracellular calcium stores. The human sperm has at least two membrane-bound stores: the acrosome surrounding the nucleus and the redundant nuclear envelop at the sperm neck [34]. We have good evidence that the release of calcium from the acrosome through IP_3-sensitive channels is required for the final step of the acrosomal exocytosis (references in [11]). However, we have observed that progesterone promotes acrosomal swelling in the presence of XP, an inhibitor of these channels. This would suggest that IP_3-sensitive channels are not involved in the signal cascade that triggers swelling. IP_3 is not the only second messenger capable of inducing calcium mobilization from intracellular stores. Calcium itself can promote calcium-mediated calcium release. Cyclic-adenosine diphospho-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) are two compounds found in the sperm that can activate calcium channels present in intracellular stores [34]. In a recent report, it has been proposed that progesterone activates SOC channels in the human sperm neck downstream a calcium signal mediated by CatSper [37]. In addition, TRP channels at the plasma membrane can be activated by diacylglycerol and trigger calcium influx independently from intracellular stores [7]. We speculate that a complex signal cascade causes a cytosolic calcium increase that activates acrosomal swelling.

Swelling was induced by some stimulators of the acrosomal exocytosis that do not require extracellular calcium. We have previously proposed a signaling cascade that is triggered by calcium and activates cAMP production [11;13]. At this point, extracellular calcium is dispensable. Exocytosis requires a calcium release from the acrosome to trigger the opening of fusion pores. However, swelling does not require acrosomal calcium. Moreover, we show that cAMP analogs promoted swelling in the presence of 5 mM BAPTA and 20 µM BAPTA-AM to chelate extracellular and intracellular calcium. This observation indicates that swelling is a cAMP-dependent, calcium-independent process; it also rules out the possibility that the effect was due to CatSper activation that would only have an effect in the presence of extracellular calcium. cAMP has been implicated in several aspects of sperm physiology. The fact that swelling was efficiently promoted by 8-pCPT, a nucleotide that activates EPAC but not PKA [38], suggests that the signaling pathway is independent of this kinase. We are currently analyzing the cAMP targets implicated in granule swelling. We propose the following cascade of events that result in acrosomal exocytosis (Fig. 5). Progesterone activates a CatSper-dependent calcium signal in the principal piece of the flagellum that promotes a calcium-induced calcium release at the neck of the sperm that opens SOC channels [34;37]. Progesterone can also induce the production of diacylgycerol that activates TRP channels at the plasma membrane. The increase of cytosolic calcium boosts the synthesis of cAMP that activates three processes: i) acrosomal swelling (this report), ii) assembly of the membrane fusion machinery [11], and iii) activation of IP_3 production [13;39]. Under these conditions, SNARE complexes can be assembled in trans, "docking" the edge of outer acrosomal membrane invaginations to the plasma membrane. IP_3 opens IP_3-sensitive channels at the acrosomal membrane, causing a local increase of cytosolic calcium that activates synaptotagmin and the downstream events leading to the opening of fusion pores [15]. It is worth noticing that the observations from our group reported here and in previous papers support the model shown in Fig. 5 downstream the opening of SOC

channels. More experiments will be necessary to characterize the connection between the signal cascade triggered by progesterone and acrosomal swelling.

The role of the soluble isoform of adenylyl cyclase in acrosomal exocytosis is controversial. The enzyme participates in the acrosome reaction in sea urchin [40] and human sperm [32]. However, the $sAC^{-/-}$ mouse has a normal ZP- and A23187- stimulated acrosomal exocytosis [41]. A recent study implicates sAC in PKA-activation in the tail of mouse sperm and tmACs in the acrosome reaction by promoting an increase of cytosolic calcium [42]. We have previously shown that in human sperm, KH7 inhibits acrosomal exocytosis stimulated by A23187 and progesterone, but not by 8-pCPT [32]. Moreover, KH7 blocks exocytosis in permeabilized sperm stimulated with calcium, suggesting that sAC is playing a role downstream an increase in cytosolic calcium. KH7 is a very specific inhibitor of sAC [43], although, other effects due to some unknown target for this reagent cannot be ruled out. In the present report, we confirmed by electron microscopy that KH7 inhibits acrosomal exocytosis in human sperm stimulated by progesterone. Swelling was also blocked, consistent with a pivotal role of cAMP in this process. The reason for the discrepancies between human and mouse sperm awaits further characterization of the role of the different adenylyl cyclase isoforms in sperm physiology.

Fusion pore opening and expansion in most secretory processes lead to the release of the granule content and incorporation of its membrane into the plasma membrane. In contrast, in mammalian sperm, pore opening and expansion cause the vesiculation of the acrosome. We propose that acrosomal swelling and outer membrane deformation are important to delineate the membrane domains where pore expansion will lead to the release of hybrid vesicles. More experimental work will be necessary to understand the molecular mechanisms causing the remarkable deformation of the acrosomal granule occurring during the acrosome reaction.

ACKNOWLEDGMENTS

The authors thank M. Furlán and A. Medero for excellent technical assistance, and C. Tomes, S. Belmonte, and G. De Blas for critically reading the manuscript.

REFERENCES

[1] Yanagimachi R. Mammalian Fertilization. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2 ed. New York: Raven Press; 1994: 189-281.

[2] Ikawa M, Inoue N, Benham AM, Okabe M. Fertilization: a sperm's journey to and interaction with the oocyte. J Clin Invest 2010; 120: 984-994.

[3] Brenker C, Goodwin N, Weyand I, Kashikar ND, Naruse M, Krahling M, Muller A, Kaupp UB, Strunker T. The CatSper channel: a polymodal chemosensor in human sperm. EMBO J 2012; 31: 1654-1665.

[4] Alasmari W, Costello S, Correia J, Oxenham SK, Morris J, Fernandes L, Ramalho-Santos J, Kirkman-Brown J, Michelangeli F, Publicover S, Barratt CL. Ca^{2+} signals generated by CatSper and Ca^{2+} stores regulate different behaviors in human sperm. J Biol Chem 2013; 288: 6248-6258.

[5] Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt C, Kirkman-Brown J, Publicover S. Ca^{2+}-stores in sperm: their identities and functions. Reproduction 2009; 138: 425-437.

[6] Darszon A, Nishigaki T, Beltran C, Trevino CL. Calcium channels in the development,

maturation, and function of spermatozoa. Physiol Rev 2011; 91: 1305-1355.
[7] Darszon A, Sanchez-Cardenas C, Orta G, Sanchez-Tusie AA, Beltran C, Lopez-Gonzalez I, Granados-Gonzalez G, Trevino CL. Are TRP channels involved in sperm development and function? Cell Tissue Res 2012; 349: 749-764.
[8] Lefievre L, Nash K, Mansell S, Costello S, Punt E, Correia J, Morris J, Kirkman-Brown J, Wilson SM, Barratt CL, Publicover S. 2-APB-potentiated channels amplify CatSper-induced Ca^{2+} signals in human sperm. Biochem J 2012; 448: 189-200.
[9] Sanchez-Cardenas C, Servin-Vences MR, Jose O, Trevino CL, Hernandez-Cruz A, Darszon A. Acrosome reaction and Ca^{2+} imaging in single human spermatozoa: new regulatory roles of $[Ca^{2+}]$I. Biol Reprod 2014; 91: 67.
[10] Mayorga LS, Tomes CN, Belmonte SA. Acrosomal exocytosis, a special type of regulated secretion. IUBMB Life 2007; 59: 286-292.
[11] Tomes CN. The proteins of exocytosis: lessons from the sperm model. Biochem J 2015; 465: 359-370.
[12] Bustos MA, Lucchesi O, Ruete MC, Mayorga LS, Tomes CN. Small GTPases in acrosomal exocytosis. Methods Mol Biol 2015; 1298: 141-160.
[13] Ruete MC, Lucchesi O, Bustos MA, Tomes CN. Epac, Rap and Rab3 act in concert to mobilize calcium from sperm's acrosome during exocytosis. Cell Commun Signal 2014; 12: 43.
[14] De Blas GA, Roggero CM, Tomes C, Mayorga LS. Dynamics of SNARE assembly and disassembly during sperm acrosomal exocytosis. PLoS Biology 2005; 3: e323.
[15] Roggero CM, De Blas GA, Dai H, Tomes CN, Rizo J, Mayorga LS. Complexin/synaptotagmin interplay controls acrosomal exocytosis. J Biol Chem 2007; 282: 26335-26343.
[16] Yudin AI, Gottlieb W, Meizel S. Ultrastructural studies of the early events of the human sperm acrosome reaction as initiated by human follicular fluid. Gamete Res 1988; 20: 11-24.
[17] Flechon JE, Harrison RA, Flechon B, Escaig J. Membrane fusion events in the Ca^{2+}/ionophore-induced acrosome reaction of ram spermatozoa. J Cell Sci 1986; 81: 43-63.
[18] Green DP. The osmotic properties of the acrosome of guinea-pig sperm. J Cell Sci 1978; 32: 165-176.
[19] Franklin LE, Barros C, Fussell EN. The acrosomal region and the acrosome reaction in sperm of the golden hamster. Biol Reprod 1970; 3: 180-200.
[20] Barros C, Bedford JM, Franklin LE, Austin CR. Membrane vesiculation as a feature of the mammalian acrosome reaction. J Cell Biol 1967; 34: C1-C5.
[21] Russell L, Peterson RN, Freund M. Morphologic characteristics of the chemically induced acrosome reaction in human spermatozoa. Fertil Steril 1979; 32: 87-92.
[22] Zanetti N, Mayorga LS. Acrosomal swelling and membrane docking are required for hybrid vesicle formation during the human sperm acrosome reaction. Biol Reprod 2009; 81: 396-405.
[23] Pocognoni CA, Berberián MV, Mayorga LS. ESCRT (Endosomal Sorting Complex Required for Transport) machinery is essential for acrosomal exocytosis in human sperm. Biol Reprod 2015; 93:124.
[24] Sosa CM, Pavarotti MA, Zanetti MN, Zoppino FC, De Blas GA, Mayorga LS. Kinetics of human sperm acrosomal exocytosis. Mol Hum Reprod 2015; 21: 244-254.
[25] Lopez CI, Belmonte SA, De Blas GA, Mayorga LS. Membrane-permeant Rab3A triggers acrosomal exocytosis in living human sperm. FASEB J 2007; 21: 4121-4130.
[26] Mendoza C, Carreras A, Moos J, Tesarik J. Distinction between true acrosome reaction

and degenerative acrosome loss by a one-step staining method using Pisum sativum agglutinin. J Reprod Fertil 1992; 95: 755-763.

[27] De Blas G, Michaut M, Trevino CL, Tomes CN, Yunes R, Darszon A, Mayorga LS. The intraacrosomal calcium pool plays a direct role in acrosomal exocytosis. J Biol Chem 2002; 277: 49326-49331.

[28] Harper C, Wootton L, Michelangeli F, Lefievre L, Barratt C, Publicover S. Secretory pathway Ca^{2+}-ATPase (SPCA1) Ca^{2+} pumps, not SERCAs, regulate complex $[Ca^{2+}](i)$ signals in human spermatozoa. J Cell Sci 2005; 118: 1673-1685.

[29] Rossato M, Di Virgilio F, Rizzuto R, Galeazzi C, Foresta C. Intracellular calcium store depletion and acrosome reaction in human spermatozoa: role of calcium and plasma membrane potential. Mol Hum Reprod 2001; 7: 119-128.

[30] O'Toole CM, Arnoult C, Darszon A, Steinhardt RA, Florman HM. Ca^{2+} entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. Mol Biol Cell 2000; 11: 1571-1584.

[31] Meizel S, Turner KO. Initiation of the human sperm acrosome reaction by thapsigargin. J Exp Zool 1993; 267: 350-355.

[32] Branham MT, Bustos MA, De Blas GA, Rehmann H, Zarelli VE, Trevino CL, Darszon A, Mayorga LS, Tomes CN. Epac activates the small G proteins Rap1 and Rab3A to achieve exocytosis. J Biol Chem 2009; 284: 24825-24839.

[33] Branham MT, Mayorga LS, Tomes CN. Calcium-induced acrosomal exocytosis requires cAMP acting through a protein kinase A-independent, Epac-mediated pathway. J Biol Chem 2006; 281: 8656-8666.

[34] Correia J, Michelangeli F, Publicover S. Regulation and roles of Ca^{2+} stores in human sperm. Reproduction 2015; 138: 425-437.

[35] Roberts-Thomson SJ, Peters AA, Grice DM, Monteith GR. ORAI-mediated calcium entry: mechanism and roles, diseases and pharmacology. Pharmacol Ther 2010; 127: 121-130.

[36] Tamburrino L, Marchiani S, Minetti F, Forti G, Muratori M, Baldi E. The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. Hum Reprod 2014; 29: 418-428.

[37] Morris J, Jones S, Howl J, Lukanowska M, Lefievre L, Publicover S. Cell-penetrating peptides, targeting the regulation of store-operated channels, slow decay of the progesterone-induced $[Ca^{2+}]i$ signal in human sperm. Mol Hum Reprod 2015; 21: 563-570.

[38] Christensen AE, Selheim F, de RJ, Dremier S, Schwede F, Dao KK, Martinez A, Maenhaut C, Bos JL, Genieser HG, Doskeland SO. cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. J Biol Chem 2003; 278: 35394-35402.

[39] Pelletan LE, Suhaiman L, Vaquer CC, Bustos MA, De Blas GA, Vitale N, Mayorga LS, Belmonte SA. ADP ribosylation factor 6 (ARF6) promotes acrosomal exocytosis by modulating lipid turnover and Rab3A activation. J Biol Chem 2015; 290: 9823-9841.

[40] Beltran C, Vacquier VD, Moy G, Chen Y, Buck J, Levin LR, Darszon A. Particulate and soluble adenylyl cyclases participate in the sperm acrosome reaction. Biochem Biophys Res Commun 2007; 358: 1128-1135.

[41] Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M, Miyamoto C, Zippin JH, Kopf GS, Suarez SS, Levin LR, Williams CJ, Buck J, Moss SB. The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. Dev Cell 2005; 9: 249-259.

[42] Wertheimer E, Krapf D, de la Vega-Beltran JL, Sanchez-Cardenas C, Navarrete F, Haddad D, Escoffier J, Salicioni AM, Levin LR, Buck J, Mager J, Darszon A, Visconti PE. Compartmentalization of distinct cAMP signaling pathways in Mammalian sperm. J Biol Chem 2013; 288: 35307-35320.

[43] Bitterman JL, Ramos-Espiritu L, Diaz A, Levin LR, Buck J. Pharmacological distinction between soluble and transmembrane adenylyl cyclases. J Pharmacol Exp Ther 2013; 347: 589-598.

FIGURE LEGENDS

Figure 1: Kinetics of acrosomal swelling after stimulation with A23187 and progesterone (Pg). **A**, **B**) Capacitated sperm were stimulated with 10 µM A23187 (**A**) or 15 µM Pg (**B**) and incubated for different periods of time before glutaraldehyde fixation. The samples were processed for electron microscopy and the percentage of sperm with intact acrosomes (black circles), swollen acrosomes (gray squares) and reacted sperm (dark gray rhomboids), was quantified for each condition (at least 200 cells per sample were analyzed). The data represent the mean ± range of two experiments. **C**) Sperm were incubated in non-capacitating (modified HTF without BSA) or capacitating medium (HTF with BSA) during (1 h) and after the swim-up (3 hs). Cells were stimulated with 15 µM progesterone for 15 min at 37°C (Pg). As control, some aliquots were incubated without the hormone (control). Data represent the percentage of sperm with swollen acrosomes (mean ± SEM) in 3 independent experiments. The means were compared with the corresponding control condition by Student's t test (ns, not significantly different; **, $P<0.01$). **D-G**) Electronmicrographs illustrating the morphology of unstimulated sperm (D) or after different times of stimulation with A23187 (E) or Pg (F and G). i, intact acrosome; **s,** swollen acrosome; r, reacted sperm. Bars = 1 µm.

Figure 2: Several reagents that stimulate acrosomal exocytosis by different mechanisms induce swelling. **A**) Capacitated sperm were incubated for 15 min at 37°C in the absence of stimulus (control) or stimulated with 10 µM A23187 (A23187), 15 µM progesterone (Pg), 1 µM thapsigargin (Tg), 1 mM db-cAMP (db-cAMP), 50 µM 8-pCPT (8-pCPT), or 1.5 µM membrane permeant Rab3A (R-Rab3A) in the presence of 1 µM XC (to prevent acrosomal exocytosis). Cells were fixed and processed for electron microscopy. For each condition, at least 200 acrosomes were classified as intact (morphology illustrated in **C**) or swollen (morphology illustrated in **D** and **E**). Data represent the percentage of sperm with swollen acrosomes (mean ± SEM) in independent experiments (n=20 for A23187, n=15 for Pg, n=7 for Tg, n=5 for db-cAMP, n=8 for 8-pCPT, n=3 for R-Rab3A). **B**) Sperm treated as in A (control or stimulated with 10 µM A23187, 15 µM Pg, or 1 µM Tg) were classified as having intact acrosomes (intact; illustrated in **C**), swollen acrosomes without deformations of the outer membrane (swollen, illustrated in **D**), acrosomes with a waiving outer acrosomal membrane and internal vesicles (waving with vesicles, illustrated in **E**), or without acrosome (reacted, illustrated in **F**). Data represent the mean ± SEM (n=7). Bars = 500 nm. In **A** and **B**, the means were compared with the control condition by one-way ANOVA and Dunnett posthoc test (ns, not significantly different; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Figure 3: Swelling occurs downstream the opening of SOC channels. **A**) Scheme representing two hypotheses. Black arrows: swelling promotes apposition of the outer acrosomal and plasma membranes at the edge of invaginations (white arrowheads); these close contacts stimulate

STIM/Orai-mediated calcium entry, which in turn triggers membrane fusion and exocytosis. White arrows: calcium entry through SOC channels (SOCE) initiates acrosomal swelling that promotes membrane apposition at the edge of invaginations (white arrowheads). The fusion machinery is assembled in these areas. Calcium release from the acrosome triggers membrane fusion and exocytosis. **B-D**) Capacitated sperm were incubated for 15 min at 37°C in the absence of stimulus (control) or stimulated with 1 µM thapsigargin, 15 µM progesterone, or 10 µM A23187, in the presence of 1 µM XC (to prevent exocytosis without affecting acrosomal swelling), 5 mM BAPTA (to chelate calcium from the extracellular medium), or two specific SOC channels inhibitors (1 µM YM or 50 µM SKF). Cells were fixed and processed for electron microscopy. In **B** to **D**, at least 100 acrosomes were classified as intact or swollen for each condition. Bars represent the percentage of swollen acrosomes (mean ± SEM, n=3-5). The means were compared with the control condition by one-way ANOVA and Dunnett posthoc test (ns, not significantly different; **, $P<0.01$; ***, $P<0.001$).

Figure 4: cAMP analogs induce acrosomal swelling in the absence of calcium. **A**) Capacitated spermatozoa were incubated with 20 µM BAPTA-AM and, when indicated, 5 mM BAPTA for 15 min at 37°C to chelate intra- and extra-cellular calcium, respectively. Cells were then stimulated with 1 mM db-cAMP, or 50 µM 8-pCPT, for another 15 min at 37°C, and finally fixed and processed for electron microscopy. **B**) Capacitated sperm were incubated for 15 min at 37°C with 20 µM BAPTA-AM (to chelate calcium in the cytosol and intracellular stores to prevent exocytosis), or 10 µM KH7 (to inhibit soluble adenylyl cyclase). Afterwards, cells were incubated with or without 15 µM progesterone (Pg) for 15 min at 37°C and finally fixed and processed for electron microscopy. In **A** and **B**, at least 150 acrosomes were classified as intact or swollen for each condition. Data represent the percentage of swollen acrosomes (mean ± range, n=2). The means were compared with BAPTA/BAPTA-AM (**A**), or BAPTA-AM (**B**) by one-way ANOVA and Dunnett posthoc test (ns, not significantly different; **, $P<0.01$; ***, $P<0.001$). **C**) Sperm were incubated under non-capacitating (modified HTF without BSA) or capacitating (HTF with BSA) conditions during (1 h) and after the swim-up (3 hs). Cells were stimulated with 10 µM A23187 (A23187) or 50 µM 8-pCPT (8-pCPT) for 30 min at 37°C. When indicated, the cells were preincubated with 1 µM NNC55-0396 (NNC) for 15 min at 37°C before adding 8-pCPT (NNC + 8-pCPT). As control, some aliquots were incubated without stimulation (control). **D**) Capacitated sperm were preincubated with 1 µM NNC55-0396 for 15 min at 37°C and then stimulated with 15 µM progesterone (NNC + Pg) for 15 min at 37°C. As control, some aliquots were incubated without any addition (control), without progesterone (NNC) or without the inhibitor (Pg). Acrosomal exocytosis was evaluated using FITC-PSL. In C and D, data represent the percentage of sperm (mean ± SEM) with swollen acrosomes (**C**) or reacted sperm (**D**) in 3 independent experiments. The means were compared with the corresponding control condition by one-way ANOVA and Dunnett posthoc test (ns, not significantly different; **, $P<0.01$). **E-H**) Electronmicrographs showing the morphology of sperm incubated under different conditions explained in **A** and **B**. i, intact acrosome; i*, deformed but not-swollen acrosome; s, swollen acrosome without invaginations of the outer membrane; wv, acrosome with a waiving outer acrosomal membrane and internal vesicles; r, reacted sperm. Bars = 1 µm.

Figure 5: Working model for acrosomal swelling and exocytosis. As proposed in Morris et al. [37] progesterone activates a CatSper-dependent calcium signal that promotes a calcium-induced

calcium release (CICR) at the neck of the sperm that opens SOC channels (SOCC). Progesterone can also activate the production of diacylgycerol (DAG) that can impact on TRP channels at the plasma membrane [7]. Calcium boosts the synthesis of cAMP that activates three processes: i) acrosomal swelling (this report), ii) assembly of the membrane fusion machinery [11], and iii) activation of IP_3 production [13;39]. Under these conditions, SNARE complexes can be assembled in trans, “docking” the edge of outer acrosomal membrane invaginations to the plasma membrane [22]. The IP_3 produced opens IP_3-sensitive channels (IP_3R) at the acrosomal membrane, causing a local increase of cytosolic calcium that activates synaptotagmin and the downstream events leading to the opening of fusion pores [15]. The observations from our group reported here and in previous papers support the model downstream the opening of SOC channels (brown horizontal bracket).

FIGURE 1

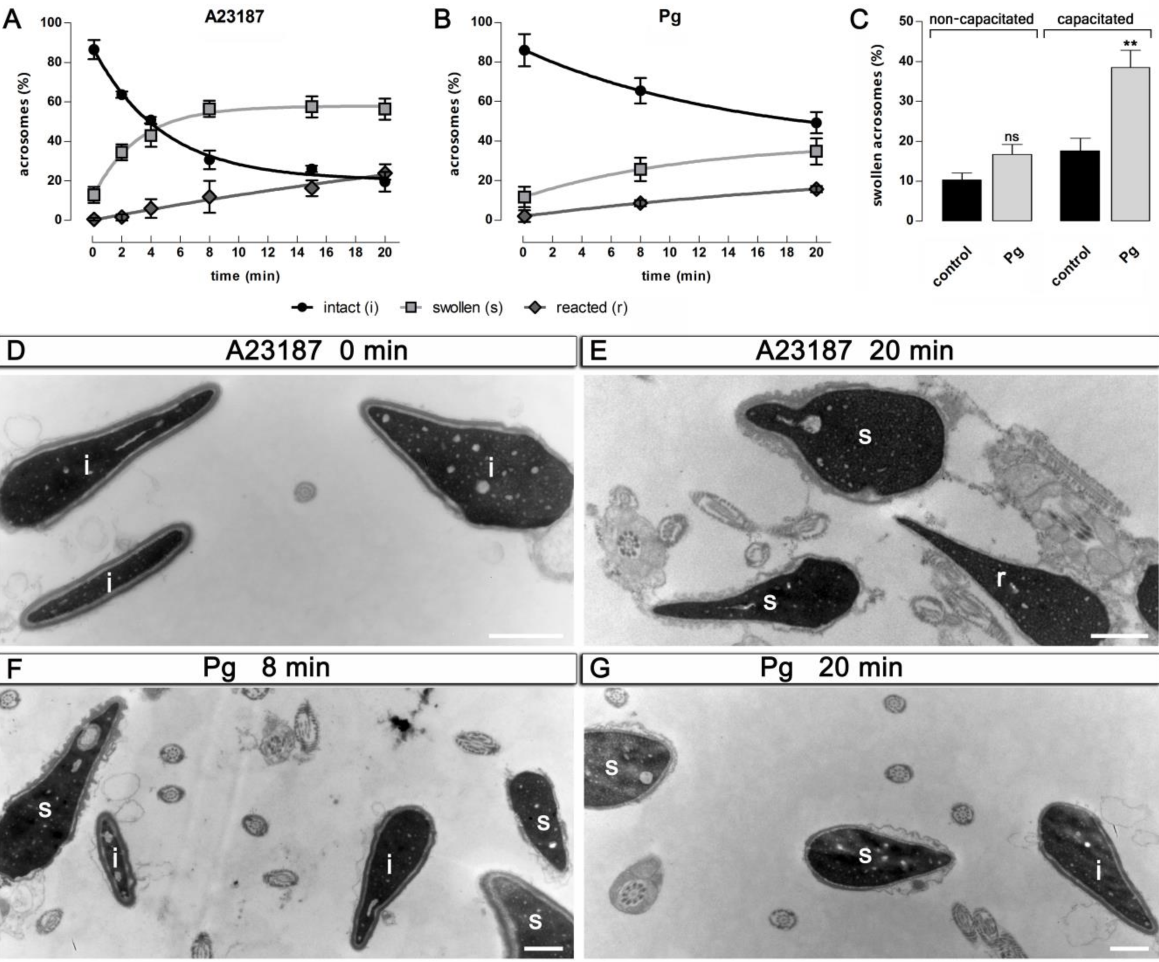


FIGURE 2

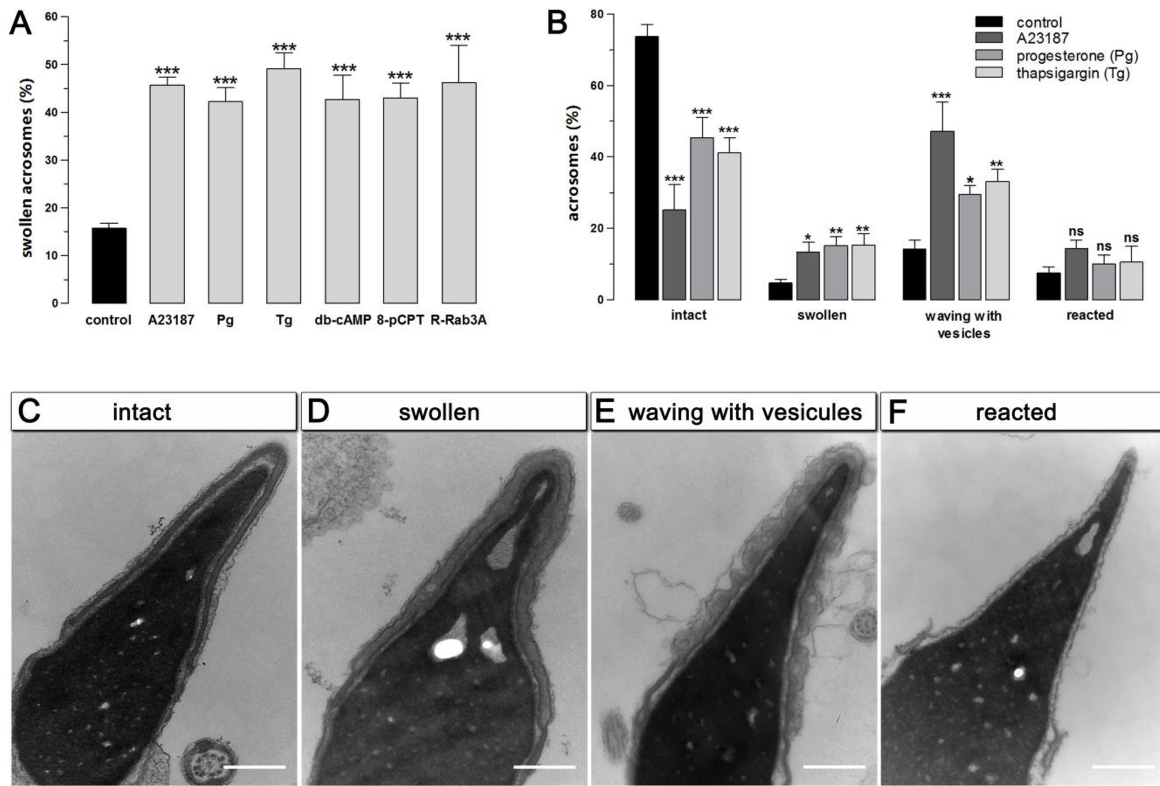


FIGURE 3

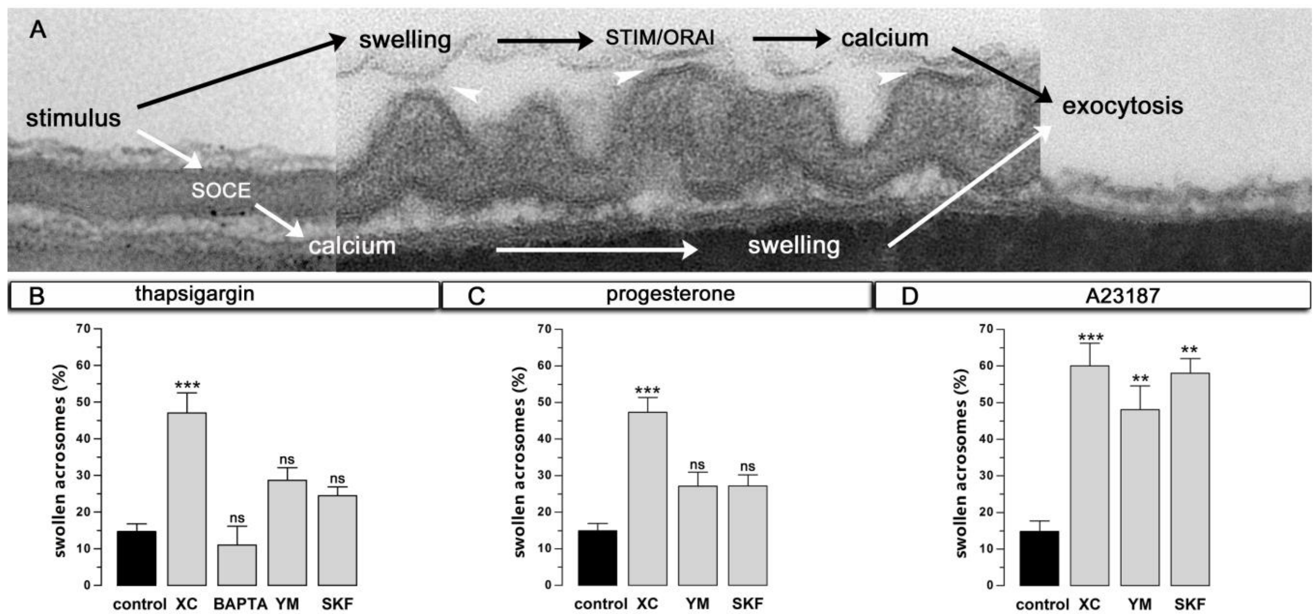
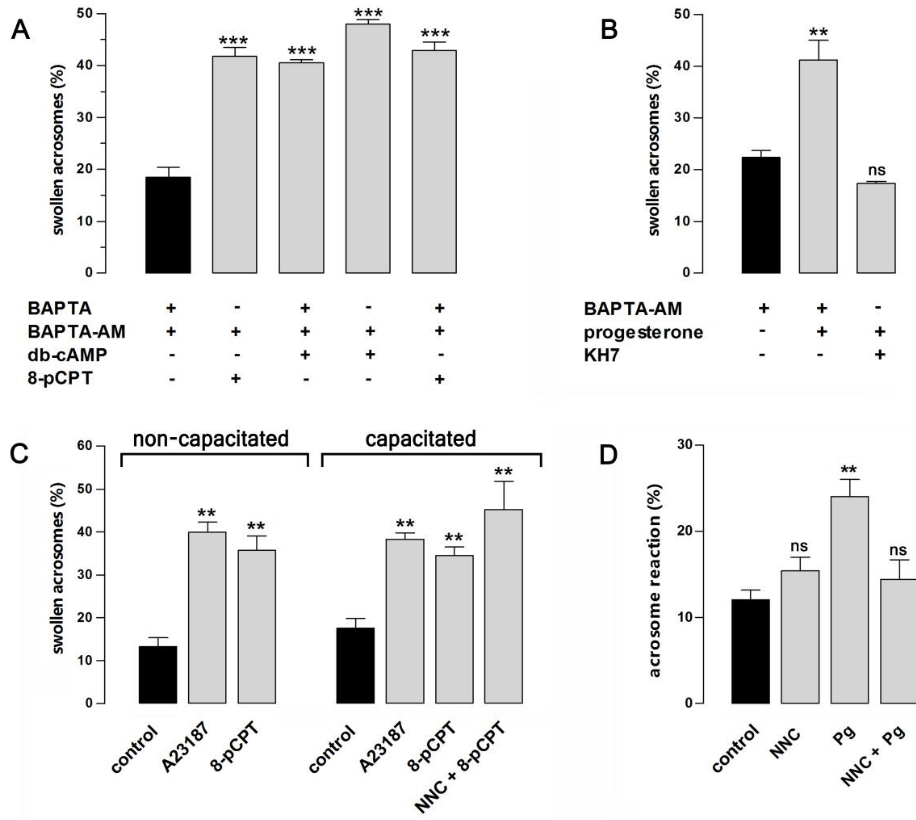


FIGURE 4



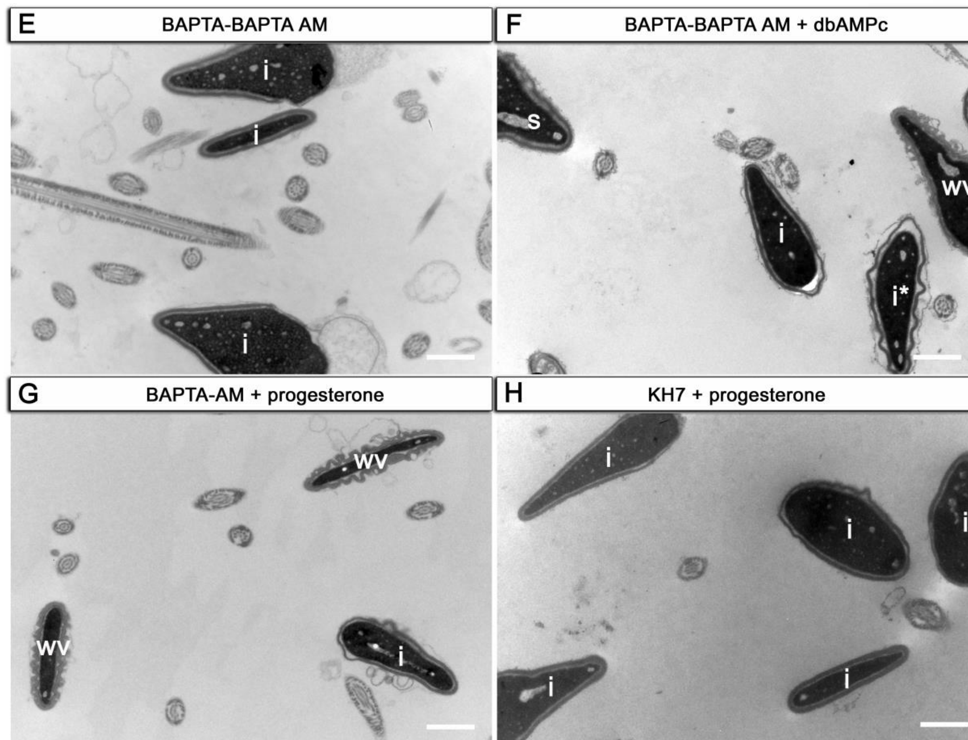


FIGURE 5