Calcium-induced Acrosomal Exocytosis Requires cAMP Acting through a Protein Kinase A-independent, Epac-mediated Pathway

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Epac, a guanine nucleotide exchange factor for the small GTPase Rap, binds to and is activated by the second messenger cAMP. In sperm, there are a number of signaling pathways required to achieve egg-fertilizing ability that depend upon an intracellular rise of cAMP. Most of these processes were thought to be mediated by cAMP-dependent protein kinases. Here we report a new dependence for the cAMP-induced acrosome reaction involving Epac. The acrosome reaction is a specialized type of regulated exocytosis leading to a massive fusion between the outer acrosomal and the plasma membranes of sperm cells. Ca\(^{2+}\) is the archetypal trigger of regulated exocytosis, and we show here that its effects on acrosomal exocytosis is fully mediated by cAMP. Ca\(^{2+}\) failed to trigger acrosomal exocytosis when intracellular cAMP was depleted by an exogenously added phosphodiesterase or when Epac was sequestered by specific blocking antibodies. The nondiscriminating dibutyryl-cAMP and the Epac-selective 8-(p-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate analogues triggered the acrosome reaction in the effective absence of extracellular Ca\(^{2+}\). This indicates that cAMP, via Epac activation, has the ability to drive the whole cascade of events necessary to bring exocytosis to completion, including tethering and docking of the acrosome to the plasma membrane, priming of the fusion machinery, mobilization of intravesicular Ca\(^{2+}\), and ultimately, bilayer mixing and fusion.

CAMP-elicited exocytosis was sensitive to anti-α-SNAP, anti-NSF, and anti-Rab3A antibodies, to intra-acrosomal Ca\(^{2+}\) chelators, and to botulinum toxins but was resistant to cAMP-dependent protein kinase blockers. These experiments thus identify Epac in human sperm and evince its indispensable role downstream of Ca\(^{2+}\) in exocytosis.

The ubiquitous second messenger cAMP regulates a large variety of crucial physiological processes, such as cell metabolism, division, growth and differentiation, secretion, memory, and neoplastic transformation. Exocytosis in several tissues is modulated by cAMP, including the release of insulin from pancreatic β-cells (1, 2), of the nonamyloidogenic soluble form of amyloid precursor protein from neurons (3), and of amylase from parotid and pancreatic acinar cells (4). In sperm, various activation pathways required to achieve egg-fertilizing ability depend on the intracellular rise of cAMP. These include changes in motility (5), capacitation (6–9), and the acrosome reaction (AR) (10, 11). The AR is a secretory event that is completed by sperm of many species at an early stage of fertilization, when sperm contact glycoproteins of the egg’s extracellular matrix, or zona pellucida (ZP) (12). Ca\(^{2+}\) is an essential mediator of the AR. Two phases of ZP-evoked Ca\(^{2+}\) responses have been described, a first, transient phase mediated by voltage-gated channels and a second, sustained phase in which Ca\(^{2+}\) permeates into the sperm cytosol through store depletion-activated channels (SOC) (13). In human sperm, the acrosome behaves as the internal store of releasable Ca\(^{2+}\) (14, 15). Ca\(^{2+}\) is released from the acrosome in two phases. The first precedes (and drives) the opening of SOC channels on the plasma membrane, whereas the second follows this opening, taking place later in the fusion cascade, once the biochemical machinery for fusion has been assembled (14, 16). In an attempt to refine our understanding of the cAMP-dependent signaling pathways during the AR, we resorted to a plasma membrane streptolysin O (SLO) permeabilization protocol developed in our laboratory (17). Incubating permeabilized sperm with Ca\(^{2+}\) resembles the physiological situation of Ca\(^{2+}\) influx through open SOC channels. Thus, ours constitutes a particularly attractive system to examine relatively late steps of the exocytic cascade, occurring after the sustained Ca\(^{2+}\) influx, while bypassing earlier pathways whose end point is the opening of SOC channels.

In mammalian cells, cAMP is synthesized by a family of nine transmembrane and one soluble adenylyl cyclase (18–20). The latter, known as sAC, appears to be the predominant form of adenylyl cyclase in sperm. Its direct activation by bicarbonate is thought to be responsible for the cAMP-induced changes in motility and AR mentioned above (21–23). sAC defines a novel means for generating cAMP, implying that the second messenger can be generated at a distance from the membrane, closer to its required site of action, and circumventing the need for diffusion to reach distant targets. Models whereby cAMP can signal in a complex consisting of both sAC and effectors have been proposed.

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2 The abbreviations used are: AR, acrosome reaction; ZP, zona pellucida; 2-APB, 2-aminoethoxydiphenylborate; BoNT/E, botulinum toxin E; Br- cAMP, dibutyryl cyclic AMP; CPA, cyclopiazonic acid; IBMX, isobutylmethylxanthine; PDE, phosphodiesterase; PKA, protein kinase A; PRI, peptide inhibitor of PKA; Rp-cAMPS, Rp-isomer of cyclic adenosine 3',5'-phosphorothioate; sAC, soluble adenylyl cyclase; SOC, store depletion-activated channels; SLO, streptolysin O; ZP, zona pellucida; 8-pCPT-cGMP, 8-(p-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate; 8-pCPT-2'-O-Me-cAMP, 8-(p-chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate; TRITC, tetramethylrhodamine isothiocyanate; NP-EGTA-AM, N,N',N'-tetraacetic acid acetoxyethyl ester; GPM, gamete preparation medium; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PVP, polyvinylpyrrolidone; PSA, Pseudotaxum sativum agglutinin; NSF, N-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.
The best characterized cAMP effectors include cAMP-dependent protein kinase (PKA), cyclic nucleotide gated channels, guanine nucleotide exchange factors, and the transcription factor cAMP-response element-binding protein (26).

For many years, major intracellular effects of cAMP were believed to be mediated by PKA. PKA is composed of two separate polypeptides, the catalytic (C) and regulatory (R) subunits that interact to form an inactive tetrameric holoenzyme (R<sub>C</sub>). PKA activation is achieved by binding of four molecules of cAMP to the R subunits, which induces a conformational change in the R subunits and leads to the dissociation of the holoenzyme into two free, catalytically active, C subunits and an R subunit homodimer (27, 28). PKA has been described in spermatozoa from several mammalian species. Furthermore, it has been implicated in sperm motility and capacitation through still undefined signaling cascades that culminate in enhanced protein tyrosine phosphorylation. Inhibitor studies have implicated PKA in the ZP-triggered AR of human sperm. It has been proposed that activation by PKA-dependent phosphorylation of Ca<sup>2+</sup> channels on the outer acrosomal membrane leads to an increase in cytosolic Ca<sup>2+</sup> and, consequently, to the AR (11).

The second well known targets for cAMP are the cyclic nucleotide-gated channels. These channels are directly opened by either cAMP of cGMP and are permeable to Ca<sup>2+</sup> ions. They form heterooligomeric complexes composed of at least two distinct subunits (α and β) (31). Both subunits have been found in the flagellum of mammalian sperm and implicated in motility (32).

The most recently described cAMP effector is Epac (exchange protein directly activated by cAMP). Epac is a Rap-specific guanine nucleotide exchange factor that is activated by the binding of cAMP to a cyclic nucleotide monophosphate-binding domain (33, 34). Two isoforms, Epac1 and Epac2, were described in mammalian cells, both containing a regulatory and a catalytic region in the N- and C-terminal portions of the protein, respectively. The regulatory domain contains the cAMP binding site, which autoinhibits the catalytic activity in the absence of cAMP (35). Evidence suggesting a crucial role for Epac in exocytosis first arose from the lack of effect of specific blockers of PKA- and cyclic nucleotide-gated channels on this process (1, 3, 36–39).

In light of the recently described multiplicity of proteins with which cAMP interacts, functions previously ascribed solely to PKA may need reevaluation. Specifically, we were interested in the possibility of an Epac-mediated, cAMP-dependent signaling pathway in the AR. Here we report a requirement for Epac in Ca<sup>2+</sup>-induced acrosomal release in human sperm. Furthermore, we demonstrate that the activation of Epac alone by a specific cAMP analogue is sufficient to achieve maximum exocytosis levels in intact and SLO-permeabilized cells. This exocytosis relies on the bona fide machinery required for fusion in all secretory cells. Epac functions in a relatively early step during the exocytosis cascade, prior to tethering by Rab3, priming by NSF/α-SNAP, docking by SNAREs, and intra-acrosomal Ca<sup>2+</sup> release.

**Experimental Procedures**

Reagents—SLO was obtained from Corgenix (Peterborough, UK). Specific rabbit polyclonal antibodies against Epac were generated by Genemed Synthesis, Inc. (San Francisco, CA) using the synthetic peptide LREDNCHFLRVDK (molecular mass 1644.8) and affinity-purified on immobilized Epac peptide. This peptide, spanning residues 285–297 in human Epac1 (33) and 438–450 in human Epac2 (40), is identical in both isoforms and corresponds to an unconserved region within the cAMP binding domain of Epac1 and the cAMP binding domain B of Epac2, located within the phosphate-binding cassette (41). The sequence is unique to Epac and bears no homology with any other mammalian proteins whose amino acid sequences are deposited in public data bases. Mouse polyclonal antibodies to recombinant α-SNAP were previously described (42). A rabbit polyclonal anti-Rab3A (purified IgG) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and a rabbit polyclonal anti-NSF (whole serum) was from Synaptic Systems (Göttingen, Germany). Horseradish peroxidase-conjugated goat anti-rabbit-IgG (Fc fragment-specific) was from Jackson ImmunoResearch (West Grove, PA). TRITC-conjugated goat anti-rabbit-IgG was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). In vitro translated, human recombinant, cAMP-specific phosphodiesterase 4D (PDE) was from Abnova Corp. (NeiHtu, Taipei, Taiwan). 8-pCPT-2′-O-Me-cAMP and 8-pCPT-cGMP were from Biolog-Life Science Institute (Bremen, Germany). Nickel-nitrotriacetic acid-agarose was from Qiagen Research Products (San Diego, CA). O-Nitrophenyl EGTA acetyoxymethyl ester (NP-EGTA-AM) and BAPTA-AM were from Molecular Probes, Inc. (Eugene, OR). Cyclopiazonic acid (CPLA), the Rp-isomer of cyclic adenosine 3′,5′-phosphorothioate (Rp-cAMPS), and 2-aminoethoxydiphenylborate (2-APB) were from Calbiochem. 3-Isobutyl-1-methylxanthine (IBMX) was from Research Biochemicals International (Natick, MA). H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoxquinolinesulfonylamine was from LC Laboratories (Woburn, MA). All other chemicals were reagent or analytical grade and were purchased from Sigma or from ICN Biochemicals, Inc. (Aurora, OH).

**Recombinant Proteins**—A plasmid encoding the light chain of botulinum toxin E (His<sub>6</sub>-BoNT/E-LC) in pQE9 was generously provided by Dr. T. Binz (Medizinische Hochschule Hannover, Hannover, Germany). The DNA was transformed into Escherichia coli XL1-Blue (Stratagene, La Jolla, CA), and protein expression was induced overnight at 20 °C with 0.2 mM isopropyl-β-D-thiogalactopyranoside. A plasmid encoding His<sub>6</sub>-NSF in pQE9 (Qiagen) was a kind gift from Dr. S. Whiteheart (University of Kentucky, Lexington, KY). This construct was transformed into E. coli M15pRep4 (Qiagen), and protein expression was induced for 4 h at 30 °C with 1 mM isopropyl-β-D-thiogalactoside. Purification of recombinant proteins was accomplished according to The QIAexpressionist (available on the World Wide Web at www.qiagen.com), except that 0.5 mM ATP, 5 mM MgCl<sub>2</sub>, and 2 mM dithiothreitol were added to all buffers involved in the purification of His<sub>6</sub>-NSF. The expression plasmid pGEX2T containing the cDNA-encoding human Rab3A was generously provided by Drs. M. Colombo and P. Stahl (Washington University, St. Louis, MO). Glutathione S-transferase-Rab3A was expressed in E. coli strain XL1-Blue (Stratagene) and purified on glutathione-Sepharose following the manufacturer’s instructions.

**SLO Permeabilization and AR Assay**—After at least 2 days of abstinence, semen samples were provided by masturbation from healthy volunteer donors who were free from sexually transmitted diseases. Semen was allowed to liquefy for 30–60 min at 37 °C. Highly motile sperm were recovered by swim-up separation for 1 h in gamete preparation medium (GPM; Serono, Aubonne, Switzerland) at 37 °C in an atmosphere of 5% CO<sub>2</sub>, 95% air. The composition of GPM is based on Earle’s balanced salt solution (0.2 g/liter Ca<sub>Cl</sub><sub>2</sub>, 0.4 g/liter KCl, 0.097 g/liter MgSO<sub>4</sub>, 6.8 g/liter NaCl, 2.2 g/liter NaHCO<sub>3</sub>, 0.14 g/liter NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 1 g/liter d-glucose, 0.01 g/liter phenol red) supplemented with 0.1 g/liter sodium pyruvate and 1 mg/ml human serum albumin. GPM contains no antibiotics. The pH and osmolality were maintained within the ranges 7.2–7.4 and 278–288 mosm, respectively. After swim up, sperm concentration was adjusted to 5–10 × 10<sup>6</sup>/ml, and the cells were incubated for at least 2 h under conditions that support capacitation (GPM; 37 °C, 5% CO<sub>2</sub>, 95% air). Permeabilization was
accomplished as previously described (17). Briefly, washed spermatozoa were resuspended in cold PBS containing 0.4 units/ml SLO for 15 min at 4 °C. Cells were washed once with PBS, resuspended in ice-cold sucrose buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-K, pH 7.0) containing 2 mM dithiothreitol, inhibitors were added when indicated, and cells were further incubated for 15 min at 37 °C. After the addition of stimulants to the sperm suspensions, incubation proceeded at 37 °C for 15 min. For the experiments with the photoinhibitable Ca\(^{2+}\) chelator NP-EGTA-AM, SLO-permeabilized sperm were preloaded with 10 µM NP-EGTA-AM before incubating in the presence of inhibitors and stimulants as described, except that all procedures were carried out in the dark (supplemental Fig. S1A). Alternatively, NP-EGTA-AM preloaded sperm were incubated first with 0.5 mM CaCl\(_2\) and second with the inhibitors to test, also in the dark (supplemental Fig. S1B). In all cases, photolysis of the chelator was induced at the end of the second incubation by 2-min exposure to a UV transilluminator FBTIV-614 (Fisher). Incubations proceeded for an additional 5 min at 37 °C. 10 mM nitrocellulose membranes (Schleicher & Schuell). Nonspecific reactivity was blocked by incubation for 1 h at room temperature with 2% bovine serum albumin in PBS/PVP for 1 h at room temperature, 12 nM (0.02 µg/ml) immunogenic peptide for 60 min at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as secondary antibody (0.25 µg/ml) with 45-min incubations. Excess first and second antibodies were removed by washing five times for 10 min each in washing buffer. Detection was accomplished with an enhanced chemiluminescence system (ECL; Amersham Biosciences) and subsequent exposure to Eastman Kodak Co. XAR film for 5–30 s.

**Indirect Immunofluorescence**—Cells maintained in GPM for 3 h under capacitating conditions were washed twice with PBS and allowed to air-dry on polylysine-coated, 9-mm round coverslips before fixing/permeabilizing in 2% paraformaldehyde, 0.1% Triton X-100 in PBS for 10 min at room temperature. Sperm were incubated in PBS containing 50 mM glycine for at least 30 min at room temperature and washed twice with PBS containing 0.4% polyvinylpyrrolidone (PVP; average Mr = 40,000; ICN) (PBS/PVP). Nonspecific staining was blocked by incubation in 2% bovine serum albumin in PBS/PVP for 1 h at room temperature. Anti-Epac antibodies were diluted in blocking solution (7.6 g/ml) preblocked or not with 12 nM (0.02 µg/ml) immunogenic peptide for 60 min at room temperature. Blots were incubated with the anti-Epac antibody (0.92 µg/ml) preblocked or not with 12 nM (0.02 µg/ml) immunogenic peptide for 60 min at room temperature. After washing as before, samples were mounted with Gelvatol and stored at 4 °C in the dark. Slides were examined with an Eclipse TE3000 Nikon microscope equipped with a Plan Apo 60×/1.40 oil objective and a Hamamatsu Orca 100 camera (Hamamatsu Corp., Bridgewater, NJ) operated with MetaMorph 6.1 software (Universal Imaging, Downingtown, PA). Background was subtracted, and brightness/contrast were adjusted to render an all-or-nothing labeling pattern using Jasc Paint Shop Pro 6.02 (Jasc Software; available on the World Wide Web at www.corel.com).

**Protein Determination**—Protein concentrations were determined by the Bio-Rad Protein assay in 96-well microplates. Bovine serum albumin was used as a standard, and the results were quantified on a Bio-Rad 3550 Microplate Reader.

**Statistical Analysis**—Data were evaluated using one-way analysis of variance. The Tukey-Kramer test was used for pairwise comparisons. The results are listed as supplemental data (Tables S1, S2, S3, S4, S5, and S6 corresponding to data depicted in Figs. 1, 2, 3, 4, 5, and 6, respectively). Only significant differences (p < 0.05) are discussed.

## RESULTS

**Reagents that Mimic cAMP Trigger AR in SLO-permeabilized Sperm**—If the opening of Ca\(^{2+}\) channels elicited by PKA (11) was the only cAMP-dependent signaling pathway during the AR, it would be expected that reagents that augment the intracellular concentration of cAMP would not have an effect in cells where the plasma membrane is permeabilized by SLO, bypassing any requirement for ion channel opening. However, when SLO-permeabilized human sperm were incubated with 1 mM Bt2cAMP they underwent acrosomal exocytosis of a magnitude similar to that achieved by 10 µM Ca\(^{2+}\) (Fig. 1A). Interestingly, the cAMP endogenously synthesized by sperm must be enough to cause a maximal response, given that 100 µM IBMX (a PDE inhibitor) elicited the same level of exocytosis as either Ca\(^{2+}\) or Bt2cAMP (Fig. 1A). These data suggest that an alternative mechanism, not related to PKA-induced opening of Ca\(^{2+}\) channels, operates during the cAMP-elicited AR. This cAMP-dependent step must be located downstream of the influx of Ca\(^{2+}\) into the cytosol through SOC channels.

**cAMP-induced AR Does NotRequire Extracellular Ca\(^{2+}\)**—The addition of micromolar concentrations (10–500 µM) of Ca\(^{2+}\) triggers acrosomal exocytosis in permeabilized spermatozoa. The same effect is observed without Ca\(^{2+}\) addition when Bt2cAMP is used as inducer (Fig. 1A). Under these conditions, free Ca\(^{2+}\) concentration in the reaction mixture, which contains 0.5 mM EGTA, is on the order of 10–7 M (14). When 5 mM EGTA is added to the system, free Ca\(^{2+}\) concentration drops to less than 10 nM. Even at this very low Ca\(^{2+}\) concentration, sperm underwent exocytosis in response to Bt2cAMP (Fig. 1B). These results show that the AR elicited by cAMP proceeds without an influx of Ca\(^{2+}\) into the cytosol from the extracellular milieu, lending support to the hypothesis that a mechanism other than the opening of Ca\(^{2+}\) channels by PKA mediates the exocytosis brought about by cAMP.

**Ca\(^{2+}\) Induces AR in Permeabilized Sperm through a cAMP-mediated Pathway**—Next, we asked if cAMP and Ca\(^{2+}\) initiate independent cascades or achieve acrosomal release through shared signaling pathways. Hence, we investigated whether Ca\(^{2+}\)-triggered exocytosis requires cyclic nucleotides. To this end, we destroyed endogenous sperm cAMP by loading permeabilized cells with 2 µg/ml catalytically active, cAMP-specific PDE before inducing the AR. This pretreatment caused an 80% reduction of the exocytotic response compared with untreated samples (Fig. 1C), suggesting that Ca\(^{2+}\) requires cAMP to stimulate exocytosis. In other words, Ca\(^{2+}\) and cAMP do not operate independently to activate the AR cascade.

**AR in Permeabilized Sperm Is Insensitive to PKA Inhibitors**—To eliminate the possibility that cAMP is acting through PKA to modulate the AR, we selected drugs that inhibit the activity of the catalytic subunit of PKA through different mechanisms of action. Preincubation with 10 µM H89 did not affect the AR triggered by 1 mM Bt2cAMP (Fig. 2A, gray
Given that Ca\(^{2+}\) signals through cAMP (Fig. 1C) and that the latter accomplishes exocytosis through a mechanism that does not rely on PKA (Fig. 2A), it would be expected that the Ca\(^{2+}\)-induced AR would also be independent of this kinase. In fact, when we preincubated permeabilized sperm with 10 \(\mu\)M H89, 15 \(\mu\)g/ml PKI (Fig. 2B), or 500 \(\mu\)M Rp-cAMPs (Fig. 3B, see below) but challenged with Ca\(^{2+}\) instead of Bt\(_2\)cAMP or IBMX, all of the inhibitors had no or very little effect on exocytosis. These data indicate that, as is the case with cAMP, Ca\(^{2+}\) does not utilize a PKA-dependent pathway to mediate acrosomal exocytosis in permeabilized sperm.

PKA inhibitors have been reported to block exocytosis, raising the possibility that the inhibitors were ineffective in our hands. When capacitated intact human sperm were stimulated with 15 \(\mu\)M progesterone, AR ensued (Fig. 2C, open bars). Exocytosis was abrogated by preincubation with 10 \(\mu\)M H89 (Fig. 2C), in agreement with previous observations of PKA dependence of the AR in intact sperm and eliminating the possibility of drug inactivity. Taken together, our data unveil a new, PKA-dependent, PKA-independent signaling pathway during acrosomal exocytosis.

Selective Epac ActivationInduces Acrosomal Exocytosis—Our findings that the AR in permeabilized sperm requires cAMP but is independent of PKA suggest that another cAMP target(s) might be mediating the effects. Epac is a plausible candidate, and we therefore turned to investigate its role in exocytosis using several different approaches. Bt\(_2\)cAMP has been linked to Epac-mediated responses in whole cells (47, 48) but also to those of PKA; therefore, we resorted to the recently designed 8-pCPT-2Me-cAMP Epac-specific cAMP analogue. When nonpermeabilized sperm were exposed to 50 \(\mu\)M 8-pCPT-2Me-cAMP, they achieved an exocytotic response similar to that brought about by...
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As expected, 50 μM 8-p-CPT-2Me-cAMP triggered exocytosis even in the virtual absence of extracellular Ca\(^{2+}\) (Fig. 3B), as shown for Bt2cAMP (Fig. 1B). These data indicate that extracellular Ca\(^{2+}\) is not required for 8-p-CPT-2Me-cAMP-triggered AR, supporting the notion that Epac governs signaling pathways unrelated to the opening of Ca\(^{2+}\) channels.

Presence and Localization of Epac in Human Sperm—Responsiveness to 8-p-CPT-2Me-cAMP is an accepted marker of the presence of Epac in cells. Thus, the inducibility of the AR by this analogue strongly implies that Epac is not only present but also functionally important for human sperm exocytosis. Specific antibodies were used to detect the presence and localization of Epac in human sperm by Western blot and indirect immunofluorescence. As shown on Fig. 3C (anti-Epac lane), the anti-Epac antibodies recognized a single protein band in sperm extracts. This band had an apparent molecular mass of 101 kDa, corresponding better with the expected molecular mass of human Epac1 (881 amino acids) than that of human Epac2 (1011 amino acids). Indirect immunofluorescence was used to localize Epac on fixed, permeabilized human sperm. The antibody reacted with the sperm head in the acrosomal region (Fig. 3D, top). This pattern was specific, since it was not observed when the antibody had been previously blocked with the synthetic pep-
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FIGURE 4. Epac is required for Ca\(^{2+}\) and cAMP-triggered human sperm AR. A, SLO-permeabilized human sperm were treated for 15 min at 37 °C in the presence of a 1 μg/ml concentration of an anti-Epac antibody-pretreated (anti-Epac→→ calcium) or not (anti-Epac→→ calcium) with 0.021 μg/ml Epac peptide or 1 μg/ml nonimmune rabbit IgG (IgG→→ calcium). Sperm were challenged with 0.5 mM CaCl\(_2\) (10 μM free Ca\(^{2+}\)) for an additional 15 min. B, same as A, except that 1 mM Bt2cAMP (gray bars) or 50 μM 8-pCPT-2′-O-Me-cAMP (dashed bars) was used as inducer. Cells were fixed, acrosomal exocytosis was evaluated by FITC-PSA binding, and data were normalized (mean ± S.E.) as described under “Experimental Procedures.” Statistical analysis is provided in Table S4.

FIGURE 5. CAMP-induced, Epac-mediated AR requires a functional fusion machinery. SLO-permeabilized human sperm were treated for 15 min at 37 °C in the presence of a 20 μg/ml concentration of an anti-Rab3A polyclonal antibody pretreated (anti-Rab3A*→→ calcium) or not (anti-Rab3A→→ inducer) with 260 nM recombinant Rab3A (A); 300 nM BoNT/E (BoNT/E→→ inducer), a 50 μg/ml concentration of an anti-α/β-SNAP antibody raised in our laboratory (purified IgG; anti-α/β-SNAP→→ inducer), or a polyclonal antibody to NSF (whole rabbit serum diluted 1:300), preincubated (anti-NSF*→→ calcium) or not (anti-NSF→→ inducer) with 44 nM recombinant NSF, or 10 μM BAPTA-AM, 10 μM CPA, or 100 μM 2-APB (C). Acrosomal exocytosis was evaluated after an additional 15 min at 37 °C in the absence (control) or presence of 0.5 mM CaCl\(_2\); 10 μM free ion; block bars), 1 mM Bt2cAMP (gray bars), or 50 μM 8-pCPT-2′-O-Me-cAMP (dashed bars). Cells were fixed, acrosomal exocytosis was monitored by FITC-PSA binding, and data were normalized (mean ± S.E.) as described under “Experimental Procedures.” Statistical analysis is provided in Table S5.

tide (Fig. 3D, bottom panels). Taken together, our data indicate that Epac is present in human sperm, localizes to the acrosomal region, and exhibits a positive role in the AR that is independent of PKA and the opening of Ca\(^{2+}\) channels.

Epac Mediates Ca\(^{2+}\) and cAMP-triggered AR—We have shown that the activation of Epac by exogenously added CAMP suffices to elicit the AR in human sperm. We have also shown that Ca\(^{2+}\) requires CAMP to achieve exocytosis. Next, we asked whether Ca\(^{2+}\) relies on Epac to fulfill its role as AR inducer. When added to SLO-permeabilized sperm, 1 μg/ml anti-Epac antibody, but not a nonimmune rabbit IgG, effectively abrogated Ca\(^{2+}\)-triggered AR (Fig. 4A). The effect of the anti-Epac polyclonal was abolished by preincubation with the synthetic peptide against which the anti-Epac antibody was raised (Fig. 4A), suggesting that the inhibitory effect of the anti-Epac was specific and due to binding to endogenous Epac. As expected, the AR elicited by both Bt2cAMP (Fig. 4B, gray bars) and 8-pCPT-2′Me-cAMP (Fig. 4B, dashed bars) was also blocked by the anti-Epac antibody. These results reveal that Epac is indispensable for human sperm acrosomal exocytosis and suggest that CAMP plays its role in Ca\(^{2+}\)-triggered AR through the activation of Epac.

cAMP-induced, Epac-mediated AR Requires a Functional Fusion Machinery—Results from our laboratory show that the AR in permeabilized sperm proceeds through a unidirectional sequence of events put in motion by Ca\(^{2+}\) stimulation (16). The next series of experiments were designed to characterize the cAMP-induced AR of human sperm, defining the place of the activation of Epac in the sequence of events leading to exocytosis.

First, we set out to determine whether the tethering of the acrosome to the plasma membrane elicited by Rab3A takes place before or after Epac activation. Specific antibodies against Rab3A (200 μg/ml) were introduced into SLO-permeabilized human sperm before challenging with 10 μM free Ca\(^{2+}\) (Fig. 5A, black bars), 1 mM Bt2cAMP (Fig. 5A, gray bars), or 50 μM 8-pCPT-2′Me-cAMP (Fig. 5A, dashed bars). Pretreatment with the anti-Rab3A antibodies inhibited Ca\(^{2+}\), Bt2cAMP-, and 8-pCPT-2′Me-cAMP-triggered AR by 98, 87, and 98% respectively. To test the specificity of this effect, the active sites of the antibodies were blocked by preincubation with recombinant Rab3A before the addition to permeabilized sperm. Inhibition was no longer observed (Fig. 5A, anti-Rab3A*→→ calcium), suggesting that their inhibitory effect was due to binding to endogenous Rab3A. These data indicate that Epac-mediated exocytosis requires Rab3A. Second, we resorted to specific anti-α-
SNAP (50 μg/ml) and anti-NSF (whole serum diluted 1:300) antibodies that prevent the AR by sequestering the endogenous proteins and therefore the priming of the fusion machinery. Ca\(^{2+}\) (Fig. 5B, black bars), Bt2cAMP (Fig. 5B, gray bars), and 8-pCPT-2Me-cAMP (Fig. 5B, dashed bars) failed to trigger exocytosis in sperm pretreated with these antibodies. Once again, the effect of the antibodies was specific, since they no longer inhibited exocytosis when preincubated with excess recombinant NSF (Fig. 5B, anti-NSF → calcium) or α-SNAP (42). BoNT/E is a potent inhibitor of exocytosis due to its highly specific proteolytic cleavage of the SNARE protein SNAP-25. When added to SLO-permeabilized sperm, 300 nM BoNT/E caused a marked inhibition of the Bt2cAMP-dependent (Fig. 5B, gray bars) and 8-pCPT-2Me-cAMP-dependent (Fig. 5B, dashed bars) AR. Since both cAMP analogues effectively induced the AR in the absence of extracellular Ca\(^{2+}\) (Figs. 1B and 3B), these results suggest that the analogues themselves, through Epac activation, are directly or indirectly able to bring about a correct priming by NSF/α-SNAP and the SNARE-dependent docking of the acrosome to the plasma membrane. Third, we investigated the requirement of Ca\(^{2+}\) mobilized from the acrosome. To this end, we chelated Ca\(^{2+}\) in the lumen of the acrosome with 10 μM BAPTA-AM, a permeant-chelating agent that accumulates in membrane-bound compartments. This treatment blocked the AR when both Bt2cAMP (Fig. 5C, gray bars) and 8-pCPT-2Me-cAMP (Fig. 5C, dashed bars) were used as inducers. Similar results were obtained when the Ca\(^{2+}\)-ATPase pump, responsible for taking up Ca\(^{2+}\) into the acrosome, was inhibited with 10 μM CPA (Fig. 5C). We then asked whether cAMP-induced AR requires the release of Ca\(^{2+}\) from the acrosomal store. Loading with 100 μM 2-APB, an IP\(_3\)-sensitive Ca\(^{2+}\) channel blocker, precluded exocytosis elicited by Bt2cAMP (Fig. 5C, gray bars) and 8-pCPT-2Me-cAMP (Fig. 5C, dashed bars). These data show that cAMP-triggered, Epac-mediated exocytosis depends on the efflux of intra-acrosomal Ca\(^{2+}\).

**cAMP/Epac Govern an Early Step during the Ca\(^{2+}\)-triggered Exocytotic Cascade**—To determine the site of action of Epac when Ca\(^{2+}\) initiates the AR, we resorted to a reversible blocker of exocytosis that prevents the AR by sequestering intra-acrosomal Ca\(^{2+}\) (14). The reversible blocker of choice was the photolabile Ca\(^{2+}\) chelator NP-EGTA-AM. UV photolysis of NP-EGTA releases the caged Ca\(^{2+}\) rapidly and with high photochemical yield (49). In our SLO-permeabilized human sperm model, the membrane-permeable compound NP-EGTA-AM crosses the plasma and outer acrosomal membranes, accumulates inside the acrosome, and thus precludes the availability of intra-acrosomal Ca\(^{2+}\). UV photolysis of NP-EGTA-AM rapidly replenishes the acrosomal Ca\(^{2+}\) pool, resuming exocytosis (see schematic in Fig. 6A and supplemental Fig. S1). In combination with AR inhibitors, NP-EGTA-AM serves to place the requirement for fusion-related factors before or after the intra-acrosomal Ca\(^{2+}\)-sensitive step. Briefly, NP-EGTA-AM allows an AR inducer to prepare the fusion machinery up to the point when intra-acrosomal Ca\(^{2+}\) is required. Inhibitors are then added, and the tubes are illuminated. Resistance to inhibitors, reflected in unaffected exocytosis, implies that their targets are required upstream of intra-acrosomal Ca\(^{2+}\) efflux. Sensitivity to inhibitors, revealed by blocked exocytosis, means their targets are located after the intra-acrosomal Ca\(^{2+}\)-sensitive step (see schematic in Fig. 6A and supplemental Fig. S1). The AR is always prevented when the inhibitors are added prior to the inducer and maintained throughout the experiment. We asked whether the cAMP/Epac-dependent step takes place before or after intra-acrosomal Ca\(^{2+}\) release by loading permeabilized sperm with NP-EGTA-AM and hydrolyzing cAMP to 5'-AMP with 2 μg/ml PDE (Fig. 6B, gray bars) or blocking Epac function with an antibody (Fig. 6C, gray bars). Both reagents inhibited exocytosis when added before, but not after, challenging with Ca\(^{2+}\). These results indicate that cAMP/Epac are necessary early, before Ca\(^{2+}\) is released from the acrosome, in the fusion cascade. This is in complete agreement with data attained when cAMP was used to elicit the AR (Fig. 5C) and suggests a role for cAMP/Epac prior to intra-acrosomal Ca\(^{2+}\) efflux.

**DISCUSSION**

Elevated cAMP concentrations serve as a switch to activate most if not all of the signaling pathways during sperm maturation, rendering the spermatozoa competent for fertilization, and also the AR. Thus, reagents that increase intracellular cAMP levels, such as the membrane-permeable analogues Bt2cAMP and 8-Br-cAMP, xanthine PDE inhibitors (to prevent cAMP hydrolysis), and the adenylyl cyclase stimulator forskolin, induce exocytosis in mammalian sperm (50). One of the mechanisms proposed to explain these observations involves PKA activation by cAMP, leading to the opening of Ca\(^{2+}\) channels (11). We now know that it is most likely that the cAMP-mediated signaling mechanism is much more complex than was believed earlier, and many cAMP-mediated effects that were previously ascribed to PKA are in fact transduced by Epac. Therefore, it is necessary to re formulate concepts of cAMP-mediated signaling to include the contribution of Epac. To this end, we have used human sperm permeabilized with the bacterial toxin SLO. Selective permeabilization of the plasma membrane was achieved by SLO binding to cholesterol at 4 °C (the toxin is inactive at this temperature (51)) and removing the excess toxin by washing and centrifugation. The fraction of SLO that remained bound to the plasma membrane was activated with dithiothreitol, and pore formation was initiated by incubation at 37 °C. This procedure not only allows access to intracellular compartments but also preserves the normal reducing cytosolic environment without causing any noticeable functional or structural damage. Furthermore, it achieves regulated acrosomal release and has been used to demonstrate the roles of Rab3A (17), NSF (52), synaptotagmin VI (53), the SNARE complex (16, 54), calmodulin (55), protein-tyrosine kinases and phosphatases (56), and α-SNAP (42) in the Ca\(^{2+}\)-dependent AR of human sperm. Furthermore, both intact and permeabilized sperm respond to challenge with cAMP analogues (Figs. 2C and 3, A and B) and BoNTs (16, 54) in a similar fashion.

In this particular study, permeabilization bestows the additional advantage of uncovering cAMP-dependent, post-SOC channel opening pathways, otherwise masked by the PKA dependence of pre-SOC channel opening in intact sperm (57). Here, we show that reagents that augment or mimic cAMP triggered the AR in a PKA-independent fashion (Figs. 2 and 3). We make such a strong assertion based on data gathered after selecting a variety of inhibitors with different mechanisms of action. Thus, H89 is a competitive inhibitor of ATP (58), whereas PKI competes with protein/peptide substrates (59). Both of these compounds block PKA activity through its catalytic subunit. In contrast, Rp-cAMPS competes with cAMP for its binding sites on the regulatory subunit but, unlike cAMP, is unable to dissociate the holoenzyme (60). In intact sperm, PKA has been claimed to exhibit a positive role in the AR by indirectly opening SOC channels on the plasma membrane (57) and thus allowing a sustained influx of Ca\(^{2+}\) into the cytosol, which ultimately leads to exocytosis (61). Consistent with these observations, PKA is required in intact (Fig. 2C) but not in permeabilized (Figs. 2, A and B, and 3, A and B) sperm. When SLO-permeabilized sperm are suspended in a solution containing Ca\(^{2+}\), the situation resembles that of open SOC channels. Therefore, we interpret any modulation of exocytosis taking place in SLO-treated cells as indicative of a post-SOC opening step in intact sperm. Given that Epac is required in per-
FIGURE 6. cAMP and Epac are required for Ca\(^{2+}\)-triggered human sperm AR before intra-acrosomal Ca\(^{2+}\) efflux. A, schematic representation of the use of NP-EGTA-AM in permeabilized sperm. Shown is the sequence of the addition of reagents. NP, NP-EGTA-AM; \(\text{h}\), photolysis of NP-EGTA-AM by UV illumination and release of intra-acrosomal Ca\(^{2+}\). B and C, permeabilized spermatozoa were loaded with 10 \(\mu\)M NP-EGTA-AM (NP) for 15 min at 37 °C to chelate intra-acrosomal Ca\(^{2+}\). Acrosomal exocytosis was then initiated by adding 0.5 mM CaCl\(_2\) (10 \(\mu\)M free Ca\(^{2+}\)) (calcium). After a further 15 min at 37 °C to allow exocytosis to proceed up to the intra-acrosomal Ca\(^{2+}\)-sensitive step, sperm were treated for 15 min at 37 °C with 2 \(\mu\)g/ml PDE4D or 1 \(\mu\)g/ml anti-Epac antibody. All of these procedures were carried out in the dark. UV flash photolysis of the chelator was induced at the end of the incubation period (\(\text{h}\)), and the samples were incubated for 5 min to promote exocytosis (NP → calcium → \(\text{h}\)). Sperm were then fixed, and AR was measured as described under “Experimental Procedures.” Several controls were included (black bars): background AR in the absence of any stimulation (control); AR stimulated by 10 \(\mu\)M free Ca\(^{2+}\) (calcium), inhibitory effect of NP-EGTA-AM in the dark (NP → calcium → dark) and the recovery upon illumination (NP → calcium → \(\text{h}\)); and inhibitory effect of the inhibitors when present throughout the experiment (NP → inhibitor → calcium → \(\text{h}\)). The data were normalized as described under “Experimental Procedures” (mean ± S.E.). Statistical analysis is provided in the corresponding Table S6.
mechamorlized sperm, we deduce that this protein displays its positive role in exocytosis downstream of SOC opening. Because PKA catalyzes a step prior to the activation of these channels, we would like to suggest that PKA and Epac act in a sequential manner to achieve sperm exocytosis. The fact that the activity of these two cAMP targets is required for exocytosis is not a sperm oddity. For instance, cAMP stimulates exocytosis of different vesicle pools in melonotaths, one through the PKA-dependent modulation of Ca^{2+} channels and the other through the Epac-dependent modulation of the secretory machinery (62). In the calyx of Held, cAMP increases the Ca^{2+} affinity for secretion (37) and enhances vesicle priming (63) and synaptic potentiation (64) through Epac activation. Epac also regulates transmitter release at the crayfish neuromuscular junction (36) and progesterone secretion by human granulosa cells (48).

The fact that IBMX brings about exocytosis means that the amount of cAMP synthesized by sperm would be high enough to support secretion, were it not hydrolyzed by endogenous PDEs. Ca^{2+}-induced AR in permeabilized human sperm was mediated by cAMP but was independent of PKA (Figs. 2B and 3B). Instead, it involved Epac. It is currently unknown whether Ca^{2+} relies on a threshold of cAMP concentrations normally present in unstimulated cells or if it somehow increases cAMP levels by altering the balance between its synthesis by cyclases and degradation by PDEs. Interestingly, Ca^{2+} stimulation of sAC activity has been well documented (65, 66). The new cAMP/Epac-related signaling pathway we have characterized also acts in nonpermeabilized cells (Fig. 2C). However, depolarizing it in nonpermeabilized cells remains difficult, because we lack Epac-related AR blockers that permeate through the plasma membrane. For instance, we used antibodies directed against Epac as specific inhibitors (Fig. 4) based on the hypothesis that the binding of an immunoglobulin on or close to an active site of a protein should alter its activity and/or its interaction with other proteins located upstream or downstream the signaling cascade, or, as the case may be here, with cAMP. This antibody was raised against a peptide mapping to within the single cAMP binding domain in Epac1 and cAMP-B in Epac2, corresponding to the highly exposed β-sheets 7 and 8 in the latter crystal structure (41). The region bears no similarity with the cAMP binding domains of human PKA RIA, PKA RIIα, PKA RIIβ, or PKA RIIβ (34) or with any other mammalian protein in public data bases, allowing us to regard our antibody as highly specific for Epac recognition. Two commercially available anti-Epac antibodies have been applied in a similar experimental strategy to inhibit the activity of H,K-ATPase in SLO-permeabilized rat kidney cells (67). In sperm, Epac exhibits acrosomal localization (Fig. 3D), consistent with its proposed role in the exocytosis of this granule. A single band corresponding to the molecular mass of Epac1 was detected by Western blot in whole sperm extracts (Fig. 3C). We would like to point out, however, that given that the peptide to which our antibody was raised is conserved in both Epac1 and Epac2, we cannot rule out the possibility that sperm contains an isomorph of Epac2 of smaller molecular mass than that described in somatic cells. To the best of our knowledge, ours is the first report on the presence and function of Epac in mammalian sperm. Indirect immunofluorescence experiments had previously suggested a subplasmalemmal localization for Epac in mouse spermatocytes and spermatids (68). Functional roles were not explored in that article. It is unknown at this point whether the apparent discrepancy between the localization of Epac reported by Berruti and us is due to the use of gametes from different species (mouse versus human), different maturation stage (spermatogonial versus mature sperm), or different antibodies (commercial versus custom made).

The first line of evidence for the requirement of Epac in the AR comes from the use of specific antibodies (see above). As shown in Fig. 4, loading of SLO-permeabilized sperm with anti-Epac antibodies reduced the exocytotic response of sperm to Ca^{2+} and cAMP analogues. We and others have previously shown that the acrosome behaves as a Ca^{2+}-storing organelle (14, 15). Release of intravesicular Ca^{2+} takes place after Rab3-elicted tethering and SNARE-mediated docking of the acrosome to the plasma membrane and is necessary for human sperm AR. By using a photosensitive Ca^{2+} chelator in combination with anti-Epac antibodies and recombinant PDE, we have been able to show that cAMP/Epac are required in sperm exocytosis before the release of Ca^{2+} from the acrosome (Fig. 6). The AR is not a wholesale, instantaneous release of components from a fluid-filled, membrane-bound sack, but rather the acrosomal contents consist of soluble proteins and an insoluble acrosomal matrix, which is the last to be released after membrane fusion. Thus, it suggests that other proteins and an insoluble acrosomal matrix, which is the last to be released after membrane fusion. It is this matrix we detect with PSA. We claim that anti-Epac antibodies actually block membrane fusion and not simply acrosomal matrix dispersal, given that exocytosis proceeds in their presence when added after releasing intra-acrosomal Ca^{2+} with NP-EGTA-AM (Fig. 6).

The second line of evidence comes from the use of the Epac-selective cAMP analogue 8-pCPT-2Me-cAMP. This compound is a very efficient stimulant of Epac in vitro and in vivo, with half-maximal activation achieved at concentrations 15 times lower than those of cAMP itself. Most importantly, this analogue is unable to activate PKA either in vitro or in vivo (69, 70). 8-pCPT-2Me-cAMP triggered the AR in permeabi-
Epac in the Acrosome Reaction

mized (Fig. 3, A and B) and intact (Fig. 2C) human sperm in concentrations similar to those used in a whole range of somatic cells. In both cases, exocytosis was resistant to H89 (Figs. 2C and 3A), in agreement with the notions that β-pCPT-2Me-cAMP does not signal through PKA and that Epac activation is sufficient to accomplish the AR. Rp-cAMPS exhibited a similar behavior (Fig. 3B). Whereas there is wide agreement on the antagonistic role of this analogue on PKA activation, its effects toward Epac are controversial. This analogue binds Epac with low affinity (70). Rehmann et al. (41) have recently referred to their own unpublished observation that Rp-cAMPS does not activate Epac; rather, it inhibits cAMP-induced Epac activation (71). Other laboratories have reported a nonantagonistic role of Rp-cAMPS toward Epac (72), and yet other studies appear to indicate that Rp-cAMPS could activate Epac-related pathways (3, 62, 73). To help resolve this controversy, we would like to contribute the information that, at least in human sperm, Epac is not activated by Rp-cAMPS. Nor does this analogue antagonize the stimulus role of cAMP on exocytosis.

β-pCPT-2Me-cAMP and Bt2cAMP were presumably acting through Epac activation and not through an influx of Ca2⁺ into the cytosol, since they elicited acrosomal exocytosis in the virtual absence of Ca2⁺ in the medium (Figs. 1B and 3B). Under standard conditions, influx of Ca2⁺ into the cell leads to the activation of Rab3A, which in turn mediates tethering of the acrosome to the plasma membrane. Next, priming by NSF/α-SNAP takes place, followed by SNARE protein assembly in trans complexes and therefore SNARE-dependent docking of the acrosome. The docking machinery contains or interacts with the Ca2⁺ sensor synaptotagmin. Upon binding Ca2⁺ mobilized from the acrosome through IP3-sensitive channels, synaptotagmin undergoes a conformational change that ultimately (and indirectly) promotes fusion. Both cAMP analogues used throughout this study required Rab3 (Fig. 5A), α-SNAP/NSF, SNAREs (Fig. 5B), and an efflux of intra-acrosomal Ca2⁺ (Fig. 5C) to bring about the AR. A model depicting our current thinking on the mechanisms underlying the AR is shown in Fig. 7, where, for simplicity, only SNAREs are drawn. Initially, SNAREs are locked in inactive cis complexes on plasma and outer acrosomal membranes. Upon Ca2⁺ entrance into the cytoplasm, sAC is stimulated, leading to an increase in cAMP levels. This cAMP binds to and activates Epac. Later on, Rab3A is activated, triggering the tethering of the acrosome to the plasma membrane. Next, α-SNAP/NSF disassemble cis SNARE complexes on both membranes. Monomeric SNAREs are free to assemble in loose trans complexes, causing the irreversible docking of the acrosome to the plasma membrane. At this point, Ca2⁺ is released from inside the acrosome through IP3-sensitive Ca2⁺ channels to trigger the final steps of membrane fusion, which require SNAREs (presumably in tight trans complexes) and synaptotagmin.

In many cell types, an increase in intracellular cAMP concentration regulates Ca2⁺-triggered exocytosis (39, 62, 74). Unlike the situation in human sperm (this work), in most of them, an elevation of cAMP alone in the absence of a Ca2⁺ rise is not sufficient to trigger exocytosis (75). Nevertheless, a limited range of cells use cAMP as a major trigger for exocytosis. The cAMP-dependent pathways coexist with Ca2⁺-dependent ones for exocytosis in these cells, and as we report here, it is likely that they use a common final SNARE-dependent mechanism. For instance, cAMP-triggered exocytosis in the parotid gland, one of the best studied, requires VAMP2. The involvement of other SNAREs or other components of the general fusion machinery in cAMP-triggered exocytosis had not, until now, been investigated (75), with the exception of the pathway cAMP-Epac2-Rim-Rab3-calcium sensor (1, 38, 76, 77). The exact mechanisms by which cAMP/Epac assemble the whole fusion machinery in preparation for exocytosis are currently under investiga-

REFERENCES
1. Kashima, Y., Miki, T., Shibasaki, T., Ozaki, N., Miyazaki, M., Yano, H., and Seino, S. (2001) J. Biol. Chem. 276, 46046–46053
2. Kung, G., Joseph, J. W., Chepurin, O. G., Monaco, M., Wheeler, M. B., Bos, J. L., Schwede, F., Genieser, H. G., and Holz, G. G. (2003) J. Biol. Chem. 278, 8279–8285
3. Maliet, M., Robert, S. J., Cacquevel, M., Gastineau, M., Vivien, D., Bergotio, J., Zugaza, J. L., Fischmeister, R., and Lezoualc’h, F. (2003) Nat. Cell Biol. 5, 633–639
4. Shimonomura, H., Imai, A., and Nashida, T. (2004) Arch. Biochem. Biophys. 431, 124–128
5. Luconi, M., Porazzi, L., Ferruzzi, P., Marchiani, S., Forti, G., and Baldi, E. (2005) Biol. Reprod. 72, 22–32
6. Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olde-Clarke, P., and Kopf, G. S. (1995) Development 121, 1139–1150
7. TarDist, S., Lefevre, L., Gagnon, C., and Bailey, J. J. (2004) Mol. Reprod. Dev. 69, 428–435
8. Leclerc, P., de Lamirande, E., and Gagnon, C. (1998) J. Androl. 19, 434–443
9. Aitken, R., Harkiss, D., Knox, W., Paterson, M., and Irvine, D. (1998) J. Cell Sci. 111, 645–656
10. Lefevre, L., Jha, K. N., de Lamirande, E., Visconti, P. E., and Gagnon, C. (2002) J. Androl. 23, 709–716
11. Breitbart, H. (2003) Cell Mol. Biol. (Noisy-le-Grand) 49, 321–327
12. Yanagimachi, R. (1994) in The Physiology of Reproduction (Knobil, E., and Neill, J. D., eds) pp. 189–317, Raven Press, New York
13. O’Toole, C. M., Arnould, C., Darszon, A., Steinhardt, R. A., and Flormann, H. M. (2000) Mol. Biol. Cell 11, 1571–1584

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