Recombinant streptolysin O (SLO) was obtained from cells) were washed twice with cold PBS, and proteins were for 20 min at 4 °C. The whole-cell BL21 (Stratagene), and expression of recombinant proteins was accomplished by incubation with 0.25 E. coli and a membrane-permeant version of this protein (R-Rab3A) (1) were generously provided by R. Regazzi (University of Lausanne, Lausanne, Switzerland). DNA encoding Rab27-GTP-binding domain of rat RIM (amino acids 11–39; RIM-RBD) (2) or 0.5 mM (His6-Rab27A) was a kind gift from D. Munafó (Scripps Research Institute, La Jolla, CA). The cDNA encoding Rab27A (containing the soluble fractions) were partitioned or not in Triton X-114 and stored at −70 °C until use. Rab3A triggers acrosomal exocytosis in living human sperm. Rab27A was a kind gift of R. Shirakawa (Kyoto University, Kyoto, Japan). The cDNAs encoding His6-Rab3As were transformed into E. coli BL21(DE3)pLysS (Stratagene), and expression of recombinant proteins was accomplished by incubation with 0.25 mM (His6-Rab3A) or 0.5 mM (His6-R-Rab3A) isopropyl-β-D-thio-galactoside (IPTG) overnight at 22 °C. Expression of GST and all GST-fused proteins was induced in E. coli BL21 (Stratagene) with 0.5 mM IPTG for 3 h at 37 °C, with the exception of GST-RIM-RBD (0.5 mM IPTG, overnight at 22 °C), Rab3A V55E (0.1 mM IPTG, overnight at 22 °C), and GST-Rab27A (1 mM IPTG, 3 h at 37 °C). The cDNA encoding His6-Rab27A was transformed into E. coli BLR(DE3) (Stratagene), and protein expression was induced with 0.5 mM IPTG for 3 h at 37 °C. Purification of His6-tagged recombinant proteins was carried out under native conditions in accordance with the protocol specified by Qiagen, except that the purification buffers contained 20 mM TrisHCl (pH 7.4) instead of 50 mM phosphate (pH 8). NaCl was 300 mM, the lysis buffer contained 8–10 mM imidazole, the washing buffer contained 20 mM imidazole, and the elution buffer contained 250 or 400 mM imidazole. GST-fused recombinant proteins were purified on glutathione-Sepharose beads following standard procedures, except for pull-down assays, for which GST-Slac2-b and GST-RIM-RBD bacterial lysates were frozen until use.

Human Sperm Subcellular Fractionation. We followed the protocol described by Bohring and Krause (4), modified as by Tomes et al. (5). In brief, capacitated sperm (100 × 10^6 cells) were incubated with or without 100 μM 2-APB followed by the AR inducers A23187 (10 μM) or 8-pCPT-2′-O-Me-cAMP (50 μM) for 15 min at 37 °C, washed twice with PBS, and diluted 1:9 in hypoosmotic swelling buffer (6). After 2 h at 37 °C in a water bath, at least 80% of the cells were swollen. We transferred the suspensions to ice, added a protease inhibitor mixture, disrupted sperm by sonication, and centrifuged once at 10,600 × g for 15 min at 4 °C and once at 20,800 × g for 10 min at 4 °C to remove cell debris. We centrifuged the resultant supernatants at 208,000 × g for 2 h at 4 °C in a Beckman Optima ultracentrifuge. The final pellets (containing the membrane proteins) and the final supernatants (containing the soluble fractions) were partitioned or not in Triton X-114 and stored at −70 °C until use.

Triton X-114 Partition. Partition experiments were conducted following standard procedures (7, 8). In brief, capacitated sperm (50 × 10^6 cells) were washed twice with cold PBS, and proteins were extracted in 1 mL of lysis buffer [20 mM TrisHCl (pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, 5 mM MgCl₂, and 1% Triton X-114] by sonication on ice (three times for 15 s each, with a 10-s interval). The lysates were rocked for 45 min at 4 °C and centrifuged at 14,000 × g for 20 min at 4 °C. The whole-cell detergent extracts were then incubated for 15 min at 30 °C. The particulate fractions obtained after subcellular fractionation were dissolved in ice-cold lysis buffer and the protease inhibitor mixture by incubation for 15 min at 30 °C. Cytosols were added to Triton X-114 (1% final concentration) and incubated for 15 min at 30 °C. All samples were centrifuged at 3,000 × g for 3 min at room temperature. Hydrophilic proteins partitioned into the upper (aqueous) phase, whereas hydrophobic proteins were recovered from the lower (detergent) phase. Protein precipitation and removal of detergent were achieved via extraction with CCl₃H₂O. Precipitated proteins were dissolved in sample buffer by heating once at 60 °C for 10 min and once at 95 °C for 3 min.

Fig. S1. Specificity of anti-Rab3A and anti-Rab27 antibodies and protein cassettes. (A) 500 ng recombinant GST-Rab3A or GST-Rab27A was run on 8% SDS-Tris-glycine gels, transferred to nitrocellulose, and immunoblotted (IB) with the anti-Rab3A (monoclonal), anti-Rab27, and anti-GST antibodies as described in Materials and Methods. M, standards (× 10^3) are indicated on the left. (B) Here 50 ng of purified GST and Slac2-b were electrophoresed in 10% Tris-glycine gels and transferred to nitrocellulose. Nonspecific binding was blocked by incubation for 1 h at room temperature in blocking solution. (Upper) Blots were overlaid with 2 μg/mL of GST-Rab27 loaded with either GTP-γ-S or GDP in blocking solution for 2 h at room temperature, washed three times in washing buffer, and probed with anti-Rab27 as described for standard Western blot analysis. (Lower) Equal loads on the gel were assessed with anti-GST antibodies after stripping. Shown is an experiment representative of two repetitions. Quantification is shown to the right. (C) 300 ng of recombinant His6-Rab3 loaded with either GTP-γ-S or GDP was incubated with GST-RIM-RBD immobilized on glutathione-Sepharose beads as detailed for the pull-down assays. The immobilized proteins were analyzed by Western blot analysis with the anti-Rab3A monoclonal antibody. Shown is an experiment representative of two repetitions. Quantification is shown to the right.
Fig. S2. The anti-Rab3A and anti-Rab27 antibodies used in this study do not cross-react when blocking the AR. (A–C) SLO-permeabilized human sperm were treated for 15 min at 37 °C in the presence of 7 nM anti-Rab27 antibody pretreated with 14 nM recombinant GST-Rab27A (A, black bar) or 14 nM recombinant GST-Rab3A (C, black bar). In (B), sperm were loaded with 133 nM anti-Rab3A polyclonal antibody pretreated with 270 nM recombinant GST-Rab27A (black bar). (D and E) Control experiments conducted exactly as described in Fig. 4 E and F, except that heat-inactivated (5 min at 95 °C) GST-Rab27A (7 nM, Rab27A*) substituted for the native protein (D) and the mutant GST-Rab3A V55E (140 nM) replaced WT Rab3A (E). The inactive proteins rescued the inhibition imposed by anti-Rab27/3A antibodies (black bars). Controls (gray bars) included background AR in the absence of any stimulation (control), AR stimulated by 0.5 mM CaCl_2 (calcium), lack of effect of 14 nM recombinant 27A alone, lack of inhibition of 270 nM Rab3A (Rab3A→calcium), inhibition by anti-Rab3A/27 antibodies (anti-Rab3A/27→calcium), and lack of effect of 14/140 nM heat-inactivated Rab27A/recombinant Rab3A V55E alone (Rab27A*/3A V55E). Sperm were fixed, and AR was measured by FITC-PSA binding as described in Materials and Methods. The data represent the mean ± SEM of at least three independent experiments.
**Fig. S3.** Description of a method to detect the localization of Rab3A-GTP and Rab27-GTP. Capacitated, SLO-permeabilized sperm were incubated with 2-APB and EDTA as described in Materials and Methods. (Upper) Cells were loaded with 40 μM GDP-β-S or GTP-γ-S in a buffer with low Mg²⁺ concentrations for 10 min at 37 °C, and bound nucleotides were stabilized with 15 mM MgCl₂ for 5 min at 37 °C. Cell suspensions were fixed in 2% (wt/vol) paraformaldehyde, attached to poly-L-lysine–coated coverslips, and overlaid with 140 nM GST, GST-RIM-RBD, or GST-Slac2-b in blocking solution. Cells were triple-stained with an anti-GST antibody as a readout for the activity probes that detect active Rab3 and Rab27 (red); with FITC-PSA, to confirm that the AR was effectively prevented by 2-APB (green); and with Hoechst 33342, to visualize all cells in the field (blue). Superposition of the three fluorescence channels is shown in the “merge” column. Quantifications (mean ± SEM of at least three independent experiments) are shown to the right of each relevant group of micrographs. We scored the presence of acrosomes by PSA-FITC staining (gray bars) and that of active Rab by GST decoration of the activity probes GST-RIM-RBD bound to endogenous Rab3 (black bars; we counted 753 cells loaded with GDP-β-S and 312 cells loaded with GTP-γ-S) and Slac2-b for Rab27 (black bars; we counted 640 cells loaded with GDP-β-S and 201 cells loaded with GTP-γ-S). (Lower) Immunostaining with the anti-Rab27 antibody. (Scale bars: 5 μm.)