

Extended Experimental Procedures.

Reagents:

Chemicals were obtained from the following sources: Pisum Sativum agglutinin (PSA), Bovine Serum Albumin (BSA) A7906, Ca²⁺ ionophore A23187 and 3-isobutyl-1-methylxanthine (IBMX), anti- β -Tubulin T4026, Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), BaCl₂, TEA (tetraethylammonium chloride), Ouabain (Ou) and 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) were purchased from Sigma (St. Louis, MO); anti-phosphotyrosine (anti-pY) clone 4G10 from Millipore Corporation (Temecula, USA); anti-phospho PKAs substrates (anti-pPKA) clone 100G7E and horseradish peroxidase-conjugated (HRP) anti-rabbit IgG from Cell Signaling, (Danvers, USA); HRP anti-mouse IgG from Vector Laboratories (Burlingame, CA). 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, acetoxymethyl ester (BCECF-AM), 8-bromo adenosine 3':5'-cyclic monophosphate (sodium salt), H89, S0859, Amiloride, Bumetanide and inh-172 from Cayman Chemicals (Ann Arbor, MI). Propidium iodide (PI) and Anti ENaC- β sc-25354 from Santa Cruz Biotechnology (Santa Cruz, USA), 3,3-dipropylthiadicarbocyanine iodide (DISC₃(5)) and CoroNa Red from Invitrogen (Carlsbad, USA) and Eosin-Y from Biopack (Buenos Aires, Argentina).

Culture media:

The non-capacitating medium for human sperm used in this study was HEPES-buffered human tubal fluid (HTF) containing KCl 4.7 mM, KH₂PO₄ 0.3 mM, NaCl 90.7 mM, MgSO₄ 1.2 mM, Glucose 2.8 mM, CaCl₂ 1.6 mM, Sodium Pyruvate 3.4 mM, Sodium Lactate 60 mM and HEPES 23.8 mM from (Sigma, St. Louis, MO). Final pH was adjusted to 7.4 with NaOH (Sigma, St. Louis, MO).

To study the role of Na⁺ in capacitation, Sodium Lactate was not included in the incubation medium and NaCl was replaced by choline chloride. Final pH of the solution was adjusted to 7.4 with KOH (Sigma, St. Louis, MO). This non-capacitating medium with different concentrations of choline chloride was used for cells washes. For capacitating condition, medium was supplemented with 25 mM NaHCO₃ and 0.5% w/v BSA.

The non-capacitating medium used in this study for mouse sperm was a modified Toyoda–Yokoyama–Hosi (modified TYH) containing 119.3 mM NaCl, 4.7 mM KCl, 1.71 mM CaCl₂.2H₂O, 1.2 mM KH₂PO₄, 1.2

mM MgSO₄, 0.51 mM sodium pyruvate, 5.56 mM glucose, 20 mM HEPES, 10 µg/ml gentamicin and phenol red 0.0006%. For capacitating conditions 15 mM NaHCO₃ and 0.5% w/v BSA were added.

Mouse sperm capacitation

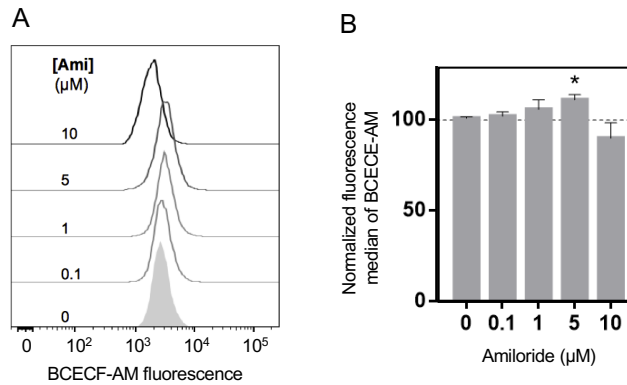
Hybrid F1 (C57BL/6 x Balb/C) mature (10–12 weeks-old) were housed in groups of 4 or 5 in a temperature-controlled room (23°C) with lights on at 07.00 h and off at 19.00 h, and had free access to tap water and laboratory chow. Animals were euthanized using Carbon Dioxide. After removal, both cauda epididymis were placed in 1 ml of modified TYH medium (without BSA and NaHCO₃). Sperm were allowed to swim out during 15 min at 37 °C (swim-out). Then, epididymis were removed, and sperm were resuspended to a final concentration of 1×10^7 cells/ml in 100 µl of the appropriate medium depending on the experiment performed. When required, sperm were pre-incubated in 100 µl of non-capacitating medium containing inhibitors or vehicle (DMSO) for 10 min. An equal volume (100 µl) of non-capacitating medium or two-fold concentrated capacitating medium (30 mM NaHCO₃, 1% w/v BSA) was added, and sperm were incubated for different time periods at 37°C.

Extraction of sperm proteins and immunoblotting

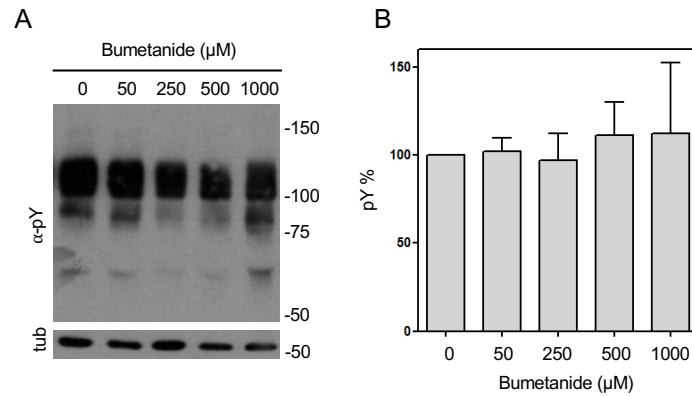
Sperm were washed by centrifugation (5 min, 400 g) in 0.5 ml of non-capacitating media, resuspended in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% v/v glycerol) and boiled for 5 min. After centrifugation for 5 min at 13,000 g, 5% v/v β-mercaptoethanol and 0.0005% w/v bromophenol blue was added to the supernatants which were boiled again for 5 min. Protein extracts equivalent to $2-4 \times 10^6$ sperm per lane were separated by SDS-PAGE in gels containing 10% w/v polyacrilamide and transferred onto nitrocellulose membranes. Blots were blocked in 5% w/v non-fat dry milk in PBS containing 0.1% v/v Tween 20 (T-PBS) for 30 min at room temperature and incubated for 1 h at room temperature with the first antibody and then and then with the secondary antibody. Antibodies were diluted in 2% w/v non-fat dry milk in T-PBS: 1:5,000 for anti-pY, 1:3,000 for anti-pPKA and 1:5,000 for anti-β-Tubulin. The corresponding secondary antibodies were diluted in 2% w/v nonfat dry milk in T-PBS: 1:3,000. In all cases the reactive bands were visualized using a chemiluminescence detection solution consisting of 100 mM Tris-HCl buffer pH 8, 205 µM coumaric acid, 1.3 mM luminol, and 0.01% v/v H₂O₂ (Sigma, St. Louis, MO) and were exposed for 10 s to CL-XPosure film (Pierce, Waltham, MA). When

necessary, membranes were stripped for 10 min in 2% w/v SDS, 0.67% v/v β -mercaptoethanol and 62.5 mM of buffer Tris pH 6.8 at room temperature. In all experiments, molecular masses were expressed in kiloDaltons (kDa). ImageJ 1.48k (National Institute of Health, USA) was used for analysis of the western blot images following the specifications of ImageJ User Guide, IJ 1.46r. The optical density of all bands including 150 kDa to 50 kDa were quantified and normalized, first to the β -Tubulin band and then to the capacitating condition of each membrane.

For ENaC- β subunit detection, after swim-up 6×10^6 cells were washed at 400 g for 5 min with PBS and then resuspended in 50 μ l of RIPA buffer solution (50 mM Tris, 150 mM NaCl, 0.5% w/v sodium deoxycholate, 1% w/v NP-40, 0.1% w/v SDS adjusted to pH 8 with HCl) with protease inhibitor (P8340, Sigma, St Louis). The solution was sonicated using 3 pulses of 10 s every 1 min, then incubated on ice/water for 1 h and vortexed 3 times. The solution was centrifugated 5 min at 700 g at 4°C and 5 μ l of sample buffer, 5% v/v β -mercaptoethanol, 0.0005% w/v bromophenol blue was added to supernatant, then boiled for 5 min. SDS-PAGE separation in gels containing 8% w/v polyacrilamide was performed as previously described. Blots were blocked with 5% w/v BSA in T-PBS for 30 min at room temperature and incubated for 1 h at room temperature with the primary and secondary antibody. The anti-primary β -antibody ENaC was diluted in 2% w/v BSA in 1: 50 T-PBS and the anti-mouse antibody in 2% w/v BSA in 1: 10000 T-PBS. Reactive bands were visualized using ECL Select™ western blotting detection reagent (GE, Healthcare).



Supplementary figure 1: Amiloride did not affect intracellular pH. (A) Human sperm were incubated in the presence (CAP) or absence of HCO_3^- for 3 h. Then, the cells were loaded with BCECF-AM to evaluate pH_i and exposed to increasing concentrations of amiloride (Ami). The intracellular pH as judged by BCECF fluorescence was not affected in the range of 0.1 – 5 μM amiloride. (B) Quantification of results presented in A. * $p < 0.05$ ($n=4$).



Supplementary figure 2: Inhibitors of NKCC such as bumetanide did not abolish pY in human sperm. (A) Human sperm were incubated in capacitating medium for 180 min with increasing concentrations of the bumetanide. Aliquots from each condition were processed for western immunoblotting with anti-pY antibodies and then membranes were reblotted with an anti- β -tubulin antibody for loading control. (B) Blots were quantified as described in “Materials & Methods”. Quantification of blot values represents the mean \pm SEM of 3 experiments.