Sodium and Epithelial Sodium Channels Participate in the Regulation of the Capacitation-associated Hyperpolarization in Mouse Sperm*

Enrique O. Hernández-González‡*, Julian Sosnik†, Jennifer Edwards‡, Juan José Acevedo‡‡, Irene Mendoza-Lujambio***, Ignacio López-González‡*, Ignacio Demarco⁵, Eva Wertheimer⁵, Alberto Darszon***, and Pablo E. Visconti‡‡

From the Department of Veterinary and Animal Science, Paige Laboratories, University of Massachusetts, Amherst, Massachusetts 01003, the Department of Cell Biology, Center for Research and Advanced Studies, Instituto Politécnico Nacional, 07300 México City, México, the Department of Cell Biology, University of Virginia, CITY, Charlottesville, Virginia 22908, the Department of Physiology and Pathophysiology, School of Medicine, Universidad Autónoma de Morelos, 62210 Cuernavaca, México, the Department of Developmental Genetics and Molecular Physiology, Institute of Biotechnology, Universidad Nacional Autónoma de México, 62210 Cuernavaca, México, and the Molecular Biomedicine Postgraduate Program, National Medicine and Homeopathy School, Instituto Politécnico Nacional, 07320 México City, México.

In a process called capacitation, mammalian sperm gain the ability to fertilize after residing in the female tract. During capacitation the mouse sperm plasma membrane potential (Em) hyperpolarizes. However, the mechanisms that regulate sperm Em are not well understood. Here we show that sperm hyperpolarize when external Na+ is replaced by N-methyl-glucamine. Readaptation of external Na+ restores a more depolarized Em by a process that is inhibited by amiloride or by its more potent derivative 5-(N-ethyl-N-isopropyl)amiloride hydrochloride. These findings indicate that under resting conditions an electrogenic Na+ transporter, possibly involving an amiloride-sensitive Na+ channel, may contribute to the sperm resting Em. Consistent with this proposal, patch clamp recordings from spermatogenic cells reveal an amiloride-sensitive inward Na+ current whose characteristics match those of the epithelial Na+ channel (ENaC) family of epithelial Na+ channels. Indeed, ENaC-α and -δ mRNAs were detected by reverse transcription-PCR in extracts of isolated elongated spermatids, and ENaC-α and -δ proteins were found on immunoblots of sperm membrane preparations. Immuno-staining indicated localization of ENaC-α to the flagellar midpiece and of ENaC-δ to the acrosome. Incubations known to produce capacitation in vitro or induction of capacitation by cell-permeant cAMP analogs decreased the depolarizing response to the addition of external Na+*. These results suggest that increases in cAMP content occurring during capacitation may inhibit ENaCs to produce a required hyperpolarization of the sperm membrane.

Mammalian sperm are not able to fertilize after ejaculation. They acquire this ability only after residing in the female uterine tract for a finite period of time that varies depending on the species. The molecular, biochemical, and physiological changes that occur in sperm while in the female tract are collectively referred to as capacitation (1). Capacitation is associated with changes in membrane properties, enzyme activities, and motility that prepare the sperm for the acrosome reaction and for penetration of the egg vestments prior to fertilization. The molecular basis of capacitation has been partially defined and includes: the removal of cholesterol from the sperm plasma membrane by cholesterol acceptors such as bovine serum albumin (2, 3), modifications in plasma membrane phospholipids, fluxes of HCO3− (4) and other intracellular ions, and increased tyrosine phosphorylation of proteins (5–7). These events are likely to play a role in the induction of hyperactivated motility and the ability of the sperm to undergo a regulated acrosome reaction (for review see Ref. 8).

Bovine and mouse sperm capacitation is also accompanied by a plasma membrane hyperpolarization. Em decreases in mouse sperm from −38 to −55 mV (4, 9, 10) and in bovine sperm from −33 to −66 mV (9). Because capacitation prepares sperm for the acrosome reaction, the capacitation-associated hyperpolarization may regulate the ability of sperm to generate transient Ca2+ elevations during the acrosome reaction induced by physiological agonists (e.g. zona pellucida) (11). In this respect, low voltage-activated T-type Ca2+ channels have been detected in mouse spermatogenic cells (12, 13), and these channels are also present in mature mouse sperm (14, 15). One unique property of low voltage-activated Ca2+ channels is that they inactivate at the resting Em of sperm prior to capacitation (around −35 mV) (12, 14). Thus, if low voltage-activated Ca2+ channels are involved in the regulation of the acrosome reaction, the capacitation-associated sperm hyperpolarization may be required to remove this inactivation (11, 16, 17).

Although the molecular mechanisms by which the sperm Em hyperpolarizes during capacitation are not clear, there exist several potential candidates. Muñoz-Garay et al. (10) demonstrated with patch clamp techniques that inward rectifying K+ channels are expressed in mouse spermatogenic cells and proposed that these channels may contribute to the capacitation-associated sperm membrane hyperpolarization. An increase in sperm K+ permeability should lead to an Em hyperpolarization, according to the K+ equilibrium potential (18). Alternatively, the sperm plasma membrane may become less permeable to Na+. The relatively depolarized mammalian sperm resting Em before capacitation could be explained, at least in part, by a relatively high Na+ permeability. A capacitation-dependent decrease of this permeability would result in a sperm hyperpolarization. It has been reported that human sperm sus-
ENaC during Sperm Membrane Hyperpolarization

pended in 0 Ca\textsuperscript{2+} medium undergo a Na\textsuperscript{+}-dependent depolarization. Li\textsuperscript{+} can replace Na\textsuperscript{+}; thus, these findings suggest that a Na\textsuperscript{+} - and Li\textsuperscript{+}-permeable electrogenic pathway may be present in mammalian sperm (19).

The present work explores this later possibility. We observed that replacement of external Na\textsuperscript{+} by nonpermeable cations resulted in sperm \(E_{m}\) hyperpolarization. The addition of external Na\textsuperscript{+} to these sperm produced a depolarization that was potently inhibited by amiloride and its analog EIPA,\textsuperscript{2} high pH, and the incubation of mouse sperm under capacitating conditions. Moreover, high pH and amiloride were also capable of hyperpolarizing sperm in the presence of Na\textsuperscript{+}. Altogether, these results suggest that epithelial Na\textsuperscript{+} channels (ENaCs) are present in mouse sperm and that they may contribute to the capacitation-associated hyperpolarization. Consistent with this hypothesis, we detected the transcripts for both ENaC-\(\alpha\) and ENaC-\(\delta\) subunits in mouse spermatogenic cells and the respective proteins in mature sperm. This is the first time ENaC-\(\delta\) is reported in mouse. Furthermore, we used electrophysiological techniques to demonstrate the presence of ENaC type currents in spermatogenic cells, the precursors of sperm. Finally, we present evidence that reduction of the Na\textsuperscript{+} sperm permeability does contribute to the capacitation-associated \(E_{m}\) hyperpolarization.

EXPERIMENTAL PROCEDURES

Materials—Achlorid, dibutyryl cAMP, \(m\)-chlorophenylhydrazone (carbonyl cyanide \(3\)-chlorophenylhydrazone), valinomycin, choline chloride (choline \(Cl\)\textsuperscript{+}), choline bicarbonate (choline \(HCO_{3}\)\textsuperscript{−}), \(N\)-methyl-\(d\)-glucamine, and water for embryo transfer (used to make Whitten’s medium) were purchased from Sigma. EIPA, \(3,3’\)-dipropylthiadicarbocyanine iodide (DiSC\textsubscript{3}(5), \(2,7’\)-bis-(2-carboxyethyl)-\(5\)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM), and Sodium Green tetraacetate were obtained from Molecular Probes (Eugene, OR). Sp-cAMPS, and 3-isobutyl-1-methylxanthine were purchased from Biomol (Butler Pike, PA). Polyclonal antibodies against ENaC-\(\alpha\) and ENaC-\(\delta\) were purchased from Chemicon International (Temecula, CA). Donkey anti-rabbit IgG biotin-conjugated and Avidin fluorescein isothiocyanate-conjugated antibodies were from Pierce.

The following compounds were prepared in Me\textsubscript{2}SO at the stock concentrations noted between parentheses and stored at \(-20\) °C except when otherwise stated: DiSC\textsubscript{3}(5), BCECF-AM, and \(m\)-chlorophenylhydrazone and valinomycin (1 mM stocks). Other compounds were prepared on the day of the experiment and dissolved in Whitten’s medium and added at the final concentration indicated between parentheses: Sp-cAMPS (100 \(\mu\)M), Rp-cAMPS (100 \(\mu\)M), dibutyryl cAMP (1 mM), and 3-isobutyl-1-methylxanthine (100 \(\mu\)M).

Sperm Preparation—Experimental protocols were approved by the University of Massachusetts Animal Care Committee. In most experiments, cauda epididymal mouse sperm were collected from CD1 retired male breeders by placing munced cauda epididymis in a modified Krebs-Ringer medium (Whitten’s HEPES-buffered (WH) medium) (20). This medium, which does not support capacitation, was first prepared in the absence of bovine serum albumin and Na\textsubscript{HCO}_{3} and contains 1 \(\text{mM}\) polyvinyl pyrrolidone (average molecular weight, 40,000). After 5 min, sperm in suspension were washed in 10 ml of the same medium by centrifugation at 800 \(\times g\) for 10 min at room temperature. The sperm were then resuspended to a final concentration of \(2 \times 10^{7}\) cells/ml and diluted 10 times in the appropriate medium depending on the experiment performed. In experiments where capacitation was investigated, 5 mg/ml of bovine serum albumin and 24 \(\mu\)M of Na\textsubscript{HCO}_{3} were added. The pH was maintained at 7.2 except when the role of extracellular pH (pH\textsubscript{e}) was evaluated. To study the role of Na\textsuperscript{+} in capacitation and in the regulation of \(E_{m}\), NaCl was replaced by either choline \(Cl\)\textsuperscript{−} or \(N\)-methyl-\(d\)-glucamine \(Cl\)\textsuperscript{−} up to the concentration indicated in the respective experiment.

Membrane Potential Assay in Sperm Populations—\(E_{m}\) was measured as previously described (4). Briefly, sperm were collected as indicated above and, after dilution in the appropriate medium, capacitated for different time periods depending on the experiment. Eight min before the measurement, 1 \(\mu\)M DiSC\textsubscript{3}(5) (final concentration) was added to the sperm suspension and further incubated for 5 min at 37 \(^\circ\)C. One \(\mu\)M \(m\)-chlorophenylhydrazone (final concentration) was then added to collapse mitochondrial potential, and the sperm was incubated for 2 additional min. After this period, 1.5 ml of the suspension was transferred to a gently stirred cuvette at 37 \(^\circ\)C, and the fluorescence (620/670 nm excitation/emission) was recorded continuously. Calibration was performed as described before (4) by adding 1 \(\mu\)M valinomycin and sequential additions of KCl (21).

To analyze changes provoked by the addition of Na\textsuperscript{+}, sperm were recovered as described above and incubated for different time periods depending on the experiment. The cells were then transferred to a gently stirred cuvette at 37 \(^\circ\)C, and the fluorescence (620/670 nm excitation/emission) was recorded continuously. After reaching steady state fluorescence, different Na\textsuperscript{+} concentrations were added while the fluorescence was recorded. After a new fluorescence steady state was reached, calibration was performed as indicated above (4). The changes in \(E_{m}\) elicited by NaCl were quantified taking into consideration the calibration curve and the initial steady state fluorescence before NaCl addition.

Intracellular pH and Na\textsuperscript{+} Measurements in Sperm Populations—[Na\textsuperscript{+}], and pH\textsubscript{i} measurements were conducted as described before (4). Briefly, sperm (1 \(\times\) 10\textsuperscript{6} cell/ml) in WH medium were incubated at 37 \(^\circ\)C for 30 min in 10 \(\mu\)M Sodium Green tetraacetate or 4 \(\mu\)M BCECF-AM, the cell-permeant nonfluorescent precursor of Sodium Green and BCECF, respectively. After incubation, the cells were washed in fresh medium once (400 \(\times g\) for 5 min) and resuspended, and 1.5 ml of this suspension was placed in a gentle stirring cuvette for fluorescence measurements. Changes in fluorescence were expressed in arbitrary units of fluorescence. When the effects of [Na\textsuperscript{+}], on pH\textsubscript{i} or [Na\textsuperscript{+}], were assayed, sperm were collected, loaded, and washed in Na\textsuperscript{−}-free WH medium.

RNA Isolation and Reverse Transcription-PCR Experiments—Total RNA was prepared from isolated mouse elongated spermatids (22) using TRIzol reagent (Sigma) according to the manufacturer’s instructions. cDNA was synthesized from total RNA samples by random hexamer-primed reverse transcription (Superscript II RNase H-Reverse Transcriptase; Invitrogen). cDNA was then subjected to PCR amplification using Tag DNA polymerase (Invitrogen). The ENaC-\(\alpha\) subunit primers were designed using the mouse reported nucleotide sequence for this gene (Scnn1a, NM_011324). Primers for the ENaC-\(\delta\) subunit were designed using the mouse genomic clone sequence AL670236. Primer sequences are ENaC-\(\alpha\), forward, \(5’\)-AAG CCC AAC AAG GTG GTA GAG T-3’, and reverse, \(5’\)-GAT GAG CCG AAC CAG AGG-3’; ENaC-\(\delta\), forward, \(5’\)-CCC AGC CAT AAA CTC-3’, and reverse, \(5’\)-ATC TCC ACC ATC AGC-3’. The absence of genomic contamination in the RNA samples was confirmed with reverse transcription-negative controls (no cDNA) for each experiment (not shown). Ampli-
fied products were analyzed by DNA sequencing to confirm their identity.

**SDS-PAGE and Immunoblotting**—Mouse sperm membranes were obtained by the method described by Hernández-González et al. (23). The sperm membranes were concentrated by centrifugation (100,000 × g) and resuspended in sample buffer (23) without 2-mercaptoethanol and boiled for 5 min. After centrifuging, the supernatants were saved, and 2-mercaptoethanol was added to a final concentration of 5% (v/v); the sample was boiled for 5 min, and then subjected to 10% SDS-PAGE (24). Electrophoretic transfer of proteins to Immobilon P (Bio-Rad) and immunodetection of ENaC subunits were carried out as previously described (25). Immunoblots were developed with the appropriate secondary antibody conjugated to horseradish peroxidase (Sigma) and an ECL kit (Amersham Biosciences) according to the manufacturer’s instructions.

**Indirect Immunofluorescence**—Sperm suspensions were fixed in formaldehyde (1.5% final concentration) for 30 min at room temperature, washed by centrifugation at 800 × g for 5 min, permeabilized in PBS-Triton X-100 (0.05% final concentration) for 15 min at room temperature and washed three times with PBS. Specific primary antibodies were added to sperm samples and incubated overnight at 4 °C, washed three times with PBS, and then incubated with the appropriate secondary antibody (Biotin-conjugated anti-rabbit IgG) for 1 h at 37 °C. The secondary antibody was then subjected to three consecutive washes with PBS and developed by incubation with avidin-fluorescein isothiocyanate diluted in HEPES-saline buffer (20 mM HEPES and 100 mM NaCl, pH 8.2) for 1 h at 37 °C. Finally, the samples were washed and mounted in PBS-glycerol (SlowFade, Molecular Probes) and examined using an epifluorescence microscope.

**Single Cell Fluorescence Analysis of Changes in [Na+]i**—Cauda epididymal mouse sperm incubated in WH medium or Na+-free WH medium were loaded with 10 μM Sodium Green tetraacetate for 30 min at 37 °C and immobilized on a poly-l-lysine-coated glass. To withdraw the dye excess, sperm were washed three times with fresh medium. Fluorescence images were collected for 1 s every 10 s using the excitation/emission pair 470/490 nm on an inverted microscope (IX-70 Olympus) through a 40× objective with a digital CCD camera (Hamamatsu C4742-95, MA). The experiments were performed at 37 °C employing a heating chamber regulated on-line with the system acquisition control. Offline analysis of the collected data were performed using Open Lab (Improvision). At least 30 cells were analyzed in each experiment.

**Electrophysiology**—Spermatogenic cells were obtained following the procedure described by Santi et al. (26). In ~3-month-old mice individual meiotic pachytene spermatocytes and spermatids or their sympathetic mainly constitute the spermatogenic cell suspension. Recordings were performed only on these sytams. Na+ currents were recorded according to the whole cell patch clamp technique (27). In brief, all of the recordings were done at room temperature using an Axopatch 200A amplifier (Axon Instruments) and 2–4 MΩm micropipettes. The cells were clamped at a holding potential of −50 mV. Currents were evoked by 200-ms voltage steps to test potentials ranging from −100 to +40 mV. The currents were captured on-line and digitized at a sampling rate of 10 kHz following filtering of the current record (5 kHz) using a computer attached to a Digidata 1200 interface (Axon). Pulse protocols, data capture, and analysis of recordings were performed using pCLAMP software (Axon). To isolate Na+ currents, the cells were bathed in a solution containing 130 mM NaCl, 3 mM KCl, 10 mM CaCl2, 2 mM MgCl2, 1 mM NaHCO3, 0.5 mM NaH2PO4, 5 mM HEPES, 10 mM glucose (pH 7.4). The internal solution consisted of 110 mM CsMeSO4, 15 mM CsCl, 4.6 mM CaCl2, 10 mM EGTA, HEPES buffer 5 (pH 7.3/CsOH). ENaC channel blockers amiloride and EIPA were prepared as 100 mM stock solutions in Me2SO and diluted in the bath solution for each experiment (Me2SO final concentration < 0.1%).

**Statistical Analysis**—The data are expressed as the means ± S.E. The means were compared using paired Student’s t test, and p < 0.05 was considered to be statistically significant.

**RESULTS**

**Mouse Sperm Resting Membrane Potential Is Na+-dependent**—To investigate whether the Na+ permeability contributes to the resting Erest, Na+ in the incubation medium was replaced by nonpermeant cations such as choline+ or N-methyl-d-glucamine+. Sperm were then diluted in WH medium with differing final Na+ concentrations; the sum of the concentrations of either Na+ and choline+ or Na+ and N-methyl-d-glucamine+ were maintained constant in all cases. Reduction of the extracellular Na+ concentration ([Na+]o) in the incubation medium leads to an Erest hyperpolarization in a concentration-dependent manner (Fig. 1, A and B). These results indicate that Na+ participates in the regulation of the resting Erest in mouse sperm. To directly assay how [Na+]o influences sperm Erest, these cells were recovered in Na+-free WH medium, and increasing concentrations of Na+ were added while the Erest was recorded continuously as described under “Experimental Procedures” (Fig. 1, C and D). Under these conditions a Na+ concentration-dependent depolarization was observed, consistent with the hypothesis that electrogenic Na+ uptake occurs in mouse sperm.

The Na+-induced Depolarization Is Inhibited by Amiloride and Regulated by pH. —The electrogenic Na+ uptake that seems to occur in noncapacitated mouse sperm could be due to Na+-permeable channels or to the Na+/Ca2+ antiporter. Tetrodotoxin and pyrethroid, which both affect voltage-dependent Na+ channels (28, 29), did not alter the Na+-induced depolarizing current when used at 1 and 50 μM, respectively (data not shown). Similarly, a well known inhibitor of Na+/Ca2+ antiporters, KB-R7943 (30), at concentrations up to 10 μM, did not inhibit the Na+ permeability (data not shown). Therefore, it is unlikely that voltage-dependent Na+ channels or the Na+/Ca2+ antiporter are responsible for the Na+-induced depolarization in noncapacitated sperm.

On the other hand, amiloride and the amiloride analog EIPA inhibited the Na+ depolarizing current in a concentration-dependent manner (Fig. 2, A and B). Amiloride and EIPA are known to potently inhibit the ENaC family of Na+ channels with IC50 values similar to those obtained for the inhibition of the Na+-induced depolarization (31, 32). Although known Na+ /H+ antiporters are not electrogenic (33, 34) and cannot be directly responsible for the Na+-induced depolarization, an intracellular pH (pHi) change could modulate other channels. This possibility was discarded by showing that addition of 20 mM [Na+]o to sperm loaded with a pH-sensitive dye (BCECF) and incubated in Na+-free WH medium did not alter pHi (Fig. 2C, left panel). As expected, the addition of NH4Cl increased pHi (Fig. 2C, right panel). Altogether these findings are consistent with the hypothesis that a member of the ENaC family is present in mature mouse sperm.

The activity of ENaC family members is dependent on pH (35–37), and thus we investigated the effect of pHi on the Na+-induced depolarization current. Mouse sperm were incubated for 10 min in Na+-free WH medium buffered at different pH (6.8–7.6), and the Na+-induced depolarization was recorded (Fig. 2, D and E). The observation that the Na+ depolarization current is significantly activated at low pH (6.8) is consistent with ENaC being present in sperm.
To confirm that the depolarization induced by Na\(^+\) is due to Na\(^+\) influx, sperm were loaded with Sodium Green, a Na\(^+\)-specific fluorescent dye. The addition of Na\(^+\) resulted in an increase in the concentration of intracellular Na\(^+\) ([Na\(^+\)]\(_i\)) in the sperm population (Fig. 3, A and B) that could be inhibited by 1 \(\mu\)M EIPA (Fig. 3C). Moreover, the addition of Li\(^+\) did not increase cell fluorescence (Fig. 3D), even though, as shown below, it causes a larger \(E_m\) depolarization than Na\(^+\) (Fig. 4).

Furthermore, [Na\(^+\)]\(_i\), was examined in individual Sodium Green-loaded sperm suspended in Na\(^+\)-free WH medium before (Fig. 3E) and after (Fig. 3E') adding 50 mM NaCl. The relative fluorescence \(R_i\) was quantified independently in the heads and flagella and expressed as \(F/F_0\), where \(F\) = fluorescence intensity after Na\(^+\) addition, \(F_0\) = basal fluorescence intensity. The addition of NaCl increased the \(R_i\) levels in both, flagella and heads (Fig. 3, E, E', and G). This increase was significantly inhibited when sperm were incubated with 1 \(\mu\)M EIPA (Fig. 3, E, F, F', and G). These results are consistent with a role of ENaC channels in the regulation of the sperm resting \(E_m\) and [Na\(^+\)]\(_i\).

The Ion Selectivity of the Amiloride-sensitive Sperm Depolarization Is Consistent with That of ENaCs—ENaCs are more permeable to Li\(^+\) than to Na\(^+\) and are effectively impermeable to Cs\(^+\) or organic ions such as NH\(_4\)Cl (38, 39). In mouse sperm suspended in Na\(^+\)-free WH medium, Li\(^+\) does produce a larger depolarization than Na\(^+\) at the same concentration (Fig. 4), although as expected it does not modify [Na\(^+\)]\(_i\), as measured with Sodium Green (Fig. 3D). This depolarization is amiloride-sensitive (Fig. 4). In contrast, Cs\(^+\) causes a smaller depolarization that is insensitive to amiloride. This shows that sperm possess Cs\(^+\)-permeable ENaC during Sperm Membrane Hyperpolarization

FIGURE 1. Membrane potential (\(E_m\)) in mouse sperm is Na\(^+\)-dependent. A, fluorescence traces showing the values of the sperm resting \(E_m\) obtained at different [Na\(^+\)]\(_e\). Each experiment displays its calibration curve. B, summary of relationship between resting \(E_m\) and [Na\(^+\)]\(_e\) (mean \(\pm\) S.E., \(n\) = 3). C, fluorescence traces and values of the sperm \(E_m\) depolarization induced by increasing [Na\(^+\)]\(_e\). \(\Delta E_m\) represents the difference between the \(E_m\) after Na\(^+\) addition (\(E_{m,n}\)) and the resting \(E_m\) (\(E_{m,0}\)) (\(\Delta E_m = E_{m,n} - E_{m,0} - E_m\)). D, summary of relationship between \(\Delta E_m\) and [Na\(^+\)]\(_e\) (mean \(\pm\) S.E., \(n\) = 3).
The Na\textsuperscript{+}-induced membrane depolarization is inhibited by two epithelial Na\textsuperscript{+} channel antagonists and regulated by extracellular pH. Caudal epididymal mouse sperm were recovered in Na\textsuperscript{+}-free WH medium, and $E_m$ was recorded, and membrane depolarization was induced by adding 20 mM NaCl. Changes in sperm $E_m$ are expressed as $\Delta E_m$, as described in the legend to Fig. 1 and under “Experimental Procedures.” A, records of Na\textsuperscript{+}-induced membrane depolarization in the presence of different EIPA concentrations. B, relationship between EIPA (upper graph) and amiloride (lower graph) concentration and $\Delta E_m$ (mean $\pm$ S.E., $n = 3$). C, the Na\textsuperscript{+}-induced depolarization does not increase pH, (measured with BCECF). Two additions of 20 mM NaCl are shown (left trace) and as control pH, was increased by adding 20 mM NH\textsubscript{4}Cl (right trace). D, the Na\textsuperscript{+}-induced sperm membrane depolarization is regulated by pH\textsubscript{i} (Na\textsuperscript{+}-free WH medium calibrated at different pHs). E, relationship between pH\textsubscript{i} and $\Delta E_m$ (mean $\pm$ S.E., $n = 3$).
ENaC during Sperm Membrane Hyperpolarization

FIGURE 3. [Na\(^{+}\)]\(_i\) increases as a result of the Na\(^{+}\)-induced depolarization. Mouse caudal epididymal sperm were recovered in Na\(^{+}\)-free WH medium and loaded with Sodium Green. Cell population measurements show [Na\(^{+}\)]\(_i\), increases upon the addition of 20 mM (A) or 50 mM NaCl (B), C, the [Na\(^{+}\)]\(_i\), increase caused by adding 50 mM NaCl is inhibited by 1 μM EIPA. D, the addition of 50 mM LiCl does not alter Sodium Green fluorescence. A–D are representative records of three independent experiments. Single cell analysis of Sodium Green loaded sperm before and after adding 20 mM of NaCl, LiCl, or CsCl as indicated in Figs. 1 and 2 and repeated for each cation in the presence of 1 μM EIPA (mean ± S.E., n = 100 cells, from three different experiments). *p < 0.01; **, p < 0.001.

FIGURE 4. Effects of different cations on sperm E\(_m\). Caudal epididymal mouse sperm were recovered in Na\(^{+}\)-free WH medium and their E\(_m\) recorded. E\(_m\) changes (ΔE\(_m\)) were determined before and after adding 20 mM of NaCl, LiCl, or CsCl as indicated in Figs. 1 and 2 and repeated for each cation in the presence of 1 μM EIPA (mean ± S.E., n = 3).

channels insensitive to micromolar concentration of amiloride and indicates that the ion selectivity of the amiloride-sensitive depolarization is in agreement with those of ENaCs.

High pH\(_e\) and Amiloride-related Compounds Hyperpolarize the Mouse Sperm Resting E\(_m\).—The previous results suggest that ENaCs are present in mouse sperm and that they contribute to the resting mouse sperm E\(_m\). We investigated further by incubating these cells in complete WH medium in the absence or in the presence of either amiloride or EIPA. As anticipated, these compounds produced a concentration-dependent hyperpolarization of the sperm E\(_m\) (Fig. 5, A and B). Furthermore, pH\(_e\) also modulated the resting E\(_m\) of sperm suspended in complete WH medium. High pH\(_e\) hyperpolarized sperm (Fig. 5, C, bottom records, and E), whereas low pH\(_e\) increased the depolarized state of resting sperm E\(_m\) (Fig. 5, C, top records, and E). In contrast, mouse sperm incubated in Na\(^{+}\)-free WH medium did not undergo E\(_m\) changes as a function of pH\(_e\) (Fig. 5, D and E). Altogether, these results strongly suggest the presence and involvement of ENaCs in the regulation of the resting E\(_m\) of mouse sperm.

Transcripts of ENaC\(_{\alpha}\) and \(\delta\) Subunits Are Present in Mouse Spermatogenic Cells.—We searched for the presence of the transcripts of the two ENaC conducting subunits, \(\alpha\) and \(\delta\). Primers for the ENaC-\(\alpha\) subunit were designed using the reported mouse nucleotide sequence for this gene (Scnn1a, NM_011324). A fragment for the ENaC-\(\alpha\) subunit of the expected length, 374 bp, was detected, and its identity was confirmed by sequencing (Fig. 6A). However, there is no mouse nucleotide or protein complete sequence reported in any gene data base for ENaC-\(\delta\). To amplify this murine gene we used the human ENaC-\(\delta\) subunit nucleotide sequence (SCNN1D, NM_002978) as a template and searched the different genome data bases. Two relevant sequences were found: a mouse genomic clone sequence (AL670236) from chromosome 4 (region syntenic to 1p36.3-p36.2 where the human ENaC-\(\delta\) subunit is localized) and a short mouse expressed sequence tag similar to the ENaC-\(\delta\) subunit (XM_487838). Reverse transcription-PCR experiments using primers for the ENaC-\(\delta\) subunit designed using these two sequences yielded a fragment of the expected size (282 bp), whose identity was confirmed by sequencing (Fig. 6A).

ENaC-\(\alpha\) and \(\delta\) Subunits Are Present in Mouse Sperm.—We confirmed the presence of Na\(^{+}\) channels of the ENaC type in sperm by Western blot analysis using polyclonal antibodies against the ENaC-\(\alpha\) and ENaC-\(\delta\) subunits. Both antibodies detected bands with molecular masses 80 kDa in kidney extracts (control), testis, and sperm (Fig. 6B). The same antibodies were then used to immunolocalize the ENaC subunits in sperm. Although ENaC-\(\alpha\) was observed in the flagellum mid-piece, ENaC-\(\delta\) antibodies stained the anterior acrosome (Fig. 6, C, and D, respectively). These results and the fact that secondary antibody alone did not stain sperm (Fig. 6E) are compatible with antibody specificity. Neither subunit changed its localization following sperm capacitation (data not shown).

Presence of an Amiloride-sensitive Na\(^{+}\) Inward Current in Spermatogenic Cells.—Because the small size and flattened shape of sperm hinders the direct electrophysiological characterization of their ionic channels, spermatogenic cells, the progenitors of sperm, were used for patch clamp studies (12, 26, 27). In these experiments symplasts of mainly pachytene spermatocytes or round spermatids were employed. Fig. 7A shows a family of representative Na\(^{+}\) currents recorded on such a symplast. The currents were evoked by 200-ms test pulses between −100 and −40 mV in 10-mV increments from a holding potential of −50 mV. Test pulses elicited rapidly activating and nonactivating Na\(^{+}\) currents (control, upper traces). Na\(^{+}\) current amplitude was significantly reduced (~50%) by adding amiloride (1 μM) to the external solution (middle traces), and this inhibition was completely reversible. Fig. 7B shows the current-voltage (I-V) relationship of the Na\(^{+}\) currents above described. Amiloride (1 μM) decreased the normalized I-V curve (Fig.
ENaC during Sperm Membrane Hyperpolarization

7B, control, closed circles, versus amiloride, open circles). The normalized amiloride-sensitive component of the whole cell Na\(^+\) currents in spermatogenic cells (Fig. 7B, inverted open triangles) has a mean reversal potential \( (V_{\text{rev}}) \) of +5 mV when recorded using isotonic (≈300 mOsm) NaCl bath and CsCl pipette solutions, which is consistent with the biophysical properties of other ENaC channels (40). The amiloride-induced inhibition reverses upon washing (Fig. 7B, inverted closed triangles). EIPA, an amiloride analog and more specific ENaC channel blocker, also consistently inhibited the Na\(^+\) currents in spermatogenic cells by ~30% at 1 \( \mu \text{M} \) (data not shown). Amiloride inhibition revealed

FIGURE 5. Antagonists of ENaCs induce membrane hyperpolarization in noncapacitated sperm. Mouse caudal epididymal sperm were recovered in regular WH medium and the sperm \( E_{\text{m}} \), determined as in Figs. 1 and 2. A, addition of 1 \( \mu \text{M} \) EIPA hyperpolarizes noncapacitated sperm. B, relationship between EIPA (left) and amiloride (right) concentration and resting \( E_{\text{m}} \) (means ± S.E., n = 3). C, pH\(_{\text{e}}\) regulates the resting \( E_{\text{m}} \) of noncapacitated sperm in WH medium. D, representative records of the resting \( E_{\text{m}} \) of sperm incubated in the absence of NaCl at different pH\(_{\text{e}}\). E, relationship between pH\(_{\text{e}}\) and resting \( E_{\text{m}} \) (means ± S.E., n = 3) in the presence (▲) or absence (○) of 100 mM NaCl.
ENaC during Sperm Membrane Hyperpolarization

**FIGURE 6.** Presence of ENaC-α and ENaC-δ transcripts in mouse spermatogenic cells and corresponding proteins in mature sperm. A, reverse transcription-PCR showing ENαC-α (374 bp) and ENαC-δ (282 bp) transcripts in RNA isolated from elongated spermatids. The identity of both PCR fragments was confirmed by sequencing. B, Western blots detecting ENαC-α and ENαC-δ in 100-μg samples of sperm membranes (S), 30 μg of testis extract (T), and 30 μg of kidney extract (K). Bands of ~80 kDa were detected in mouse sperm, testis, and kidney using polyclonal antibodies against rat ENαC-α and human ENαC-δ. C–E, presence and localization of ENαC-α and ENαC-δ subunits in fixed mouse sperm revealed with a Biotin-labeled secondary antibody/Avidin-fluorescein isothiocyanate detection system. ENαC-α is localized in the midpiece (C), whereas ENαC-δ is present in the acrosomal region (D), E, control with the secondary antibody. C', D', and E' are the phase contrast images of C, D, and E, respectively. B–E are representative of at least three independent experiments.

**FIGURE 7.** Spermatogenic cells express an amiloride-sensitive component of their Na⁺ currents. A, representative whole cell current traces in absence (control, upper traces), presence (middle traces) and after washing (lower traces) of 1 μM amiloride in mouse spermatogenic cells. The currents were evoked by 200-ms test pulses from a holding potential of −50 mV to voltages between −100 to +40 mV in 10-mV increments. B, mean whole cell current-voltage (I-V) relationships recorded as in A. The addition of 1 μM amiloride (open circles) reduced the normalized current ∼50% with respect to the control (closed circles). The current inhibition by amiloride is completely reversible (inverted closed triangles). The amiloride-sensitive component of spermatogenic cell currents is represented as inverted open triangles. C, amiloride inhibition revealed two spermatogenic cell subpopulations of differing sensitivity. In the more sensitive subgroup (left bars), Na⁺ currents were inhibited ∼50% by 1 μM amiloride. Adding 20 μM amiloride or 50 μM EIPA, a more specific amiloride analog for ENαC channels, did not significantly increase current inhibition. In the less sensitive group (right bars), the addition of either 1 or 20 μM of amiloride or 50 μM of EIPA inhibited ∼20% of the Na⁺ currents. In all plots, the symbols and bars represent the means ± S.E. (n = 8).

two spermatogenic cell populations with differential sensitivity. As previously mentioned, in the more sensitive category, the Na⁺ currents were inhibited ∼50% by 1 μM amiloride (Fig. 7C, Subgroup 1). Adding 20 μM amiloride or 50 μM EIPA did not cause a statistically significant increase in Na⁺ current inhibition, suggesting that 1 μM amiloride produces close to maximal inhibition in this spermatogenic cell subpopulation. Adding 1 or 20 μM amiloride or 50 μM EIPA inhibited only ∼20% of the Na⁺ currents in the less sensitive cellular subpopulation (Fig. 7C, Subgroup 2). The difference in inhibition between these ENαC blocker concentrations was not statistically significant. Taken together, these findings indicate that ENαC channels are responsible for an important component of Na⁺ currents in germ cells and are consistent with their presence in mature sperm.

Decreased Na⁺ Permeability Is Involved in the Regulation of the Capacitation-associated Hyperpolarization—If a decrease in Na⁺ permeability mediates the capacitation-associated hyperpolarization, the Na⁺-induced depolarization observed in Fig. 1C should be reduced in capacitated sperm when compared with noncapacitated cells. However, sperm incubated in Na⁺-free WH medium would not undergo capacitation (4). Therefore, to test this hypothesis two alternative approaches were assayed. First, because permeable cAMP analogs are able to induce capacitation in the absence of [Na⁺]e, dibutyryl-cAMP (1 mM), and 3-isobutyl-1-methylxanthine (100 μM) were added to sperm incubated in Na⁺-free WH medium (4). Under these conditions, the Na⁺-induced depolarization was significantly inhibited (Fig. 8, A and B). Second, the sperm were incubated under capacitating and noncapacitating conditions for 1 h; thereafter, they were centrifuged and diluted >20 times in Na⁺-free WH medium (final [Na⁺]e, ∼5 mM). Under these conditions, the Na⁺-induced depolarization was significantly inhibited in capacitated but not in the noncapacitated sperm populations (Fig. 8, C and D).
Altogether, these observations indicate that Na\(^+\) influx is inhibited in capacitated sperm.

**DISCUSSION**

In the present work, we have analyzed whether a decrease in Na\(^+\) permeability contributes to the capacitation-associated hyperpolarization. We measured sperm plasma membrane \(E_m\) with a fluorescent dye that has previously been extensively employed in studies of mammalian sperm (4, 9, 10, 21). The addition of \(m\)-chlorophenylhydrazone, a mitochondrial uncoupler, 2 min before the fluorimetric measurements precludes the contribution of mitochondrial \(E_m\) to the final calibration. The calibration procedure followed in this study compensates for variation in sperm concentration and viability and assures an accurate comparison of the average sperm population \(E_m\) between different experimental conditions. Using this methodology, we have demonstrated that replacing Na\(^+\) with nonpermeant cations such as choline\(^+\) or N-Met-Glut\(^+\) significantly hyperpolarizes the sperm \(E_m\). Moreover, the addition of Na\(^+\) to sperm incubated in Na\(^-\)free medium resulted in an increase in [Na\(^+\)], as measured with Sodium Green and a depolarization, further indicating that an electrogenic Na\(^+\) uptake system is present and active in mouse sperm. Interestingly, the Na\(^+\)-induced depolarization is long lasting, implying the presence of a sustained Na\(^+\) permeability in non-capacitated sperm.

Because of existing evidence for their presence in sperm, the main electrogenic Na\(^+\) transport systems considered as candidates for the Na\(^+\)-induced depolarization were: voltage-dependent Na\(^+\) channels (41), Na\(^+\)/Ca\(^2+\) exchangers (42), Na\(^+\)/K\(^+\) ATPases (43), transient receptor potential channels of the C type (44–46) and polycystins (PCs) (47, 48), and Na\(^+\) channels from the degenerin/ENaC superfamily (49). The Na\(^+\)-induced depolarization observed in mouse sperm has many of the signature properties of ENaC channels: 1) it is blocked by submicromolar amiloride and its analog EIPA, whereas the IC\(_{50}\) values for PC1–PC2 and transient receptor potential channels are greater than 50 \(\mu M\) (50, 51); 2) it is activated by external acidification (pH\(_i\) < 7.4); in contrast, PC1–PC2 and transient receptor potential channels are not activated by external acidification; and 3) it has a similar ion selectivity as ENaCs in other cell types; namely Li\(^+\) > Na\(^+\) > Cs\(^+\) or K\(^+\) (38, 39). Although sperm in Na\(^-\)free WH medium underwent a small depolarization upon Cs\(^+\) addition, this \(E_m\) change was insensitive to EIPA, suggesting that ENaCs are not responsible. Moreover, such electrogenic Na\(^+\) uptake could be due to Na\(^-\)-permeable channels or to the Na\(^+\)/Ca\(^2+\) antiporter. Tetrodotoxin and pyrethroid, both of which affect voltage-dependent Na\(^+\) channels (28, 29), did not alter the Na\(^+\)-induced depolarizing current when used at 1 and 50 \(\mu M\) respectively (data not shown). Similarly, a well known inhibitor of Na\(^+\)/Ca\(^2+\) antiporters, KB-R7943 (30), at concentrations up to 10 \(\mu M\), did not inhibit the Na\(^+\) permeability (data not shown). Therefore, it is unlikely that voltage-dependent Na\(^+\) channels or Na\(^+\)/Ca\(^2+\) antiporters are responsible for the Na\(^+\)-induced depolarization in non-capacitated sperm. Finally, the addition of Na\(^+\) to sperm does not increase their pH\(_i\), indicating that this depolarization is not related to a Na\(^+\)/H\(^+\) antiporter activity. Thus, these measurements and their pharmacological profile are consistent...
ENaC during Sperm Membrane Hyperpolarization

with the hypothesis that ENaCs are functionally present in mature sperm. Interestingly, ENaCs are modulated by diverse mechanisms such as phosphorylation, pH, and insertion into the plasma membrane, where when open, they regulate the resting $E_m$ (31, 38). This ENaC property is consistent with the depolarized resting $E_m$ observed in non-capacitated sperm.

ENaC is a heteromultimeric channel that can be formed by the combination of four subunits: $\alpha$, $\beta$, $\gamma$, and $\delta$, where $\alpha$ and $\delta$ are directly involved in forming the channel pore (38, 39). A previous investigation reported the presence of the ENaC-$\delta$ subunit in human testis (52), although this subunit has not previously been reported in mouse. We have found a sequence similar to the human $\delta$ subunit in the mouse genome; in a BLAST search, this sequence matched a mouse testis $\delta$ subunit, indicating the presence of ENaC-$\delta$ in mouse testis. A previous investigation reported the presence of ENaC-$\alpha$ and ENaC-$\gamma$ localize to the midpiece and the anterior head of mouse sperm, respectively. To the best of our knowledge, this is the first report showing the presence of the ENaC-$\delta$ subunit at the transcript or protein level in mouse, specifically in spermatogenic cells and mature sperm. Furthermore, adding Na$^+$ as a permeant in Na$^+$-free WH medium revealed an amiloride-sensitive [Na$^+$]$_i$, increase both in suspensions and in single cells in the head and midpiece, the regions where ENaC subunits were found. However, ENaC-$\alpha$ and ENaC-$\gamma$ subunits detected in testis, ovary, brain, and pancreas (37, 52–54), suggesting that ENaCs in mouse sperm might be homomeric channels constituted by ENaC-$\alpha$ or ENaC-$\gamma$. It should be pointed out that homomeric channels formed by ENaC-$\alpha$ or ENaC-$\gamma$ are able to conduct Na$^+$ (37, 53). Why do mouse spermatozoa display different ENaC channels associated to distinct sperm regions? Sperm could require local [Na$^+$]$_i$ to modulate specific ion transporters, a possibility worth investigating further.

Are ENaCs involved in the regulation of the capacitation-associated hyperpolarization? As mentioned, the sperm $E_m$ hyperpolarizes when Na$^+$ is replaced by a nonpermeable cation. In addition, the conditions that inhibit the Na$^+$-induced depolarization, such as the presence of amiloride or high pH$_e$, induce a sustained sperm hyperpolarization with values similar to those observed in a capacitated sperm population (4, 9, 16). Closing of Na$^+$ channels during capacitation would explain, at least in part, the observed sperm hyperpolarization that accompanies this process. Furthermore, incubation of sperm under capacitating conditions inhibited the Na$^+$-induced depolarization. Moreover, the addition of cAMP-permeable agonists to sperm incubated in Na$^+$-free WH medium, an experimental setting that would overcome the absence of Na$^+$ in the capacitation medium (4), also inhibited the Na$^+$-induced depolarization. On the other hand, mouse sperm incubated under conditions that do not support capacitation maintain a depolarized $E_m$ and Na$^+$ influx is not inhibited.

The mechanisms that regulate ENaC activity during capacitation are unknown. Because cAMP analogs are able to inhibit the Na$^+$ depolarization current, it is possible that the cAMP pathway regulates ENaCs. Capacitation is associated with an elevation of the cAMP levels as well as with an increase in protein tyrosine phosphorylation. It is noteworthy that elevated cAMP levels inhibit ENaCs in other cell types. This inhibition can be caused by activation of the cystic fibrosis transmembrane regulator, which increases Cl$^-$ uptake (55, 56). Cystic fibrosis transmembrane regulator mRNA has been detected in testis and more specifically in spermatogenic cells (57, 58). However, immunohistochemical analysis performed with cystic fibrosis transmembrane regulator-specific antibodies revealed immunoreactivity in round and elongated spermatids, but not in mature sperm (58). This matter deserves further examination in the future, although other Cl$^-$ transporters or mechanisms could be involved in the regulation of ENaCs in sperm.

The electrogenic Na$^+$ transport produced by ENaCs depends on the extracellular environment; changes in [Na$^+$]$_e$ or pH$_e$ affect ENaC activity (35, 38). A recent report shows that a decrease in pH$_e$ activates the human ENaC-$\delta$ channel (36, 37). These data together with the pH$_e$ dependence and pharmacology of the sperm Na$^+$ permeability are consistent with the presence of ENaC-$\delta$ in mouse sperm. The pH$_e$ and Na$^+$ dependence of $E_m$ in noncapacitated sperm could also be explained by the participation of acid-sensitive degenerin/epithelial Na$^+$ channels. Such acid-sensing ion channels are only transiently activated by a drop in pH$_e$ (59, 60). In contrast, the sperm depolarization induced by increasing pH$_e$ is sustained, indicating the presence of an active Na$^+$ permeability, possibly because of ENaC-$\delta$. In whichever case, the molecular events that regulate ENaCs in mammalian sperm and their participation in capacitation require further investigation.

Acknowledgment—We thank Dr. Christopher Wood for suggestions and Andres Saralegui for help with the confocal microscopy.

REFERENCES

1. Yanagimachi, R. (1994) in The Physiology of Reproduction (Knobil, E., and Neill, J. D., eds) Vol. 1, pp. 189–317, Raven Press, Ltd., New York.
2. Visconti, P. E., Galantino-Homer, H., Ning, X., Moore, G. D., Valenzuela, J. P., Jorgez, C. J., Alvarez, J. G., and Kopf, G. S. (1999) J. Biol. Chem. 274, 3235–3242.
3. Visconti, P. E., Ning, X., Forbes, M. W., Alvarez, J. G., Stein, P., Connors, S. A., and Kopf, G. S. (1999) Dev. Biol. 214, 429–443.
4. Demarco, I. A., Espinosa, F., Edwards, J., Sosnik, J., De La Vega-Beltran, J. L., Hockensmith, J. W., Kopf, G. S., Darszon, A., and Visconti, P. E. (2003) J. Biol. Chem. 278, 7001–7009.
5. Visconti, P. E., Bailey, J. L., Moores, G. D., Pan, D., Olsds-Clarke, P., and Kopf, G. S. (1995) Development 121, 1129–1137.
6. Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olsds-Clarke, P., and Kopf, G. S. (1995) Development 121, 1139–1150.
7. ficarro, S., Chertiinh, O., Westbrook, V. A., White, F., Jones, F., Kalab, P., Marto, J. A., Shabanowitz, J., Herr, J. C., Hunt, D., and Visconti, P. E. (2005) J. Biol. Chem. 278, 11579–11589.
8. Visconti, P. E., Westbrook, V. A., Chertiinh, O., Demarco, I., Sleight, S., and Diekman, A. B. (2002) J. Reprod. Immunol. 53, 133–150.
9. Zeng, Y., Clark, E. N., and Florman, H. M. (1995) Dev. Biol. 171, 554–563.
10. Muñoz-Garay, C., De la Vega-Beltran, J. L., Delgado, R., Labarca, P., Felix, R., and Darszon, A. (2001) Dev. Biol. 234, 261–274.
11. Florman, H. M., Arnould, C., Kazam, J. G. L., Li, C., and O’Toole, C. M. (1998) Biol. Reprod. 59, 12–16.
12. Liewano, A., Santti, C. M., Serrato, C. J., Trevino, C. L., Bellve, A. R., Hernandez-Cruz, A., and Darszon, A. (1996) FEBS Lett. 381, 150–154.
13. Arnould, C., Villaz, M., and Florman, H. M. (1998) Mol. Pharmacol. 53, 1104–1111.
14. Arnould, C., Cardullo, R. A., Lemos, J. R., and Florman, H. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13004–13009.
