IN Volvement of a Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{-} Cotransporter in Mouse Sperm Capacitation

Ignacio A. Demarco\textsuperscript{1}, Felipe Espinosa\textsuperscript{3}, Jennifer Edwards\textsuperscript{1}, Julián Sosnik\textsuperscript{1}, José Luis de la Vega-Beltrán\textsuperscript{3}, Joel W. Hockensmith\textsuperscript{2}, Gregory S. Kopf\textsuperscript{4,6}, Alberto Darszon\textsuperscript{3} and Pablo E. Visconti\textsuperscript{1,5}.

\textsuperscript{1}Center for Res. in Contraception and Reprod. Health (CRCRH), Dept. of Cell Biol., \textsuperscript{2}Dept. of Biochemistry and Molecular Genetics, U. of Virginia, Charlottesville, VA 22908. 

\textsuperscript{3}Universidad Autonoma de Mexico. \textsuperscript{4}Center for Research in Reproduction and Women’s Health. U. of Pennsylvania, Philadelphia, PA 19104.

**Running title:** Role of Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{-} cotransporter in sperm

Keywords: sperm, capacitation, bicarbonate, sodium, cotransporter, membrane potential.

\textsuperscript{5}Correspondence to: Pablo E. Visconti  
Dep. of Cell Biology  
U. of Virginia  
1300 Jefferson Park Ave.  
Charlottesville, VA 22908  
USA  
Tel: (434) 243-4825  
FAX: (434) 982-3912  
Email: pv6j@virginia.edu

\textsuperscript{6}Present address of GSK: Wyeth Ayerst P.O. Box 8299, Philadelphia, PA 19101-8299
SUMMARY

Mammalian sperm are incapable of fertilizing eggs immediately after ejaculation; they acquire fertilization capacity after residing in the female tract for a finite period of time. The physiological changes sperm undergo in the female reproductive tract that render sperm able to fertilize constitute the phenomenon of "sperm capacitation". We have demonstrated that capacitation is associated with an increase in the tyrosine phosphorylation of a subset of proteins and that these events are regulated by a HCO$_3^-$/cAMP-dependent pathway involving protein kinase A (PKA).

Capacitation is also accompanied by hyperpolarization of the sperm plasma membrane. Here we present evidence that, in addition to its role in the regulation of adenylyl cyclase, HCO$_3^-$ has a role in the regulation of plasma membrane potential in mouse sperm. Addition of HCO$_3^-$ but not Cl$^-$ induces a hyperpolarizing current in mouse sperm plasma membranes. This HCO$_3^-$-dependent hyperpolarization was not observed when Na$^+$ was replaced by the non-permeant cation choline$^+$. Replacement of Na$^+$ by choline$^+$ also inhibited the capacitation-associated increase in protein tyrosine phosphorylation as well as the zona pellucida-induced acrosome reaction. The lack of an increase in protein tyrosine phosphorylation was overcome by the presence of cAMP agonists in the incubation medium. The lack of a hyperpolarizing HCO$_3^-$ current and the inhibition of the capacitation-dependent increase in protein tyrosine phosphorylation in the absence of Na$^+$ suggest that a Na$^+$/HCO$_3^-$ cotransporter is present in mouse sperm and is coupled to events regulating capacitation.
INTRODUCTION

Upon ejaculation, mammalian sperm are not able to fertilize; they become fertilization competent during transit through the female reproductive tract (1). The molecular, biochemical, and physiological changes that occur in sperm while in the female tract are collectively referred to as capacitation. During capacitation, changes in membrane dynamics and properties, enzyme activities, and motility render spermatozoa responsive to stimuli that induce the acrosome reaction and prepare these cells for penetration of the egg vestments prior to fertilization. These changes are facilitated by the activation of sperm cell signaling cascades during residence of these cells in the female reproductive tract in vivo or in defined media in vitro. Molecular events implicated in the initiation of capacitation have been partially defined, and include the removal of cholesterol by cholesterol acceptors such as bovine serum albumin (BSA) from the sperm plasma membrane, modifications in plasma membrane phospholipids, fluxes of $\text{HCO}_3^-$ and other intracellular ions, and increased tyrosine phosphorylation of proteins. These events are likely involved in the induction of hyperactivation and the acrosome reaction (for review see Visconti et al., (2)).

Mammalian sperm capacitation is also accompanied by the hyperpolarization of the sperm plasma membrane (3). Hyperpolarization is observed as an increase in the intracellular negative charges when compared with the extracellular environment. Although it is not clear how sperm plasma membrane potential is regulated during capacitation, it appears that membrane hyperpolarization may be partially due to an enhanced $\text{K}^+$ permeability as a result of a decrease in inhibitory modulation of $\text{K}^+$ channels (3). Recently, Muñoz-Garay et al. (4) demonstrated with patch clamp techniques that inward rectifying $\text{K}^+$ channels are expressed in mouse spermatogenic cells and proposed that these channels may be responsible for the
capacitation-associated membrane hyperpolarization. Interestingly, Ba$^{2+}$ blocks these K$^{+}$ channels with an IC$_{50}$ similar to that shown to inhibit the capacitation-associated hyperpolarization and the zona pellucida-induced acrosome reaction (4). The functional role of sperm plasma membrane hyperpolarization during capacitation is at present not well understood. However, one may speculate that since capacitation prepares the sperm for the acrosome reaction, capacitation-associated membrane hyperpolarization may regulate the ability of sperm to generate transient Ca$^{2+}$ elevations during acrosome reaction by physiological agonists (e.g. zona pellucida of the egg or progesterone) (5). This hypothesis is consistent with the presence of low voltage-activated (LVA) Ca$^{2+}$ T channels in spermatogenic cells (6,7) that may also be present in mature sperm.

Numerous studies have demonstrated that capacitation is a HCO$_3^-$-dependent process (8-12). However, little is known about the mechanisms of HCO$_3^-$ transport in sperm. The ability of 4,4'-diiodothiocyanatostilbene-2,2'-disulfonic acid (DIDS), a well-known inhibitor of anion transporters, to inhibit the actions of HCO$_3^-$ on various sperm functions suggests that sperm contain functional anion transporters (13-16). The transmembrane movement of HCO$_3^-$ has been associated with the increase in intracellular pH (pHi) observed during capacitation (17,18) and with the regulation of cAMP metabolism (19). Adenylyl cyclase, the enzyme responsible for cAMP synthesis, is markedly stimulated by physiologically relevant HCO$_3^-$ concentrations (15,20,21) and is likely to be the testicular HCO$_3^-$-dependent adenylyl cyclase (sAC) (22) (23,24).

In the present work, we have investigated the role of bovine serum albumin (BSA) and HCO$_3^-$ in plasma membrane hyperpolarization of mouse sperm during capacitation. We have demonstrated that both BSA and HCO$_3^-$ in the incubation media are necessary for the
capacitation-associated membrane hyperpolarization. In addition, we have shown that HCO₃⁻ directly induced hyperpolarization of the mouse sperm plasma membrane. This HCO₃⁻-induced hyperpolarization is Na⁺-dependent, results in an increase in pHi and is inhibited by DIDS. Altogether these data demonstrate the presence of a Na⁺/HCO₃⁻ cotransporter in mouse sperm and suggest its involvement in the regulation of sperm plasma membrane hyperpolarization and additional events leading to the capacitation state.

EXPERIMENTAL PROCEDURES

**Materials.** Chemicals were obtained from the following sources: dibutyryl cyclic AMP (dbcAMP), Rp-cAMPS, 3-isobutyl-1-methylxanthine (IBMX), cholesterol-3-sulfate (cholesterolSO₄⁻), 2-hydroxy-propyl-beta-cyclodextrin (2-OH-p-β-CD), carbonyl cyanide m-chlorophenylhydrazone (CCCP), valinomycin, nigericin, ouabain, H89, 4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid (DIDS), 4-acetamido-4’-isothiocyanatostilbene-2,2’-disulfonic acid (SITS), choline chloride (choline+Cl⁻), choline bicarbonate (choline+HCO₃⁻), trimethylamine hydrochloride (TMA), and water used to make Whitten’s media (water for embryo transfer) were purchased from Sigma; the fluorescent dyes 3,3’-dipropylthiadicarbocyanine iodide [DiSC₃(5)], bis-(1,3-diethylthiobarbituric acid trimethine oxonol [DiSBAC₂(3)], and 2’,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) were purchased from Molecular Probes.

Procedures for preparing stock solutions varied according to solubility and the desired final concentrations. The following were prepared in dimethyl sulfoxide (DMSO) at the stock concentrations noted between parentheses and stored at −20 °C except when otherwise stated: cholesterolSO₄⁻ (100 and 10 mM), H89 (10 mM), IBMX (200 mM made fresh the day of the experiment), DiSC₃(5), DiSBAC₂(3), BCECF-AM and CCCP (all 1 mM), valinomycin (2 mM),
ouabain (1, 5, and 10 mM), and DIDS and SITS (both 100 mM). Rp-cAMPS (100 mM), and dbcAMP (100 mM made fresh the day of the experiment) were prepared in stock solutions with Whitten’s medium. 2-OH-p-β-CD was added directly to Whitten’s medium for a final concentration of 1 mM. Solutions of 800 mM TMA, choline⁺Cl⁻, choline⁺HCO₃⁻, and NaHCO₃ were made the day of the experiment in deionized water and buffered with 50 mM HEPES. Nigericin was dissolved in ethanol to a stock concentration of 5 mM.

**Preparation of Sperm.** In most experiments, cauda epididymal mouse sperm were collected from CD1 retired male breeders by placing minced cauda epididymis in a modified Krebs-Ringer medium (Whitten's-HEPES-buffered) (25). This medium, which does not support capacitation, was first prepared in the absence of bovine serum albumin (BSA) and NaHCO₃, and contains 1 mM polyvinyl pyrrolidone (average MW: 40,000). After 5 min. sperm in suspension were washed in 10 ml of the same medium by centrifugation at 800 x g for 10 min. at room temperature (RT)(24°C). Sperm were then resuspended to a final concentration of 2 x 10⁷ cells/ml and diluted ten times in the appropriate medium depending on the experiment performed.

In experiments where capacitation was investigated, 5 mg/ml of BSA and 24 mM of NaHCO₃ were added; to compensate for the addition of NaHCO₃, 24 mM NaCl was added to the control medium. In all cases pH was maintained at 7.2. To study the role of Na⁺ in capacitation and in the regulation of the membrane potential, NaCl was replaced by choline⁺Cl⁻ up to the concentration indicated in the respective experiment. Other Na⁺ salts present in Whitten's-HEPES were replaced with their respective K⁺ salts and the total K⁺ concentration was maintained at 5.9 mM. Since β-cyclodextrins are able to replace BSA as a cholesterol sink and capacitate mouse sperm (26), in some experiments these compounds were used to analyze the role of plasma membrane cholesterol release in different signaling events.
**SDS-PAGE and immunoblotting.** After incubation under different experimental conditions, the sperm were concentrated by centrifugation, washed in 1 ml of phosphate buffered saline (PBS) containing 1 mM sodium orthovanadate, resuspended in sample buffer (Laemmli, 1970) 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%. The samples were boiled for 5 min. and subjected to 10% SDS-PAGE (27). Electrophoretic transfer of proteins to Immobilon P (Bio-Rad, Hercules, CA) and immunodetection of tyrosine phosphorylated proteins and hexokinase type 1 were carried out using anti-phosphotyrosine (α PY) monoclonal antibodies (Clone 4G10, UBI, Lake Placid) and a polyclonal anti-hexokinase type 1 (α HK1) as previously described (28). Immunoblots were developed with the appropriate secondary antibody conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) and an ECL kit (Amersham corp., Arlington Heights, IL) according to the manufacturer’s instructions.

**Membrane Potential Assay in Cell Populations.** A number of fluorescent indicator dyes have been commonly used by several laboratories to evaluate membrane potential. DiSBAC$_2$(3) and DiSC$_3$(5) have been successfully used in mammalian sperm (3,29). These fluorescent dyes are charged and distribute across cellular membranes in response to electrochemical gradients. As a result of binding to intracellular proteins, the dyes undergo quenching of the fluorescent emission and exhibit a slight shift in their spectrum (30). Because DiSBAC$_2$(3) is an anionic oxonol and DiSC$_3$(5) is a cationic carbocyanine dye, they distribute in opposite directions across the cell membrane at equivalent membrane potentials. Neither dye has toxic effects on sperm function at the concentrations used (< 5 µM) (3). When constant concentrations of sperm and probe are used, these dyes provide reproducible estimates of plasma membrane potential.
Both fluorescent dyes possess advantages and disadvantages in sperm. For the most part, we have used DiSC₃(5) due to its greater signal and the convenience of calibration using valinomycin. One problem with the use of DiSC₃(5) is that this cationic dye binds to mitochondria in their normal energized state; this can contribute up to 20-30% of the fluorescence signal (3). To alleviate this problem, recordings were initiated after dissipating the mitochondrial membrane potential with 1 µM CCCP. Under these conditions, the plasma membrane potential of valinomycin-treated mouse sperm responds to the extracellular K⁺ concentration following the Nernst equation. In selected experiments, a set of parallel membrane potential determinations were carried out using DiSBAC₂(3), which is negatively charged, distributes across the membrane in the opposite direction, and does not bind to mitochondria; these experiments were performed as controls to the determinations performed with the cationic DiSC₃(5). DiSBAC₂(3) has the disadvantage that it forms an insoluble complex with valinomycin, thus complicating calibration of plasma membrane potential.

Sperm were collected as described above and after dilution in the appropriate medium, capacitated for different time periods. Eight min. before the measurement, 1 µM DiSC₃(5) (final concentration) was added to the sperm suspension and further incubated for 5 min. at 37°C and, when used, 1 µM CCCP (final concentration) was added and the sperm incubated for 2 additional min. After this period, 1.5 ml of the suspension was transferred to a gently stirred cuvette at 37°C and the fluorescence (620/670 nm excitation/emission) was recorded continuously. After reaching a steady fluorescence (usually after 1-2 min.), calibration was performed by adding 1 µM valinomycin and sequential additions of KCl (29). The initial suspension contains 5.9 mM KCl; additional KCl was added to the final concentrations of 8, 12, 20 and 36 mM KCl, corresponding to plasma membrane potentials of -80, -72, -61, -47 and -32 mV respectively.
These values were obtained using the Nernst equation, assuming an intracellular K⁺ concentration of 120 mM (31) and considering that, in the presence of CCCP and valinomycin, the membrane potential corresponds to the K⁺ equilibrium. The sperm membrane potential in each case was linearly interpolated using these data as plasma membrane potential vs arbitrary units of fluorescence (Fig. 1). The fluorescence emission of DiSC₃(5) depends on variables such as sperm number, dye concentration, sperm viability, fluorimeter cuvette dimensions and presence of compounds that interact with DiSC₃(5). The internal calibration of each single determination compensates for variables that influence the absolute fluorescence values. As mentioned before, in some cases membrane potential was measured using DiSBAC₂(3). In these cases, sperm were incubated as described above, except that 0.5 µM of DiSBAC₂(3) was added 5 min. before the fluorescence measurements and fluorescence (535/560 nm excitation/emission wavelength pair) was recorded.

**Intracellular pH measurements.** For this purpose, the pH-sensitive dye BCECF was used (32). Sperm (1x 10⁶) in Whitten’s medium were incubated for 5 min. in 4 µM BCECF-AM, the permeant nonfluorescent precursor of BCECF. Once within the cell, nonspecific esterases hydrolyze the acetoxy methyl ester (AM), yielding the non-permeant fluorescent indicator. The low leakage rate of BCECF and the small intracellular volume result in the final intracellular concentration being much higher than the external incubation concentration. After incubation, cells were washed in fresh medium once (400 x g for 5 min.) and 1.5 ml of this suspension was placed in a gentle-stirring cuvette for fluorescence measurements. The pH-dependent spectral shift exhibited by BCECF allows calibration of the pH response in terms of the ratio of fluorescence intensities measured at two different excitation wavelengths (510/450 nm, fixed emission at 540 nm). Calibration was performed by the null point determination as described by
Babcock (31). In those experiments in which the effect of the Na⁺ concentration was assayed, the sperm were collected and washed in Na⁺-free medium (Whitten’s/choline⁺).

Assay for acrosome reaction. On the premise that only capacitated sperm will undergo exocytosis in response to zona pelludidae, the zona pellucida-induced acrosome reaction in sperm was analyzed as end point of capacitation. Zona pellucidae were prepared from homogeneized ovaries of virgin female 22-day old outbred CD1 mice (Charles River) as described (33) and solubilized for all experiments by the procedures outlined previously (11). The percentage of acrosome reaction was measured using Coomasie blue G-250 as described (34). Briefly, sperm were incubated at 37°C for 45 min. followed by the addition of 5 zona pellucida equivalents/µl. After additional 30 min. of incubation at 37°C, an equal volume of 2X fixative solution (10% formaldehyde in phosphate-buffered saline) was added to each tube. Following fixation, 10 µl aliquots of suspension were spread onto glass slides and air-dried. The slides were then stained with 0.22 % Coomasie blue G-250 in 50 % methanol and 10 % glacial acetic acid for 3-5 min., gently rinsed with deionized H₂O, air-dried, and mounted with 50 % (v/v) glycerol in PBS. To calculate the percentage of acrosome reaction, at least 100 sperm were assayed per experimental condition for the presence or absence of the characteristic dark blue acrosomal crescent.

RESULTS

Cholesterol acceptors and HCO₃⁻ are necessary for the capacitation-associated membrane hyperpolarization

As mentioned above, HCO₃⁻ and BSA are necessary for capacitation. BSA has been hypothesized to function as a cholesterol acceptor for release of this sterol from the sperm plasma membrane, and HCO₃⁻ has a role in the regulation of sperm pHᵢ and cAMP metabolism. Hyperpolarization of the sperm plasma membrane has also been associated with capacitation.
However, it is not established whether hyperpolarization is upstream, downstream or independent of the other signaling pathways involved in capacitation.

β-cyclodextrins are cyclic hepta saccharides known for their ability to bind cholesterol; we have previously demonstrated that in the presence of these compounds cholesterol is released from the sperm plasma membrane. This cholesterol-efflux initiates signaling events leading to capacitation (26). When BSA is replaced by 1 mM 2-OH-p-β-CD in complete medium, cauda epididymal mouse sperm are hyperpolarized after 45 min. of incubation, similarly to the control using 5 mg/ml of BSA (Fig. 2 and data not shown). In contrast, in media lacking BSA or β-cyclodextrin the capacitation-associated hyperpolarization did not occur (Fig. 2). In addition, when the cholesterol-binding sites of BSA were saturated with 5 µM cholesterolSO₄⁻, the capacitation-associated hyperpolarization of the sperm plasma membrane was inhibited (Fig. 3). This concentration of cholesterolSO₄⁻ has already been demonstrated to inhibit other capacitation-dependent processes such as the increase in protein tyrosine phosphorylation and the zona pellucida-induced acrosome reaction (34). This experiment further suggests that cholesterol efflux is involved in the regulation of the sperm plasma membrane potential.

To evaluate the role of HCO₃⁻ in the sperm plasma membrane hyperpolarization, cauda mouse sperm were incubated in HCO₃⁻-free medium in the presence of 1 mM 2-OH-p-β-CD or 5 mg/ml of BSA, and the plasma membrane potential was measured at different periods. In the absence of HCO₃⁻, the sperm plasma membrane potential remained constant in both cases (+ 2-OH-p-β-CD – HCO₃⁻: Fig. 2 A, D; + BSA – HCO₃⁻: data not shown). Altogether, these results suggest that similarly to other capacitation-related processes, cholesterol acceptors and HCO₃⁻ play a role in the regulation of the sperm plasma membrane potential.

**HCO₃⁻ produces a hyperpolarization in cauda epididymal mouse sperm**
Since HCO₃⁻ is negatively charged, we evaluated whether the addition of HCO₃⁻ to the sperm was able to directly modulate the membrane potential. As shown in Fig. 4 A and B, addition of HCO₃⁻ to a final concentration of 5 mM induced a hyperpolarization of 15 ± 3 mV in a sperm population. Other anions such as Cl⁻ (Fig. 4 A, B), I⁻, Br⁻, NO₃⁻ (data not shown) did not elicit hyperpolarization at the same concentration. The possibility of nonspecific interactions between HCO₃⁻ and the positive carbocyanine dye was discarded since this anion did not change the fluorescence measurement in the absence of sperm. In addition, the HCO₃⁻ elicited hyperpolarization was also observed using the oxonol DiSBAC₂(3), a negatively charged dye (Fig. 4 C). In contrast to carbocyanines, when hyperpolarization occurs, oxonol dyes are released and a decreased fluorescence is observed (Molecular Probes Manual). These data suggest that a HCO₃⁻ electrogenic permeability is present in mouse sperm.

Several hypotheses that can account for this HCO₃⁻ hyperpolarizing current are summarized in Fig. 5 A. First, the possibility of nonspecific anion channels (model 1) can be discarded since other anions are not able to hyperpolarize the sperm membrane (see above). It is possible that a HCO₃⁻/Cl⁻ antiporter (model 2) is present in sperm as previously suggested (15,16,35). Since HCO₃⁻/Cl⁻ exchanger is electroneutral, HCO₃⁻-elicited hyperpolarization could be produced indirectly by a HCO₃⁻-dependent increase in pHᵢ. This is not the case since 5 mM NH₄Cl as well as 5 mM trimethylamine increased pHᵢ (data not shown) but did not hyperpolarize the sperm plasma membrane (Fig. 5 B). Another plausible explanation is the presence of a HCO₃⁻/Na⁺/Cl⁻/H⁺ antiporter (model 3) (18). Similarly to the HCO₃⁻/Cl⁻, this exchanger is electroneutral. Nevertheless, hyperpolarization could occur by coupling this antiporter with the electrogenic Na⁺/K⁺ ATPase. To analyze this possibility, cauda mouse sperm were incubated for 5 min. with 10 µM ouabain or in the absence of extracellular K⁺. Although both treatments are able to inhibit
the Na⁺/K⁺ pump, neither of them inhibited the HCO₃⁻-induced hyperpolarization (Fig. 5 C) suggesting that this ATPase is not involved in this regulation. Since HCO₃⁻ is also able to modulate sperm adenylyl cyclase (model 5) (36), we assayed whether cAMP antagonists were able to inhibit the HCO₃⁻ induced hyperpolarization. Neither H89 nor Rp-cAMPS inhibited the change in plasma membrane potential suggesting a direct electrogenic role of HCO₃⁻.

**Na⁺/HCO₃⁻ cotransporter activity is present in mouse sperm**

Another option in Fig. 5A is the presence of an electrogenic Na⁺/HCO₃⁻ cotransporter (model 4). Several criteria have been set to evaluate the presence of this type of cotransporter (37): 1) HCO₃⁻-dependence, 2) electrogenicity, 3) Na⁺-dependence, 4) blockage by stilbenes such as DIDS and SITS, and 5) ability to increase pHi. The first and second criteria have already been shown to be fulfilled (Fig. 4). To study whether the HCO₃⁻-elicited hyperpolarization was Na⁺- dependent, NaCl in the *in vitro* capacitation media was replaced by choline⁺Cl⁻, and the HCO₃⁻ induced hyperpolarization measured by the addition of choline⁺HCO₃⁻ to a final concentration of 5 mM. In the absence of external Na⁺ the HCO₃⁻ elicited hyperpolarizing current was almost absent (Fig. 6). Since HCO₃⁻ is necessary for capacitation and for the capacitation-associated increase in tyrosine phosphorylation, if HCO₃⁻ transport is Na⁺ dependent, it is predicted that in the absence of this cation neither capacitation nor the increase in tyrosine phosphorylation would be observed. When Na⁺ is replaced by choline⁺, there is no increase in protein tyrosine phosphorylation (Fig. 7 A). As predicted, since HCO₃⁻ action is mediated by a cAMP pathway, addition of 1 mM dbcAMP and 100 μM IBMX to the capacitation media induced the increase in tyrosine phosphorylation in Na⁺-free medium (Fig. 7 A). This experiment indicates that signaling pathways involved in the cAMP-tyrosine phosphorylation process were active in Na⁺-free medium and that the lack of tyrosine phosphorylation is likely due to the lack of HCO₃⁻ transport.
Moreover, Na\(^{+}\)-free medium was not able to capacitate sperm as shown by the inability of *zona pellucida* (ZP) to induce the acrosome reaction (Fig. 7 B). To discard a direct effect of Na\(^{+}\) on the ZP-induced acrosome reaction, epididymal mouse sperm were capacitated in the absence of Na\(^{+}\), then centrifuged and resuspended in Na\(^{+}\)-containing media and the solubilized ZP added. Under these conditions the ZP was not able to induce the acrosome reaction (Fig. 7 B), suggesting that the lack of Na\(^{+}\) is directly affecting the capacitation process and the lack of activation of the acrosome reaction is not due to an effect of Na\(^{+}\) ions on this exocytotic process.

Stilbenes in general and DIDS in particular have been shown to inhibit a variety of anionic transporters; accordingly, DIDS inhibition is one of the criteria used to describe Na\(^{+}\)/HCO\(_3\)^\(^{-}\) cotransporter activity. As shown in Fig. 8, DIDS inhibited the HCO\(_3\)^\(^{-}\)-induced hyperpolarizing current in a concentration dependent manner with an IC\(_{50}\) of 100 \(\mu\)M (Fig. 8 A-G), similar to the IC\(_{50}\) observed in the inhibition of the spontaneous acrosome reaction in hamster and in bovine sperm (14,16). DIDS also blocked the increase in protein tyrosine phosphorylation (Fig. 8 I); the high molecular weight product observed in the presence of DIDS (arrows in Fig. 8 I) is a tyrosine phosphorylated isoform of hexokinase type I (28) as demonstrated in the right portion of the panel using specific antibodies against this enzyme. The appearance of this high molecular weight product in the presence of DIDS is likely to be due to the crosslinking properties of this compound (38). Another stilbene compound, SITS, had a similar inhibitory action on the HCO\(_3\)^\(^{-}\)-dependent hyperpolarization (Fig. 8 H). After washing the sperm with stilbene-free media, the inhibitory effect was not observed, suggesting that these compounds acted in a reversible way as shown by others (39). As an additional criterion, pH\(_i\) was measured using a carboxyfluorescein dye (BCECF) (18). In these experiments, addition of HCO\(_3\)^\(^{-}\) produced an increase in pH\(_i\) that was not observed when sperm were incubated in Na\(^{+}\)-free media (Fig. 9).
DISCUSSION

Mammalian sperm do not acquire full fertilizing capacity immediately after ejaculation; the ability to fertilize is achieved in the female tract in a process known as capacitation. Capacitation is associated with a cAMP/PKA-dependent pathway that is upstream of an increase in protein tyrosine phosphorylation (2). Capacitation is also associated with the hyperpolarization of the sperm plasma membrane. The functional role of sperm plasma membrane hyperpolarization during capacitation is at present not well understood. Since the presence of low voltage-activated (LVA) Ca\(^{2+}\) T channels has been demonstrated in mouse spermatogenic cells (6,7), Florman and coworkers have hypothesized that hyperpolarization of the sperm plasma membrane is required for the activation of these LVA Ca\(^{2+}\) T channels by physiological agonists (e.g. zona pellucida or progesterone)(40). A signature property of LVA Ca\(^{2+}\) channels is a low threshold for voltage-dependent inactivation. This means that these Ca\(^{2+}\) channels are inactive when the plasma membrane is depolarized, such as is observed before capacitation, thus suppressing premature exocytosis until the completion of capacitation. After capacitation, hyperpolarization of the sperm plasma membrane will allow the agonist-dependent opening of LVA Ca\(^{2+}\) T channels during the acrosome reaction (5,40).

In the present work we have analyzed whether components of the capacitation media that regulate the cAMP pathway and the increase in protein tyrosine phosphorylation have a role in the regulation of the sperm plasma membrane potential. The central observations of this study are that (1) HCO\(_3^-\) and cholesterol acceptors are necessary for the capacitation-associated hyperpolarization; and (2) a Na\(^+\)/HCO\(_3^-\) cotransporter activity is present in mouse sperm. These data suggest that HCO\(_3^-\) in the capacitation medium and cholesterol efflux may have a direct as
well as an indirect function in controlling events leading to the hyperpolarization of the sperm plasma membrane.

Previously, an essential role of HCO₃⁻ in the capacitation process has been demonstrated. HCO₃⁻ is necessary in the sperm incubation media for the sperm to undergo a zona pellucida-dependent acrosome reaction and for the cAMP/PKA-dependent increase in protein tyrosine phosphorylation observed during capacitation (11,36,41). Interestingly, during epididymal transit, and subsequently in the female tract, sperm are exposed to significant changes in extracellular HCO₃⁻ concentrations. For example, caudal epididymal sperm are stored in an environment that contains very low HCO₃⁻ concentrations due, in part, to reabsorption by the epididymis (42,43). After ejaculation, in the seminal fluid and then in the female tract, there is an increase in HCO₃⁻ concentration that has been associated with the stimulation of sperm motility (9) and which has been shown to be necessary for capacitation (11,12). The regulatory role of HCO₃⁻ on capacitation is likely to be mediated by a testis soluble adenylyl cyclase (sAC), a HCO₃⁻-dependent adenylyl cyclase present in mammalian sperm (23). Although this enzyme was originally purified from testis cytosol, it is noteworthy that in sperm, adenylyl cyclase activity is recovered only in the particulate fraction. An attractive hypothesis is that sAC and Na⁺/HCO₃⁻ cotransporters colocalize in mammalian sperm. This idea could be further extended to other systems, since sAC has been recently shown to be present in kidney and other tissues known to possess Na⁺/HCO₃⁻ cotransporters (23).

BSA is also required for capacitation; its role is linked to its ability to bind cholesterol and facilitate its efflux from the sperm plasma membrane. Supporting this hypothesis, BSA can be replaced in the capacitation media by other protein and non-protein cholesterol acceptors such as high density lipoprotein (HDL) and the high affinity cholesterol-binding cyclic sugars, β-
cyclodextrins (26,34). In the present work, we have demonstrated that 2-OH-p-CD is able to induce the capacitation-associated hyperpolarization in the absence of BSA. Moreover, when sperm are incubated in the presence of the cholesterol analogue, cholesterolSO₄⁻, in order to saturate the cholesterol binding sites of BSA, the capacitation-associated hyperpolarization is inhibited. These data suggest that cholesterol efflux has a role in the regulation of the sperm plasma membrane potential.

Sperm plasma membrane potential was measured using fluorescent dyes; this method gives reliable measurements of the plasma membrane potential and has already been used to measure membrane potential in mammalian sperm (3,29). The addition of CCCP two minutes before the fluorimetric measurements precludes the contribution of mitochondrial membrane potential to the final calibration. The calibration procedure followed in this study compensates for variation in sperm concentration and viability, and assures an accurate determination of the average sperm population plasma membrane potential. Using this methodology, we have demonstrated that addition of HCO₃⁻ to the sperm suspension induces a rapid hyperpolarization, suggesting that a HCO₃⁻ transporter activity is present in mouse sperm.

The functional family of HCO₃⁻ transporters includes Cl⁻/HCO₃⁻ exchangers, a Na⁺-driven Cl⁻/HCO₃⁻ exchanger, a K⁺/HCO₃⁻ cotransporter and Na⁺/HCO₃⁻ cotransporters with as many as three different stoichiometries (37). The HCO₃⁻ hyperpolarizing permeability observed in mouse sperm has all the signature properties of a Na⁺/HCO₃⁻ cotransporter recently defined by Romero and Boron (37): 1) it is electrogenic, 2) Na⁺-dependent, 3) HCO₃⁻ dependent, 4) increases pHi and 5) it is blocked by stilbenes such as DIDS. Moreover, the HCO₃⁻ induced increase in pHi was blocked in Na⁺-free medium. In addition, since HCO₃⁻ is necessary for capacitation, if this Na⁺/HCO₃⁻ cotransporter has a role in this extratesticular sperm maturational process, we can hypothesize that
capacitation as well as the capacitation-associated increase in tyrosine phosphorylation would be Na⁺-dependent processes. Supporting this hypothesis we have demonstrated that neither capacitation measured by the ability of zona pellucida to induce the acrosome reaction nor the increase in tyrosine phosphorylation was observed in the absence of Na⁺. These data suggest that a Na⁺/HCO₃⁻ cotransporter activity is necessary for the regulation of capacitation.

How the Na⁺/HCO₃⁻ cotransporter activity is regulated during capacitation is not clear. It is possible that since HCO₃⁻ is necessary but not sufficient for capacitation, other upstream events are required for the activation of this HCO₃⁻ transporter. On the one hand, cholesterol efflux could be involved in the regulation of HCO₃⁻ transport. On the other hand, it is not possible to discard the idea that cholesterol efflux and HCO₃⁻ transport are interdependent since capacitation-associated alterations in the transbilayer phospholipid behavior resulting in membrane lipid disorder were reported to occur through a cAMP-dependent pathway after exposure of boar sperm to HCO₃⁻ (44).

Intracellular pH has been implicated in the control of several mammalian sperm functions such as the development of progressive motility, capacitation, and the acrosome reaction. However, the transport mechanisms that regulate pHi in mammalian sperm are not well understood. A Na⁺-dependent Cl⁻/HCO₃⁻ exchanger activity has been described in mouse sperm (18). This work from 1996 was performed before the cloning and characterization of Na⁺/HCO₃⁻ cotransporters (45) and some of its conclusions could be explained by the presence of a Na⁺/HCO₃⁻ cotransporter activity in mouse sperm. Although in the present work it is not possible to completely discard the presence of a Na⁺-dependent Cl⁻/HCO₃⁻ non electrogenic exchanger, our results demonstrate the presence of Na⁺/HCO₃⁻ cotransporter activity and suggest that this cotransporter is involved in the regulation of sperm pHi. Several related proteins constitute the
emerging Na⁺/HCO₃⁻ cotransporter family (NBC1-3). This family of HCO₃⁻ transporters is found in a variety of epithelial and non-epithelial tissues (37). Consistent with our results, Western blots using antibodies against NBC1 recognized a protein in rat epididymis and in rat sperm (42). That protein has a higher molecular weight than the one expected for NBC1; this could be due to different posttranslational modifications or to the presence of a unique member of the NBC family. These possibilities warrant further investigation.

ACKNOWLEDGEMENTS

This study was supported by NIH HD38082 (to PEV), NIH HD06274 and HD 22732 (to GSK), by CONACyT and DGAPA IN201599 (to AD) and by the Mellon Foundation.
REFERENCES

1. Yanagimachi, R. (1994) in *The Physiology of Reproduction* (Knobil, E., and Neill, J. D., eds) Vol. 1, pp. 189-317, 2 vols., Raven Press, Ltd., New York

2. Visconti, P. E., Westbrook, V. A., Chertihih, O., Demarco, I., Sleight, S., and Diekman, A. B. (2002) *J. Reprod. Immunol.* **53**, 133-50.

3. Zeng, Y., Clark, E. N., and Florman, H. M. (1995) *Dev. Biol.* **171**, 554-63

4. Munoz-Garay, C., De la Vega-Beltran, J. L., Delgado, R., Labarca, P., Felix, R., and Darszon, A. (2001) *Dev. Biol.* **234**, 261-74.

5. Florman, H. M., Arnoult, C., Kazam, I. G., Li, C., and O'Toole, C. M. (1998) *Biol. Reprod.* **59**, 12-6

6. Lievano, A., Santi, C. M., Serrano, C. J., Trevino, C. L., Bellve, A. R., Hernandez-Cruz, A., and Darszon, A. (1996) *FEBS Lett.* **388**, 150-4

7. Arnoult, C., Cardullo, R. A., Lemos, J. R., and Florman, H. M. (1996) *Proc. Natl. Acad. Sci. U S A* **93**, 13004-9

8. Lee, M. A., and Storey, B. T. (1986) *Biol. Reprod.* **34**, 349-56

9. Neill, J. M., and Olds-Clarke, P. (1987) *Gamete Res.* **18**, 121-40.

10. Shi, Q. X., and Roldan, E. R. (1995) *Biol. Reprod.* **52**, 540-6

11. Visconti, P. E., Bailey, J. L., Moore, G. D., Pan, D., Olds-Clarke, P., and Kopf, G. S. (1995) *Development* **121**, 1129-37.

12. Boatman, D. E., and Robbins, R. S. (1991) *Biol. Reprod.* **44**, 806-13

13. Okamura, N., Tajima, Y., and Sugita, Y. (1988) *Biochem. Biophys. Res. Commun.* **157**, 1280-7

14. Spira, B., and Breitbart, H. (1992) *Biochim. Biophys. Acta* **1109**, 65-73
15. Visconti, P. E., Muschietti, J. P., Flawia, M. M., and Tezon, J. G. (1990) *Biochim. Biophys. Acta* **1054**, 231-6

16. Visconti, P. E., Stewart-Savage, J., Blasco, A., Battaglia, L., Miranda, P., Kopf, G. S., and Tezon, J. G. (1999) *Biol. Reprod.* **61**(1), 76-84

17. Parrish, J. J., Susko-Parrish, J. L., and First, N. L. (1989) *Biol. Reprod.* **41**, 683-99

18. Zeng, Y., Oberdorf, J. A., and Florman, H. M. (1996) *Dev. Biol.* **173**, 510-20

19. Garbers, D. L., Tubb, D. J., and Hyne, R. V. (1982) *J. Biol. Chem.* **257**, 8980-4.

20. Okamura, N., Tajima, Y., Soejima, A., Masuda, H., and Sugita, Y. (1985) *J. Biol. Chem.* **260**, 9699-705

21. Garty, N. B., and Salomon, Y. (1987) *FEBS Lett.* **218**, 148-52

22. Buck, J., Sinclair, M. L., Schapal, L., Cann, M. J., and Levin, L. R. (1999) *Proc. Natl. Acad. Sci. U S A* **96**, 79-84

23. Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R., and Buck, J. (2000) *Science* **289**, 625-8

24. Jaiswal, B. S., and Conti, M. (2001) *J. Biol. Chem.* **276**, 31698-708.

25. Moore, G. D., Ayabe, T., Visconti, P. E., Schultz, R. M., and Kopf, G. S. (1994) *Development* **120**, 3313-23

26. 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**, 3235-42

27. Laemmli, U. K. (1970) *Nature* **227**, 680-5

28. Kalab, P., Visconti, P., Leclerc, P., and Kopf, G. S. (1994) *J. Biol. Chem.* **269**, 3810-7

29. Espinosa, F., and Darszon, A. (1995) *FEBS Lett.* **372**, 119-25
30. Plasek, J., and Hrouda, V. (1991) *Eur. Biophys. J.* **19**, 183-8

31. Babcock, D. F. (1983) *J. Biol. Chem.* **258**, 6380-9

32. Garcia, M. A., and Meizel, S. (1999) *Mol. Reprod. Dev.* **52**, 189-95.

33. Ward, C. R., Storey, B. T., and Kopf, G. S. (1992) *J. Biol. Chem.* **267**, 14061-7

34. Visconti, P. E., Ning, X., Fornes, M. W., Alvarez, J. G., Stein, P., Connors, S. A., and Kopf, G. S. (1999) *Dev. Biol.* **214**, 429-43

35. Parkkila, S., Rajaniemi, H., and Kellokumpu, S. (1993) *Biol. Reprod.* **49**, 326-31

36. Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, D., Olds-Clarke, P., and Kopf, G. S. (1995) *Development* **121**, 1139-50.

37. Romero, M. F., and Boron, W. F. (1999) *Annu. Rev. Physiol.* **61**, 699-723

38. Hoffmann, E. K. (1986) *Biochim. Biophys. Acta* **864**, 1-31.

39. Sabeur, K., and Meizel, S. (1995) *J. Androl.* **16**, 266-71.

40. Arnoult, C., Kazam, I. G., Visconti, P. E., Kopf, G. S., Villaz, M., and Florman, H. M. (1999) *Proc. Natl. Acad. Sci. U S A* **96**, 6757-62

41. Visconti, P. E., Johnson, L. R., Oyaski, M., Fornes, M., Moss, S. B., Gerton, G. L., and Kopf, G. S. (1997) *Dev. Biol.* **192**, 351-63

42. Jensen, L. J., Schmitt, B. M., Berger, U. V., Nsumu, N. N., Boron, W. F., Hediger, M. A., Brown, D., and Breton, S. (1999) *Biol. Reprod.* **60**, 573-9.

43. Brooks, D. E. (1983) *Aust. J. Biol. Sci.* **36**, 205-21

44. Gadella, B. M., and Harrison, R. A. (2000) *Development* **127**, 2407-20

45. Romero, M. F., Hediger, M. A., Boulpaep, E. L., and Boron, W. F. (1997) *Nature* **387**, 409-13.
FIGURE LEGENDS

Fig. 1. Membrane potential calibration using valinomycin and sequential addition of KCl.
A: Mouse caudal epididymal sperm were incubated at 37°C in Whitten’s media. After a 7 min. incubation, 1 µM DiSC3(5) was added to the sperm suspension and further incubated for 5 min., then CCCP (1 µM) was added, the sperm incubated for an additional 2 min. and 1.5 mL of the suspension was added to the fluorimeter chamber for recording the fluorescence emission. After the signal reached stability (usually within 1-2 min.), calibration was performed adding 1 µM valinomycin (val.) and sequential additions of KCl. The initial suspension contains 5.9 mM KCl and the subsequent KCl additions resulted in final concentrations (mM KCl) in the sperm suspension of 8 (a), 12 (b), 20 (c), and 36 (d), which correspond to membrane potentials of –80, -72, -65, -47, and –32 mV respectively. These values were obtained using the Nernst equation as explained in Methods. B: Plasma membrane potential values obtained with valinomycin and sequential addition of KCl versus the arbitrary units of fluorescence. Sperm membrane potential is then linearly interpolated using these data. In this case the plasma membrane potential = -36 mV.

Fig. 2. Cholesterol acceptors and NaHCO3 are necessary for the capacitation-associated membrane hyperpolarization: Mouse caudal epididymal sperm were incubated in Whitten’s media for the time periods indicated in the Figure at 37°C with only 1 mM 2-OH-p-β-CD (A, ◯), with only 24 mM NaHCO3 (B, ▲), or with both (C, ●). Sperm plasma membrane potential determination in each case was performed as described in Fig. 1. D: The data in this graph represent the mean ± s.e.m. from three independent experiments.

Fig. 3. Saturation of cholesterol binding sites by cholesterolSO4 inhibits the capacitation-associated membrane hyperpolarization. A-D: Mouse caudal epididymal sperm were incubated
in Whitten’s media containing 5 mg/ml BSA for 5 (A, C) or 60 (B, D) min. at 37°C in the presence (C, D) or absence (A, B) of 5 µM cholesterolSO₄⁻. Sperm plasma membrane potential determination in each case was performed as described in Fig. 1. E: The data in this graph represent the mean ± s.e.m. from three independent experiments including the experiment depicted in A-D.

**Fig. 4. HCO₃⁻ induces a hyperpolarization in mouse sperm.** A: Mouse caudal epididymal sperm were incubated at 37°C in HCO₃⁻ free Whitten’s media without the addition of cholesterol acceptors. Before calibration 8 µl of 800 mM NaCl in 50 mM HEPES (pH= 7.8) was added (final [NaCl] = 5 mM) and fluorescence was recorded. Following this addition, the same amount of 800 mM NaHCO₃ in 50 mM HEPES (pH =7.8) was added and fluorescence recorded until steady state. Sperm plasma membrane potential was determined after calibration as described in Fig. 1. B: The data in this graph represent the mean ± s.e.m. from three independent experiments including the experiment depicted in A. C: Mouse caudal epididymal sperm were incubated at 37°C in HCO₃⁻-free Whitten’s media. After 7 min. incubation 1 µM DiSBAC2(3) (oxonol anionic dye) was added to the sperm suspension, further incubated for 5 min. and 1.5 ml of the suspension added to the fluorimeter chamber to record the fluorescence emission. The membrane potential responses to additions of NaCl and NaHCO₃ were analyzed as described in A.

**Fig. 5. A: Different models to explain the HCO₃⁻-dependent hyperpolarization.** 1) Hyperpolarization of the plasma membrane could be effected through an anionic channel with some selectivity for HCO₃⁻; 2) HCO₃⁻ could be entering the cell through an electroneutral Cl⁻/HCO₃⁻ antiporter, resulting in an alkalinization of the intracellular compartment and in this way modulate the sperm plasma membrane potential indirectly through a pH-dependent channel; 3) HCO₃⁻ could enter the cell through a Na⁺/HCO₃⁻/Cl⁻/H⁺ antiporter and the resultant increase in
intracellular Na\(^+\) could activate the electrogenic Na\(^+\)/K\(^+\) ATPase, in this way hyperpolarizing the cell; 4) the HCO\(_3^-\)-dependent hyperpolarization could be due to an electrogenic Na\(^+\)/HCO\(_3^-\) or K\(^+\)/HCO\(_3^-\) cotransporter; 5) HCO\(_3^-\) action could be secondary to the activation of sperm HCO\(_3^-\)-dependent adenylyl cyclase. **B: Increase in pHi.** To analyze the effect of increasing intracellular pH, NH\(_4\)Cl or trimethylamine were added to a final concentration of 5 mM in independent experiments, after reaching steady state fluorescence. **C: Inhibition of Na\(^+\)/K\(^+\) ATPase.** Caudal epididymal sperm were pre-incubated in medium containing 10 \(\mu\)M ouabain, or in K\(^+\)-free medium, and the HCO\(_3^-\)-dependent hyperpolarization was then analyzed as described in Fig. 4. **D: cAMP/PKA inhibitors.** The HCO\(_3^-\)-dependent hyperpolarization was analyzed in sperm pre-incubated in medium containing either 30 \(\mu\)M H-89 or 1 mM Rp-cAMPS. All of these experiments were repeated at least three times and a representative experiment is shown. Calibration using valinomycin and increasing concentrations of KCl was performed as described in Fig. 1.

**Fig. 6. The HCO\(_3^-\)-induced hyperpolarization is Na\(^+\)-dependent.** A-F: Caudal epididymal sperm were recovered in Na\(^+\)-free Whitten’s medium (Whitten’s/choline\(^+\)) and then incubated at 37°C in the same medium (A) or with different NaCl concentrations (B-F) as indicated in the Figure. The HCO\(_3^-\)-dependent hyperpolarization was then analyzed using choline\(^+\)HCO\(_3^-\) instead of NaHCO\(_3\) as described in Fig. 4. **G:** The data in this graph represent the mean ± s.e.m. from three independent experiments including the experiment depicted in panels A-F.

**Fig. 7. Capacitation and capacitation-associated increase in protein tyrosine phosphorylation are Na\(^+\)-dependent.** **A: Na\(^+\)-dependent increase in protein tyrosine phosphorylation.** Caudal epididymal sperm were recovered in Na\(^+\)-free medium (Whitten’s/choline\(^+\)) and then incubated in the same media or in Whitten’s media with different
NaCl concentrations prepared as described. In the last lanes, sperm were incubated in Na⁺-free or in Na⁺-containing medium with the addition of 1 mM dbcAMP and 100 µM IBMX as indicated in the Figure text. After 1.5 h of incubation, the sperm were extracted and proteins subjected to SDS-PAGE, transferred to Immobilon P and probed with antiphosphotyrosine antibody (clone 4 G10). B: Zona pellucida-induced acrosome reaction is inhibited in Na⁺-free medium. Mouse caudal epididymal sperm were incubated in Whitten’s/choline⁺ with the addition of 24 mM choline⁺HCO₃⁻ and 5 mg/ml BSA or in complete Whitten’s medium for 45 min. Sperm incubated in Na⁺-free media were separated in two aliquots, one of them was not treated, the other one was centrifuged and the sperm resuspended in Na⁺-containing media. Solubilized zona pellucida was then added to the 3 aliquots to a final concentration of 5 zona pellucida/µl (white bars) or the equivalent amount of medium (black bars, control) and the sperm incubated for an additional 30 min. The status of the sperm acrosome was then evaluated as described in Materials and Methods. The data represent the mean ± s.e.m of 6 determinations.

Fig. 8. DIDS and SITS inhibit the HCO₃⁻-induced hyperpolarization and the capacitation-associated increase in tyrosine phosphorylation. A-F. Mouse caudal epididymal sperm were pre-incubated in Whitten’s media for 30 min. in the absence (A) or in the presence of 10 (B), 100 (C), 300 (D), 600 (E) or 1,000 µM (F) of DIDS. After addition of 1 µM DiSC₃(5) and 1 µM CCCP, the amplitude of HCO₃⁻-induced hyperpolarization was measured by addition of 10 µL 800 mM NaHCO₃ in 50 mM HEPES (pH =7.8). G. Effect of DIDS on the HCO₃⁻-induced hyperpolarization. Membrane potential was measured as indicated in methods without washing (white bars) or after washing the DIDS by centrifugation and resuspension in DIDS-free media (black bars). This graph represents the mean ± s.e.m of 3 experiments including those shown in panels A-F. H. Effect of SITS on the HCO₃⁻-induced hyperpolarization. Membrane potential was
measured as indicated in methods without washing (white bars) or after washing the SITS by centrifugation and resuspension in SITS-free media (black bars). The data represent the mean ± s.e.m of 3 experiments. Mouse caudal epididymal sperm were incubated in complete Whitten’s media (24 mM NaHCO₃ and 5 mg/ml BSA) in the absence or presence of concentrations of DIDS. After 1.5 h, Western blots using α PY antibodies were performed as described. The arrows show the position of hexokinase type 1 (28). The Western blot on the right was developed with α HK1. A representative experiment (out of 3) is shown.

**Fig. 9. The HCO₃⁻-dependent increase in pH is Na⁺-dependent.** Mouse caudal epididymal sperm were incubated in Whitten’s medium (left) or in Whitten’s/choline⁺ (Na⁺-free, right) for 5 min. in 4 µM BCECF-AM. After incubation, sperm were washed in fresh medium once (400 x g for 5 min.) and 1.5 ml of this suspension was placed in a gentle-stirring cuvette for fluorescence measurement at two different excitation wavelengths (510/450 nm, fixed emission at 540 nm). To assay for the effect of HCO₃⁻ on pH, choline⁺HCO₃⁻ was added to a final concentration of 5 mM as in Fig. 6. Calibration was performed by the null point determination as described by Babcock (1983).
| 15 min | 30 min | 45 min | 60 min |
|--------|--------|--------|--------|
| ![Graph A](image1.png) | ![Graph B](image2.png) | ![Graph C](image3.png) | ![Graph D](image4.png) |

| Em (mV) | time (min) |
|--------|-----------|
| -32 mV | 0         |
| -47 mV | 20        |
| -61 mV | 40        |
| -80 mV | 60        |

Graph A: Val c d
Graph B: Val a b c d
Graph C: Val a b c d
Graph D: Val a b c d

- CD + HCO₃⁻
- CD - HCO₃⁻
A

\[ \begin{align*}
1 & \quad \text{HCO}_3^- \\
2 & \quad \text{Cl}^- \\
3 & \quad \text{HCO}_3^- \\
4 & \quad \text{Na}^+ \\
5 & \quad \text{cAMP/PKA}
\end{align*} \]

HCO$_3^-$-dependent adenyl cyclase

Hyperpolarization

B

\[ \begin{align*}
\text{NH}_4\text{Cl} & \quad \text{NaHCO}_3 \\
\text{NaHCO}_3 & \quad \text{TMA}
\end{align*} \]

Increase in intracellular pH

C

ouabain

NaHCO$_3$

Na$^+$/K$^+$ ATPase inhibitors

D

H89

NaHCO$_3$

cAMP/PKA inhibitors
Involvement of a Na+/HCO3-cotransporter in mouse sperm capacitation
Ignacio A. Demarco, Felipe Espinosa, Jennifer Edwards, Julian Sosnik, Jose Luis De la Vega-Beltran, Joel W. Hockensmith, Gregory S. Kopf, Alberto Darszon and Pablo E. Visconti

*J. Biol. Chem.* published online December 19, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206284200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts