Involvement of Cystic Fibrosis Transmembrane Conductance Regulator in Mouse Sperm Capacitation*

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Mammalian sperm acquire fertilizing ability in the female tract during a process known as capacitation. In mouse sperm, this process is associated with increases in protein tyrosine phosphorylation, membrane potential hyperpolarization, increase in intracellular pH and Ca2+, and hyperactivated motility. The molecular mechanisms involved in these changes are not fully known. Present evidence suggests that in mouse sperm the capacitation-associated membrane hyperpolarization is regulated by a cAMP/protein kinase A-dependent pathway involving activation of inwardly rectifying K+ channels and inhibition of epithelial sodium channels (ENaCs). The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl− channel that controls the activity of several transport proteins, including ENaCs. Here we explored whether CFTR is involved in the regulation of ENaC inhibition in sperm and therefore is essential for the capacitation-associated hyperpolarization. Using reverse transcription-PCR, Western blot, and immunocytochemistry, we document the presence of CFTR in mouse and human sperm. Interestingly, the addition of a CFTR inhibitor (diphenylamine-2-carboxylic acid; 250 μM) inhibited the capacitation-associated hyperpolarization, prevented ENaC closure, and decreased the zona pellucida-induced acrosome reaction without affecting the increase in tyrosine phosphorylation. Incubation of sperm in Cl−-free medium also eliminated the capacitation-associated hyperpolarization. On the other hand, a CFTR activator (genistein; 5–10 μM) promoted hyperpolarization in mouse sperm incubated under conditions that do not support capacitation. The addition of dibutylryl cyclic AMP to noncapacitated mouse sperm elevated intracellular Cl−. These results suggest that cAMP-dependent Cl− fluxes through CFTR are involved in the regulation of ENaC during capacitation and thus contribute to the observed hyperpolarization associated with this process.

Sperm capacitation is a complex phenomenon required for fertilization. In mouse sperm, capacitation includes reorganization of the plasma membrane, increase in protein tyrosine phosphorylation, hyperpolarization of the plasma membrane potential ($E_m$)2, and increases in intracellular pH (pH$_i$) and Ca2+ ([Ca2+]i) (reviewed in Refs. 1 and 2). Capacitation is also associated with the appearance of hyperactivated motility (3).

All of these changes prime sperm to effectively reach and penetrate the outer layers of the egg and to undergo the acrosome reaction (AR) (4). Among the changes observed during mouse sperm capacitation, the hyperpolarization that takes place during this process has been proposed to play the important role of removing inactivation from voltage-dependent Ca2+ channels such that they open upon a zona pellucida (ZP)-induced depolarization and trigger the AR. Little is known about the molecular entities that participate in this hyperpolarization and how all of the capacitation-associated changes are combined to promote capacitation.

Several candidates have been proposed to participate in the capacitation-induced hyperpolarization. Studies from our group have demonstrated that inwardly rectifying K+ channels contribute to this hyperpolarization (5, 6). In addition, we documented that an electrogenic Na+/HCO$_3$-cotransporter hyperpolarizes mouse sperm when external HCO$_3$- is elevated (7). Furthermore, we recently reported the presence of epithelial Na+ channels (ENaCs) in mouse sperm and showed that a reduction in the sperm Na+ permeability is essential for the capacitation-associated hyperpolarization (8). This later work strongly suggests that mouse sperm (ENaCs) are constitutively active in noncapacitated sperm and close during capacitation, resulting in $E_m$ hyperpolarization. Interestingly, experiments in this work suggest that closing of ENaCs is regulated directly or indirectly by a cAMP/protein kinase A (PKA)-dependent pathway (8).

The cystic fibrosis transmembrane conductance regulator (CFTR) is a CAMP-modulated Cl− channel and a regulator of

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2 The abbreviations used are: $E_m$, plasma membrane potential; AR, acrosome reaction; ZP, zona pellucida; ENaC, epithelial sodium channel; PKA, protein kinase A; CFTR, cystic fibrosis transmembrane conductance regulator; Br2-cAMP, dibutylryl cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine; MQAE, N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide; DiSC$_3$(5), 3′,3′-dipropylthiadicarbocyanine iodide; SITS, 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid; TRITC, tetramethylrhodamine isothiocyanate; DPC, diphenylamine-2-carboxylic acid; WH, Whitten’s HEPES-buffered.

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several transporters and proteins, including K+ channels, such as ROMK1 and ROMK2 (9, 10), anion exchangers, aquaporins, and ENaCs (11). In salt-absorbing sweat ducts, activation of CFTR causes ENaC stimulation (12). In other tissues that are absorptive or secretory, depending on the physiological need, activation of CFTR reciprocally inhibits ENaCs (13). Cystic fibrosis, the most prevalent human genetic disease, is caused by CFTR mutations (14). Additionally, the involvement of CFTR in male and female infertility has long been recognized (15). Nearly all men with cystic fibrosis are infertile due to a congenital bilateral absence of the vas deference. Recently, Chan et al. (16) proposed that HCO₃⁻ permeation through CFTR (17) present in endometrial cells plays a role during in vivo sperm capacitation and may account for some cases of female cystic fibrosis infertility. This finding is consistent with the requirement of HCO₃⁻ for capacitation. Several modes of regulation of CFTR have been explored, including phosphorylation, Cl⁻ and ATP levels, E₄m, and direct protein-protein interaction (12, 18, 19). Interestingly, CFTR and ENaC colocalize and may interact in several tissues (14).

In this work, we document the presence of CFTR in both mouse and human sperm and demonstrate that diphenylamine-2-carboxylic acid (DPC), an inhibitor of CFTR, blocks the capacitation-associated hyperpolarization. Moreover, genistein, an activator of CFTR channels, hyperpolarized sperm under conditions that do not support capacitation. These genistein-induced changes in E₄m were inhibited by DPC. Since CFTR is a Cl⁻ channel, we used the Cl⁻ fluorescent probe N-(ethoxycarbonylmethyl)-6-methoxyquinolinium (MQAE) to determine the intracellular Cl⁻ concentration ([Cl⁻]) of noncapacitated mouse sperm and explored the influence of CFTR regulators on this parameter. The addition of genistein, as well as cAMP analogues, promoted Cl⁻ influx in noncapacitated sperm that was blocked by DPC. Considering that closing of ENaCs in sperm is regulated by a CAMP pathway, we hypothesized that CFTR regulation of the sperm E₄m might be mediated by ENaCs. Consistent with this hypothesis, genistein diminished the amiloride-induced hyperpolarization and the amiloride-sensitive Na⁺ permeability in noncapacitated sperm that we had reported earlier (8), suggesting that CFTR regulates the capacitation-associated hyperpolarization in mouse sperm through the inhibition of ENaCs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Amiloride, dibutyryl cyclic AMP (Bt₂cAMP), 3-isobutyl-1-methylxantine (IBMX), carbonyl cyanide m-chlorophenylhydrazone, valinomycin, nigericin, sodium gluconate, and potassium gluconate were purchased from Sigma. MQAE, 3,3'-dipropylthiadicarbocyanine iodide (DiSC₅(5)), 4-acetomido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) were obtained from Invitrogen. Polyclonal antibody against CFTR was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-goat IgG biotin-conjugated and Avidin TRITC-conjugated were from Pierce. DPC was from Sigma.

DPC (10 mM stock), genistein, DiSC₅(5), carbonyl cyanide m-chlorophenylhydrazone, and valinomycin (1 mM stocks) were prepared in Me₂SO and stored at −20°C until use. Bt₂cAMP (1 mM), H-89, and IBMX (100 μM) were prepared on the day of the experiment using a modified Krebs-Ringer medium (Whitten’s HEPES-buffered (WH) medium) (20) and used at the indicated concentration.

**Sperm Preparation**—Experimental protocols were approved by the local Animal Care and Bioethics Committee of the Instituto de Biotecnología-Universidad Nacional Autónoma de México. In most experiments, cauda epididymal mouse sperm were collected from CD1 retired male breeders by placing minced cauda epididymis in WH medium. This medium does not support capacitation unless supplemented with 5 mg/ml bovine serum albumin and 24 mM NaHCO₃. After 10 min, the sperm suspension was washed in 10 ml of the same medium by centrifugation at 800 × g for 10 min at room temperature. Sperm were then resuspended to a final concentration of 2 × 10⁷ cells/ml and diluted 10 times in the appropriate medium depending on the experiment. To study the role of Cl⁻ in capacitation and in the regulation of E₄m, NaCl and KCl were replaced by sodium gluconate and potassium gluconate, respectively.

**Human Sperm Preparation**—Semen was obtained from normal, fertile volunteers by masturbation after at least 2 days of abstinence. After liquefaction, 1 ml of Ham’s F-10 was applied to 1 ml of semen to allow the motile sperm to swim up into the upper layer of the suspension (1 h at 37°C). Swim-up sperm were collected and adjusted to 1 × 10⁶ cells/ml. Samples were used for Western blot analysis and indirect immunofluorescence analysis as described for mouse sperm.

**RNA Isolation and Reverse Transcription-PCR Experiments**—Total RNA was prepared from isolated mouse elongated spermatids (21) or ejaculated human sperm, using TRIzol Reagent (Sigma) according to the manufacturer’s instructions. cDNA was synthesized from total RNA samples with random hexamer-primed reverse transcription (Superscript II RNase H-reverse transcriptase; Invitrogen). cDNA was then subjected to PCR amplification using TaqDNA polymerase (Invitrogen). The CFTR subunit primers were designed using the human and mouse reported nucleotide sequence for these genes (human CFTR NM_020038 and mouse CFTR NM_021050, respectively). Primer sequences for human CFTR are as follows: forward, 5'TAG ATT ATG GGA GAA CTG G-3'; reverse, 5'ATG AGA AAC GGT GTA AGG T-3'. Primer sequences for mouse CFTR are as follows: forward, 5'-GGA GCA AAC CCA AAC A-3'; reverse, 5'-AGC AGC CAC CTC AAC C-3'. The absence of genomic contamination in the RNA samples was confirmed with reverse transcription-negative controls (no reverse transcription) for each experiment. Amplified products were analyzed by DNA sequencing in order to confirm their identity.

**SDS-PAGE and Immunoblotting**—Human and mouse sperm extracts were obtained by the method described by Ref. 22. Sperm were resuspended in sample buffer containing protease inhibitors without 2-mercaptoethanol, and boiled for 5 min. Sperm samples were centrifuged at 10,000 × g for 15 min. After centrifugation, the supernatants were collected, and 2-mercaptoethanol was added to a final concentration of 5% (v/v). The samples were boiled for an additional 5 min and then subjected to 10% SDS-PAGE (23). Electrotransfer of proteins to Immo-
bilon P (Bio-Rad) and immunodetection of CFTR was carried out as previously described (24). Immunoblots were incubated with anti-CFTR and developed with the appropriate secondary antibody conjugated to horseradish peroxidase (Sigma) and the chemoluminescent ECL kit (Amersham Biosciences) according to the manufacturer’s instructions.

Indirect Immunofluorescence—Sperm suspensions were fixed in paraformaldehyde (4% 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. The secondary antibody was then subjected to three consecutive washes with PBS and developed by incubation with avidin-fluorescein isothiocyanate 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 a confocal microscope.

Membrane Potential Assay in Sperm Populations—E<sub>m</sub> was measured as previously described (7). Briefly, sperm were collected as indicated above, diluted in the appropriate medium and capacitated for different time periods depending on the experiment. Eight min before the measurement, 1 μM DiSC<sub>3</sub>(5) (final concentration) was added to the sperm suspension and further incubated for 5 min at 37 °C. One μM carbonyl cyanide m-chlorophenylhydrazone (final concentration) was then added to collapse mitochondrial potential, and sperm were incubated for an additional 2 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. Calibration was performed as described before by adding 1 μM valinomycin and sequential additions of KCl.

Na<sup>+</sup>-induced Depolarization and Amiloride-induced Hyperpolarization—After reaching steady state fluorescence, 50 mM NaCl was added while fluorescence was recorded in the presence or absence of different compounds as indicated in each figure. After a new fluorescent steady state was reached, calibration was performed as indicated above. E<sub>m</sub> changes elicited by NaCl were quantified, taking into consideration the calibration curve and the initial steady state fluorescence before NaCl addition. Controls to determine if amiloride or its solvent (Me<sub>2</sub>SO) altered the fluorescence of DiSC<sub>3</sub>(5) were performed with dye only and did not show significant changes in fluorescence (data not shown).

Intracellular Cl<sup>-</sup> Measurements in Sperm Populations—[Cl<sup>-</sup>], was measured in sperm populations using a Cl<sup>-</sup>-sensitive fluorescent dye (MQAE) (25). Sperm were incubated with 10 mM MQAE for 30 min at 37 °C. Excess MQAE was removed by diluting sperm 10-fold with WH-Cl<sup>-</sup>-free medium (100 mM sodium gluconate, 4.4 mM potassium gluconate, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 5.4 mM glucose, 0.8 mM pyruvic acid, 4.8 mM lactic acid, 20 mM HEPES, pH 7.2) and centrifuging for 5 min at 300 × g. The sperm pellet was resuspended in an aliquot of 2 × 10<sup>6</sup> sperm/ml with medium under the following conditions. 1) To obtain the maximum fluorescence (F<sub>max</sub>), WH-Cl<sup>-</sup>-free medium with nigericin (10 μM) and tributyltin (10 μM) was used. 2) To determine the fluorescence at defined Cl<sup>-</sup> concentrations (F<sub>Cl</sub><sup>-</sup>), two media were mixed (WH containing different NaCl concentrations (10–100 mM) and WH-Cl<sup>-</sup>-free medium keeping Cl<sup>-</sup> + gluconate<sup>-</sup> = 100 mM); nigericin (10 μM) and tributyltin (10 μM) were also added. 3) For minimum fluorescence (F<sub>min</sub>), 150 mM KSCN buffered with 10 mM HEPES (pH 7.2) and 5 μM valinomycin was used. Samples were illuminated at 350 nm, and emission was collected at 450 nm. The relationship between Cl<sup>-</sup> concentration and MQAE fluorescence intensity expressed as the ratio F<sub>0</sub>/F<sub>Cl</sub><sup>-</sup> provides a straight line with a slope equal to K<sub>g</sub>^*^, the Stern-Volmer constant, where the total quenchable signal F<sub>0</sub> is defined by F<sub>max</sub> − F<sub>min</sub> [Cl<sup>-</sup>], was estimated using the constructed plot (25–27).

The sperm [Cl<sup>-</sup>], was also evaluated according to Garcia and Meizel (28). Mouse sperm were incubated for 30 min in WH medium containing different Cl<sup>-</sup> concentrations (0–100 mM) and 10 mM MQAE. Excess MQAE was removed as mentioned above, and emission fluorescence intensity data from sperm suspensions (2 × 10<sup>6</sup> sperm/ml) were recorded for 120 s. The intracellular and extracellular [Cl<sup>-</sup>] were equilibrated by permeabilizing the sperm membrane with digitonin (10 μM), and the fluorescence was recorded for another 120 s. The [Cl<sup>-</sup>], was calculated as described by Garcia and Meizel (28). It is important to note that the values of the [Cl<sup>-</sup>], are to be taken cautiously, since they are obtained from a heterogeneous sperm population. Cl<sup>-</sup> determinations require a calibration, which assumes that the dye behaves in sperm as in other cells (24–26) and does not correct for residual external dye, and some differences in experimental conditions. It is only possible to wash mouse sperm once by centrifugation, since they lose viability if centrifuged further.

The influence of different drugs (i.e. genistein, cAMP/IBMX, DPC, H-89, and SITS) on [Cl<sup>-</sup>], was determined using sperm suspensions loaded with MQAE as described above, after recording the basal fluorescence for 1–3 min, and measuring for a further 5–10 min. Two controls were performed: 1) drug solvents (Me<sub>2</sub>SO or water) were added while the fluorescence was recorded, and 2) MQAE fluorescence without cells was recorded, and the drugs were added. No significant fluorescence changes were observed after performing both controls (data not shown). [Cl<sup>-</sup>], changes were estimated as described above.

Acrosome Reaction Assay—Caudal epididymal mouse sperm were collected from CD1 mice and placed in capped 1.5-ml microcentrifuge tubes containing medium 199 (Sigma) supplemented with bovine serum albumin (0.4%, w/v), Na<sup>+</sup> pyruvate (30 mg/gliter), and NaHCO<sub>3</sub> (2.2 g/liter) at 37 °C (4–5 × 10<sup>6</sup> cells/ml). The swim-up method (29) was used to separate sperm with >90% motility. The sperm suspension was incubated 10 min, and the top ~1 ml was separated and capacitated for 30 min at 37 °C (30). AR was induced after capacitation (in the presence or absence of DPC) in a 30-μl aliquot by adding 5 ZP eq/μl or 15 μM A23187. ZP was obtained from mouse oocytes (31). The percentage of AR was determined in a 30-μl aliquot by adding an equal volume of 10% formaldehyde in phosphate-
buffered saline. Following fixation, 10-μl aliquots of the sperm suspension were spread onto glass slides and air-dried. The slides were stained with 0.22% Coomassie Blue G-250 in 50% methanol and 10% glacial acetic acid for ~5 min, rinsed, and mounted with 50% (v/v) glycerol in phosphate-buffered saline (5). At least 100 sperm were assayed per experimental condition to calculate the percentage of AR.

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

RESULTS

CFTR Is Present in Mammalian Sperm—CFTR modulates several cellular processes, interacting with various channels and transporters, particularly with ENaCs (14, 32). We reported the presence of αENaC and βENaC subunits in mouse sperm (8) and have now found the αENaC subunit in human sperm (data not shown). We examined if CFTR is present in mouse and human sperm. First, the presence of CFTR transcripts was analyzed using reverse transcription-PCR; cDNA was synthesized from total RNA extracted from human sperm ejaculates and purified mouse spermatogenic cells (33). Specific primers for CFTR were designed using the reported human and mouse nucleotide sequences for these genes. CFTR fragments of the expected length were detected from human sperm (476 bp) and mouse spermatogenic cells (581 bp) cDNA (Fig. 1A), and their identities were confirmed by DNA sequencing.

Because expression of CFTR transcripts in mouse spermatids and in human sperm does not imply the presence of the CFTR protein in mature sperm, Western blot and immunofluorescence experiments were conducted using an anti-CFTR polyclonal antibody. This antibody detected a protein band at the expected molecular weight in both human and mouse sperm, as well as in whole extracts from mouse lung that were included as a positive control (Fig. 1B). In all of these cases, preincubation with the CFTR antigenic peptide eliminated the Western blot signal (Fig. 1B). Using the same antibody, CFTR was immunolocalized to the midpiece of human (Fig. 1C) and mouse sperm (Fig. 1E). It is worth noting that αENaC localized to the same sperm region (8). Previous incubation of the antibody with the respective antigenic peptide blocked the immunofluorescence signal (Fig. 1, D and F).

CFTR Is Involved in the Capacitation-associated Hyperpolarization—Since CFTR is a Cl⁻ channel, it may contribute to the mouse sperm resting $E_{m}$ and to the changes it undergoes during capacitation. Interestingly, 250 μM DPC, which inhibits CFTR, was able to block the capacitation-associated hyperpolarization without affecting the $E_{m}$ of sperm incubated under conditions that do not support capacitation (Fig. 2). On the other hand, noncapacitated sperm incubated 10 min with 10 μM genistein, a compound that activates CFTR, undergo a hyperpolarization that was greater than that which accompanies capacitation. Notably, the genistein-induced hyperpolarization was blocked by DPC. These results indicate that CFTR activation influences the sperm $E_{m}$ (Fig. 2, A and B).

CFTR Participates in Capacitation—Taking into consideration that DPC blocked the capacitation-associated hyperpolarization, we explored whether CFTR inhibition was also able to block the capacitation process. Two experimental protocols were used. 1) Mouse sperm were capacitated in the presence or absence of 250 μM DPC, and then the AR was induced adding ZP. 2) Sperm were capacitated in the absence of DPC and then incubated for 5 min with DPC, just prior to the induction of the AR with ZP. The presence of DPC during sperm capacitation inhibited the ZP-induced AR ~60% compared with the control without DPC. In contrast, the AR was not affected when DPC was added after capacitation (Fig. 3A). This result is consistent with the participation of CFTR in the capacitation process of mouse sperm. On the other hand, DPC did not alter the tyrosine phosphorylation pattern associated with capacitation (data not shown), suggesting that hyperpolarization and tyrosine phosphorylation are independent processes.

External Cl⁻ Influences the Sperm Hyperpolarization Induced by Genistein or by Capacitation—Since CFTR is a Cl⁻ channel, we investigated whether [Cl⁻]c affects the capacita-
The capacitation-induced hyperpolarization was inhibited by DPC and mimicked by GNS. Also shown is the calibration obtained by adding 1 μM valinomycin followed by sequential additions of K+ ionophore. DPC inhibited the AR if added during capacitation but not if added after capacitation, suggesting a role for CFTR in the capacitation-associated hyperpolarization.

**CFTR in Sperm Capacitation**

Mouse sperm were incubated in WH medium supplemented with bovine serum albumin and NaHCO3, and AR was induced with ZP or A23187 (Ca2+ ionophore). DPC inhibited the AR if added during capacitation but not if added after capacitation, suggesting a role for CFTR in the capacitation-associated hyperpolarization.

**The Functional State of CFTR Influences Cl− Uptake in Sperm**—The previous results led us to examine how the functional state of CFTR affected the transport of Cl− in mouse sperm. Using MQAE, a Cl−-sensitive dye, we first determined the [Cl−]i changes during capacitation. Fig. 5 shows a higher [Cl−]i for capacitated sperm (40.8 ± 7.3 mM (S.D.)) compared with the [Cl−]i of noncapacitated sperm (31.4 ± 3.5 mM (S.D.)), a difference that, although small, is statistically significant (see p values).

These [Cl−]i values and the known [Cl−]o set the Cl− equilibrium potential (EC1) for noncapacitated sperm at ~−31.3 mV and at ~−24.5 mV for capacitated sperm, according to the Nernst equation (34). Similar values for the sperm [Cl−]i and ECl− were obtained using the method described by Garcia and Meizel (28): [Cl−]i for noncapacitated sperm = 28.0 ± 3.6 mM (S.D., n = 4) with an ECl− = ~−34.3 mV, and [Cl−]i for capacitated sperm = 39.2 ± 5.0 mM (S.D., n = 4) with an ECl− = ~−25.5 mV. Such a sperm [Cl−]i increase produced during capacitation was inhibited by the addition of DPC during this process (Fig. 5). In fact, DPC had an unexpected effect, producing a decrease in the sperm [Cl−]i (23.6 ± 9.1 mM (S.D.)) after 60 min of incubation in capacitating medium.

Genistein activation of CFTR in noncapacitated sperm loaded with MQAE led to an increase in [Cl−]i (35.9 ± 2.5 mM (S.D.); see Fig. 5), which was strongly inhibited by DPC (Fig. 6). Note that a decrease in the fluorescence of this dye reflects an increase in [Cl−]i (Fig. 6).
Although genistein is known to directly activate CFTR (35), it can also inhibit tyrosine kinases. Because of this, the increase in \([\text{Cl}^-]_i\) induced by genistein was examined in the presence of another tyrosine kinase inhibitor (tyrphostin 47) and of a phosphotyrosine phosphatase inhibitor (Na$_3$VO$_4$). In addition, we explored whether the effect of genistein on \([\text{Cl}^-]_i\) uptake was mediated by cAMP/PKA-dependent signaling pathways using H-89. It is worth noting that contrary to DPC, none of these compounds blocked the increase in \([\text{Cl}^-]_i\) induced by genistein (Fig. 6, A and B), suggesting that this compound directly activates CFTR in mouse sperm. Furthermore, to check whether genistein works via tyrosine kinases or phosphatases, the direct addition of tyrphostin 47 and Na$_3$VO$_4$ was assayed. Neither inhibitor had a statistically significant effect on the \([\text{Cl}^-]_i\) of noncapacitated sperm (Fig. 6, A and B).

**CFTR and Other Cl$^{-}$ Transporters Are Involved in the [Cl$^{-}$] Increase Induced by cAMP**—CFTR is a Cl$^{-}$ channel regulated by cAMP and PKA (36). As expected from the results described above, the addition of Bt$_2$cAMP and IBMX to noncapacitated sperm increased \([\text{Cl}^-]_i\) within ~30 s. This increase was only partially inhibited by sperm precubation with DPC (Fig. 7). This experiment suggests that CFTR is not the only Cl$^{-}$ transporter involved in the Cl$^{-}$ uptake. To explore alternative Cl$^{-}$ entry routes, SITS, an inhibitor of CFTR, Cl$^{-}$/HCO$_3$ exchangers, and Cl$^{-}$ channels (37), was used. SITS (20 μM) caused a stronger inhibition of the Bt$_2$cAMP/IBMX-induced \([\text{Cl}^-]_i\) increase (Fig. 7), further confirming that other Cl$^{-}$ transporters participate in this \([\text{Cl}^-]_i\) increase. As anticipated the effect of these cAMP agonists was blocked by H-89, a PKA inhibitor that prevents capacitation and the capacitation-associated increase in protein tyrosine phosphorylation (38, 39) (Fig. 7).

**Activation of CFTR Inhibits ENaC**—Previously, we have shown that ENaCs are present in mouse sperm and that amiloride, an ENaC inhibitor, hyperpolarizes noncapacitated sperm and inhibits a depolarization induced by Na$^+$ (8). As shown in the present work, genistein activation of CFTR also induces hyperpolarization of the sperm $E_m$ (Fig.
Inhibited the Na\(^+\)-induced depolarization at the same level as amiloride (Fig. 9). Since genistein does not inhibit ENaCs directly (40), this result agrees with the hypothesis that inhibition of Na\(^+\) influx through ENaCs is indirectly induced by activation of CFTR (Fig. 9).

**DISCUSSION**

The molecular basis of capacitation is not well understood. In mouse sperm, a plasma membrane hyperpolarization is associated with this process. Since low voltage-activated Ca\(^{2+}\)/H\(^+\) channels (T-type) are functionally present in mouse sperm (33, 41), it has been proposed that the capacitation-associated hyperpolarization removes inactivation of T-type Ca\(^{2+}\)/H\(^+\) channels, allowing them to respond to ZP and trigger AR (41–43).

We recently established that ENaC closure contributes to the capacitation-associated hyperpolarization. In noncapacitated sperm, these channels depolarize E\(_m\). During capacitation ENaC activity is inhibited. This condition is mimicked by adding cAMP agonists to noncapacitated sperm and eliminated by the PKA inhibitor H-89 (7). On the other hand, CFTR is known to interact with ENaCs in other systems and modulate their activity (14, 44). Moreover, it is well established that CFTR activity is regulated by a cAMP pathway involving PKA phosphorylation. In this work, we investigated the hypothesis that in mouse sperm the increase in cAMP associated with capacitation regulates ENaC activity through activation of CFTR. Consistent with this hypothesis, we have shown the following. 1) CFTR is present in mouse and human sperm. 2) Genistein, at a concentration that effectively activates CFTR (45), hyperpolarized noncapacitated sperm. The involvement of CFTR in these E\(_m\) changes was evidenced by their sensitivity to DPC, a known CFTR inhibitor. 3) DPC also blocked the capacitation-associated hyperpolarization and the ZP-induced acrosome reaction. 4) Lowering [Cl\(^-\)] or eliminating this anion inhibited the capacitation-associated hyperpolarization. Furthermore, CFTR activation by genistein in the absence of external Cl\(^-\) did not induce hyperpolarization. 5) Genistein increased Cl\(^-\) influx, and this event was blocked by DPC. 6) Genistein blocked the amiloride-sensitive depolarization induced by external Na\(^+\) addition.
CFTR is regarded as a global regulator of electrolyte transport through its ability to function as a Cl⁻ channel (9, 46). We evaluated [Cl⁻], in noncapacitated and capacitated sperm, finding that [Cl⁻], increases during capacitation. Our results show that capacitated mouse sperm have a [Cl⁻], value similar to those reported for capacitated human sperm (28). It is worth considering that the [Cl⁻] values obtained in this work and by Garcia and Meizel (28) are approximate. They are averages of a heterogeneous sperm population and were obtained using dye-specific calibration protocols, which assume that dye behavior is the same in different cell types and experimental conditions (24–26). Despite this, these Cl⁻ measurements allow comparisons between different physiological sperm states as well as the determination of changes induced by different drugs.

The high [Cl⁻], in noncapacitated sperm determines that their $E_{\text{Cl}^-}$ is $\sim 31$ mV, close to the electrochemical equilibrium ($E_{\text{eq}} = \sim 40$ mV). Therefore, as in other cell types, Cl⁻ movements are energetically cheap (37). Under these conditions, CFTR activation alone would not change $E_{\text{eq}}$ or [Cl⁻], a great deal but could act as a shut allowing Cl⁻ influx through some additional Cl⁻ transport systems, such as electroneutral cotransporters (47). This could explain the [Cl⁻], increases measured when noncapacitated sperm are exposed to genistein and Bt₂cAMP/IBMX or when they are capacitated. The required activation of CFTR for Cl⁻ uptake is consistent with the fact that it is partially inhibited by the CFTR antagonist DCP. However, the participation of other Cl⁻ transporters is revealed by the greater inhibition produced by SITS, a Cl⁻ transport inhibitor of broader specificity.

Cl⁻ homeostasis in mammalian sperm is likely to require a complex network of transporter proteins. For instance, NKCC is important to maintain high [Cl⁻], in olfactory sensory neurons and epithelial cells (48–50). On the other hand, HCO₃⁻ is necessary to activate adenyl cyclase during mammalian sperm capacitation, although how it enters sperm is not well understood. CFTR plays a crucial role in HCO₃⁻ secretion in airway, colonic, pancreatic, and kidney epithelial cells (51). Furthermore, CFTR regulates the activity of different transporters, such as ENaC, Cl⁻/HCO₃⁻, and Na⁺/HCO₃⁻ exchangers and the Na⁺/HCO₃⁻ cotransporter (9). We suggest that CFTR activation and increased [Cl⁻], could be a driving force for HCO₃⁻ influx, through the recycling of Cl⁻ during capacitation.

CFTR Cl⁻ channel activation by cAMP requires phosphorylation by PKA (52). In mammalian sperm, PKA activity levels are regulated by the cell physiological state. Sperm capacitation in media containing Ca²⁺, HCO₃⁻, and bovine serum albumin elevates cAMP and PKA activity, increasing tyrosine phosphorylation (2). These increases in cAMP levels and PKA activity most likely stimulate CFTR. This hypothesis is reinforced by two important facts. 1) Permeable cAMP analogs can activate Cl⁻ influx through PKA, since H-89 inhibited the cAMP/IBMX-induced [Cl⁻], increase. That H-89 inhibited this Cl⁻ uptake more than DPC also suggests that in addition to CFTR, other cAMP/PKA-dependent Cl⁻ transporters are involved. 2) Genistein induces a Cl⁻ - and DPC-dependent hyperpolarization and Cl⁻ influx in noncapacitated sperm. This phytoestrogen can influence CFTR function directly or indirectly through its properties as a tyrosine kinase inhibitor (36). However, the Cl⁻ influx generated by genistein in noncapacitated sperm was inhibited only by DPC and not by inhibitors of tyrosine kinases (tyrphostin 47), phosphatases (NaVO₄), or PKA (H-89). These findings altogether strongly suggest that genistein is directly activating CFTR.

We reported the presence in mouse sperm of αENaC and δENaC in the midpiece and acrosome region, respectively. Interestingly, CFTR and δENaC are found in the midpiece. Although we do not have data implying their physical interaction, their localization in the same sperm region is consistent with the hypothesis that αENaC may be regulated by CFTR. On the other hand, the interaction between CFTR and δENaC has not been reported; in sperm they are differentially localized, which excludes their physical interaction and indicates that δENaC is probably regulated by a different mechanism, as has been reported (53). CFTR in mammalian sperm could regulate...
other transport proteins, such as K+ channels, anion exchangers, and aquaporin water channels (54), as documented in other cells (55, 56). Therefore, this channel could participate in the modulation of various aspects of sperm physiology in addition to capacitation, such as motility and the AR.

As discussed above, the near parity of $E_m$ and $E_{Cl}$ in noncapacitated sperm cannot explain how genistein can cause a large hyperpolarization if only CFTR activation is considered. Two possible explanations are that 1) there is a fraction of the sperm population with distinctly different $E_m$ and $E_{Cl}$ values, where CFTR opening leads to a significant $Cl^-$ influx and hyperpolarization, and 2) more likely, as in other systems (9, 46), CFTR activation inhibits ENaC, which hyperpolarizes sperm $E_m$ and shunts $Cl^-$ transport so that other transport systems may carry out $Cl^-$ influx (14, 44). This explanation is consistent with the inhibition by genistein of the Na+-induced depolarization. Our findings altogether support the hypothesis that CFTR activation during mouse sperm capacitation results in ENaC inhibition and that this link, as anticipated, regulates the sperm $E_m$. In conclusion, we propose that CFTR is activated during capacitation upon an initial cAMP increase, leading to ENaC inhibition, hyperpolarization, and $Cl^-$ influx through other transporters. This increase in $[Cl^-]$ may be coupled to a $HCO_3^-$ increase that will further elevate cAMP, thus facilitating capacitation.

While this manuscript was being reviewed, a paper by Xu et al. (57) appeared indicating the presence and participation of CFTR in mammalian sperm capacitation. These authors suggest that $HCO_3^-$ transport through CFTR is fundamental for sperm capacitation and $HCO_3^-$-dependent cAMP production. Similar to Xu et al. (57), our findings point out that CFTR is necessary for sperm function; however, our results indicate that the role of CFTR goes beyond its ability to

**FIGURE 8.** The hyperpolarization induced by amiloride (AMLO) is inhibited by genistein (GNS). A, the addition of 0.25 mM amiloride (ENaC inhibitor) to sperm in Whitten's medium induces a hyperpolarization that is inhibited by 10 mM genistein and unaffected by DPC. All $E_m$ records were calibrated and calculated as described under “Experimental Procedures.” B, summary results presented as the mean ± S.E. ($n = 5$) of the sperm $E_m$ changes ($\Delta E_m$), where $\Delta E_m$ represents the difference between $E_m$ after amiloride addition ($E_{mD}$) and before (resting $E_m$, $E_{mR}$) ($\Delta E_m = E_{mD} - E_{mR}$).

**FIGURE 9.** The Na+-induced membrane depolarization is inhibited by genistein. A, adding 25 mM Na+ to sperm in Whitten’s medium induces a depolarization that is inhibited by 0.25 mM amiloride and 10 mM genistein (GNS). All records and $E_m$ were calibrated and calculated as described under “Experimental Procedures.” B, summary of results presented as the mean ± S.E. ($n = 5$). $\Delta E_m$ represents the difference between $E_m$ after the NaCl addition ($E_{mD}$) and before (resting $E_m$, $E_{mR}$) ($\Delta E_m = E_{mD} - E_{mR}$).
transport HCO\textsubscript{3}\textsuperscript{−} and is related to its Cl\textsuperscript{−} transport capacity. Although it is not possible to discard some contribution of HCO\textsubscript{3}\textsuperscript{−} transport in CFTR function during sperm capacitation, our results show that CFTR inhibitors (both CFTR inh-172 and DPC) are unable to inhibit the bicarbonate-dependent increase in tyrosine phosphorylation (data not shown).

Moreover, the absence of Cl\textsuperscript{−} prevented the capacitation-associated hyperpolarization in the presence of NaHCO\textsubscript{3} (capacitating conditions) and genistein-induced hyperpolarization in the absence of HCO\textsubscript{3}\textsuperscript{−} (noncapacitating conditions). Altogether, our findings point toward a role of CFTR during capacitation as a Cl\textsuperscript{−} transporter whose activation leads to ENaC inhibition that results in membrane hyperpolarization.

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