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Cl^- Is Required for HCO_3^- Entry Necessary for Sperm Capacitation in Guinea Pig: Involvement of a Cl^-/HCO_3^- Exchanger (SLC26A3) and CFTR[1]

Wen Ying Chen,[4,5] Wen Ming Xu,[5] Zhang Hui Chen,[4] Ya Ni,[4] Yu Ying Yuan,[4] Si Chang Zhou,[4] Wen Wei Zhou,[6] Lai Ling Tsang,[5] Yiu Wa Chung,[5] Pia Höglund,[7] Hsiao Chang Chan,[2,5] and Qi Xian Shi[3,4]

Unit of Reproductive Physiology,[4] Institute of Reproductive Health, Zhejiang Academy of Medical Sciences, Hangzhou 310013, China
Department of Physiology,[5] Faculty of Medicine, Epithelial Cell Biology Research Center, Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong
Experimental Animal Center of Zhejiang Province,[6] Hangzhou 310013, China
Hospital for Children and Adolescents,[7] Department of Medical Genetics, University of Helsinki, FI-00014 Helsinki, Finland

ABSTRACT

Our previous study demonstrated the involvement of cystic fibrosis transmembrane conductance regulator (CFTR) in transporting bicarbonate that is necessary for sperm capacitation; however, whether its involvement is direct or indirect remains unclear. The present study investigated the possibility of a Cl^-/HCO_3^- exchanger (solute carrier family 26, number 3 [SLC26A3]) operating with CFTR during guinea pig sperm capacitation. Incubating sperm in media with various concentrations of Cl^- resulted in varied percentages of capacitated sperm in a concentration-dependent manner. Depletion of Cl^-, even in the presence of HCO_3^-, abolished sperm capacitation and vice versa, indicating the involvement of both anions in the process. Capacitation-associated HCO_3^--dependent events, including increased intracellular pH, cAMP production, and protein tyrosine phosphorylation, also depend on Cl^- concentrations. Similar Cl^- dependence and inhibitor sensitivity were observed for sperm-hyperactivated motility and for sperm-egg fusion. The expression and localization of CFTR and SLC26A3 were demonstrated using immunostaining and Western blot analysis. Taken together, our results indicate that Cl^- is required for the entry of HCO_3^- that is necessary for sperm capacitation, implicating the involvement of SLC26A3 in transporting HCO_3^-, with CFTR providing the recycling pathway for Cl^-.

bicarbonate, CFTR, chloride, SLC26A3, sperm capacitation

INTRODUCTION

Spermatozoa must undergo an activation process called capacitation, which consists of morphological, biochemical, and physiological changes in sperm necessary to acquire fertilizing potential [1, 2]. It has been demonstrated that HCO_3^- is essential for capacitation, as prevention of HCO_3^--induced intracellular alkalization results in failure of capacitation [3–7]. Impaired HCO_3^- secretion by endometrial epithelium also reduced capacitation and fertilizing capacity in cocultured sperm [8].

Although HCO_3^- has been shown to activate soluble adenylate cyclase in the cytoplasm of sperm as the initial step in a series of events, including intracellular cAMP production and protein tyrosine phosphorylation, that lead to capacitation [5, 9–11], how HCO_3^- is transported into sperm remained unanswered. Our recent studies demonstrated a crucial role of cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel known to conduct Cl^- and HCO_3^- [12, 13] in the process of capacitation [14, 15]. Disruption of CFTR function by its inhibitors or antibodies was shown to reduce the number of capacitated sperm and to affect several capacitation-associated HCO_3^--dependent events such as increased intracellular pH (pHi), membrane hyperpolarization, and cAMP production, indicating the involvement of CFTR in the transport of HCO_3^-. Sperm from cystic fibrosis (CF) heterozygous mice also had reduced fertilizing capacity in vitro and in vivo, confirming an important role of CFTR in determining sperm-fertilizing capacity. However, it was unclear whether CFTR directly conducts HCO_3^- or acts as a Cl^- channel working in parallel with a Cl^-/HCO_3^- exchanger, thereby providing a recycling pathway for the Cl^- that is necessary to operate the anion exchanger, as is the case in pancreatic ductal HCO_3^- secretion [16–18]. We undertook the present study to test the latter possibility, as a Cl^-/HCO_3^- exchanger has been reported in sperm [19–21]. The Cl^- dependence would seem to be required for operation of the exchanger, which would not be necessary if CFTR conducts HCO_3^- directly. Therefore, the primary goals of the present study were to examine the dependence of sperm capacitation and the associated HCO_3^--dependent events on Cl^- concentrations in the minimal capacitating medium (MCM), as well as the sensitivity of these events to inhibitors of CFTR and the anion exchanger.

Capacitation is also a prerequisite for the sperm acrosome reaction (AR), an exocytic process releasing hydrolytic enzymes from the acrosome to enable sperm penetration of the egg investments and cell membrane in response to natural agonists such as progesterone and the zona pellucida (ZP) [22, 23]. Therefore, sperm capacitation can be defined as the acquisition of the capacity of sperm to undergo the AR [24,

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[2]Correspondence: FAX: 852 26035022;
e-mail: hsiaocchan@cuhk.edu.hk
[3]Correspondence: FAX: 86 571 8807 5447; e-mail: qx_shi@126.com

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25], and a practical approach to assess capacitation is to measure the ability of sperm to undergo an induced AR, even if capacitation and the AR are two separate events. This approach exploits the operational definition of capacitation [22, 26], namely, measuring the increase in the number of acrosome-reacted sperm in response to an AR inducer, which in general seems to be reliable when the natural AR inducers progesterone and ZP are used. Therefore, the present study investigates the possible involvement of a Cl^-/HCO_3^- exchanger and CFTR in transporting HCO_3^-, which is important for capacitation. The study assesses the induced AR in guinea pig sperm, a model that has been used conventionally for its ease in detection of acrosome exocytosis from the sperm head [27], in conjunction several other biochemical and fluorimetric techniques to monitor different capacitation-associated events.

MATERIALS AND METHODS

Media Preparation

The media used throughout the study comprised a low-Ca^{2+} MCM that consisted of 111.76 mM NaCl, 2.7 mM KCl, 0.49 mM $MgCl_2$, 25.07 mM $NaHCO_3$, 2.78 mM glucose, 10 mM sodium lactate, 1.0 mM sodium pyruvate, 50 μg of kanamycin monosulfate/ml, 20 mM Hepes, and 4mg/ml of bovine serum albumin (Fraction V, A3059; Sigma, St. Louis, MO). The pH was 7.9, with a final osmolality of 300–305 mOsm/kg. No Ca^{2+} was added, although when measured as described previously [28, 29], the Ca^{2+} concentration was 23 μM. This low-Ca^{2+} MCM induces capacitation of guinea pig spermatozoa under in vitro conditions but does not support the AR [29–31]. The AR can occur only with the addition of Ca^{2+} [32, 33]; therefore, 2 mM $CaCl_2$ was added when the AR was induced. This medium contained 115.3 ± 1.7 mM Cl^-, n = 3 (hereafter referred to as complete MCM). The Cl^- in this medium was replaced with gluconic acid to yield Cl^--deficient MCM (26.0 ± 0.4 μM, n = 3).

Isolation and Preparation of ZP

Ovaries were obtained from female guinea pigs (age, 21–22 days) and were homogenized in cold buffer as described previously [29]. Briefly, the homogenate was washed through a two-step Percoll gradient (10%–20%), and the zonae were isolated by examining the samples of various layers under a phase-contrast microscope. The band containing most of the ZP was located in the interphase between the homogenate and the 10% Percoll. Zonae were stored at −20°C until the day of use, when they were solubilized at 60°C for 1 h, rendering them heat stabilized.

Evaluation of Sperm Capacitation

White and black retired male breeder guinea pigs were purchased from the Laboratory Animal Service Center of The Chinese University of Hong Kong and the Experimental Animal Center of Zhejiang Province. All animal experiments were approved by the animal experimentation ethics committee of The Chinese University of Hong Kong and by the local ethics committee of Zhejiang Province. All guinea pigs were housed and then euthanized. The spermatozoa from guinea pig vas deferens and cauda epididymis were washed for 18 min at 600 × *g* through a Percoll gradient (85%-55%-35%) and were adjusted to 2–3 × 10^7 cells/ml (unless indicated otherwise), resuspended in complete or Cl^--deficient MCM, and incubated at 38.5°C for 6 h [29, 34]. Sperm capacitation was evaluated by the induction of rapid synchronization of the AR following the addition of 2 mM Ca^{2+} and progesterone or ZP [29, 35]. To assess the capacitation-dependent spontaneous or induced AR, spermatozoa were first exposed to reagents or their solvents as controls for 6 h to capacitate them and were then washed, resuspended in the desired medium, and treated with 2 mM Ca^{2+} or other agonists for 15 min before assaying the AR using phase-contrast microscopy. The percentage of AR was determined among 600 motile spermatozoa. The spermatozoa that had lost their acrosomal caps without losing motility were recorded as acrosome reacted.

Measurement of pHi in Spermatozoa

The level of pHi was determined by cells loaded with 2′,7-bis-2 (carbosyethyl)-5-(and-b)-carboxyfluorescence, acetoxymethyl ester (BCECF) as previously described [14], with minor modifications. Briefly, caudal epididymal spermatozoa were obtained from retired breeder guinea pigs and were adjusted to ~2 × 10^6 cells/ml in complete MCM and in Cl^--deficient MCM. Spermatozoa were then incubated in a 5% Co_2 incubator at 38.5°C. When needed, 5 μM BCECF was added, and the incubation continued for an additional 30 min. Following this, the cells were pelleted and washed twice to remove free dye before measurement of pHi. Fluorescence was detected by an excitation ratio of 490:440 nm (emission, 520 nm) using a luminescence spectrometer (LS50B; Perkin-Elmer, Norwalk, CT). Calibration was performed according to the method by Fraire-Zamora and Gonzalez-Martinez [36], with modification. Briefly, at the end of each trace, 10 μl of 5 mM nigericin was added to permeabilize the cells, the pH was then acidified with 10 μl of HCl, and the measured pH values (determined with a conventional pH meter) were compared with the corresponding ratio values. Usually, we make three additions before ending the experiment. These data were analyzed using Prism 3.0 software (GraphPad Software, San Diego, CA) to convert ratios to pH values.

Intracellular cAMP Measurement

Spermatozoa were incubated in complete or Cl^--deficient MCM and in HCO_3^--deficient MCM for 6 h. Fifty microliters of sperm suspension (~10^6 cells/ml) was added to an equal volume of the same medium. Incubations were ended by the addition of five volumes of ice-cold 100 mM HCl in 100% ethanol. Samples were kept on ice for 30 min and were then lyophilized and assayed for cAMP (Assay Designs, Ann Arbor, MI) using the manufacturer's protocol for acetylated samples.

Protein Tyrosine Phosphorylation of Spermatozoa

Incubated sperm were centrifuged at 5000 × *g* for 3 min at room temperature, washed once in 1 ml of PBS, resuspended in sample buffer without mercaptoethanol and boiled for 5 min, and then centrifuged and boiled in the presence of 5% 2-mercaptoethanol for 5 min. Next, SDS-PAGE was performed in 10% gels with constant 100 V for 1.5 h, followed by transfer to polyvinylidene fluoride membrane for 1 h at 4°C. The membrane was blocked and then incubated with primary antibody (1:2000), a monoclonal mouse antibody against phosphotyrosine (p-Tyrosine, 4G10; Cell Signaling Technology, Boston, MA), at 4°C overnight. After washing with Tris-buffered saline Tween-20 (TBST) four times, membrane was incubated with the secondary antibody (1:7500), peroxidase-conjugated affinity-purified anti-mouse IgG (Rockland, Gilbertsville, PA), for 1 h at room temperature and then washed four times with TBST. Positive immunoreactive bands were detected using an enhanced chemiluminescence (ECL) kit (Lumi-Light plus Western blotting detection reagents, RPN2106; GE HealthCare, Little Chalfont, England) according to the manufacturer's instructions.

Assessment of Sperm-Fertilizing Capacity

To ascertain whether Cl^- has a role in sperm-fertilizing ability, we used a zona-free hamster egg assay as a test system for appraisal of the fertilizing ability of guinea pig spermatozoa. This method has been described previously [37, 38].

Assessment of Sperm-Hyperactivated Motility

Sperm-hyperactivated motility (HAM) accompanies sperm capacitation. HAM represents a change in motility pattern, specifically an increase in the amplitude of the flagella bend and average lateral head movement. No software for analysis of guinea pig sperm motility is available, to our knowledge. In this study, three characteristic movement patterns were considered hyperactivated motility as described previously [35], with some modifications as follows: 1) sperm head exhibiting marked lateral placement, with forward motility and tail whiplash movement; 2) sperm forward progressive movement, with a nonlinear trajectory; 3) sperm head equatorial segment attachment to the slide surface, displaying quick swinging with flagella movements that are much greater in the flagellum than in the head, without cell progression [39, 40].

Immunofluorescence Staining

For indirect immunofluorescence studies of CFTR and SLC26A3 (solute carrier family 26, number 3) localization, spermatozoa were washed by centrifugation through a three-layer gradient of Percoll as already described. Sperm were fixed in 4% paraformaldehyde overnight at 4°C and were then smeared on slides previously coated with poly-L-lysine and air dried. After washing with PBS three times, the slides were blocked with 10% normal goat serum at room temperature for 1 h. After further washing in PBS twice for 5 min each, sperm were incubated with primary CFTR antibody (CF3, ab2784, 1:500; Abcam, Cambridge, England), mouse isotype-specific IgM (Rockland) as a CFTR negative control, or SLC26A3 antibody (1:250; a gift from Dr. P.

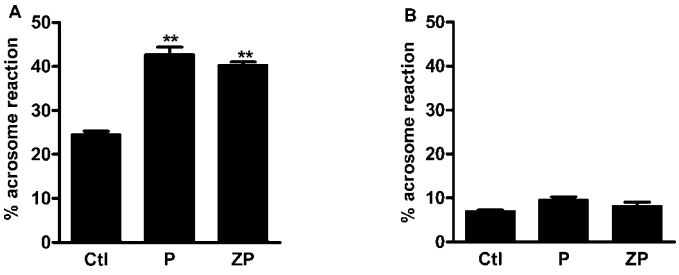

FIG. 1. Dependence of sperm capacitation on Cl^- in MCM. Capacitation in guinea pig sperm was assessed by their ability to undergo the agonist-induced AR after incubation for 6 h in MCM with or without Cl^-. Results are means ± SEM (n = 3). **A**) Sperm were initially incubated in MCM for capacitation, then washed and resuspended in Cl^--deficient MCM (Ctl), and stimulated with progesterone (P, 10 μM) or ZP (ZP, 1 ZP/μl) for 15 min before the AR assessment. **B**) The progesterone- or ZP-induced AR was greatly suppressed when sperm were incubated in Cl^--deficient MCM for 6 h and switched to complete MCM for the AR induction, indicating the dependence of capacitation but not the AR on Cl^-. **Compared with the control and Cl^--deficient MCM, respectively, $P < 0.001$.

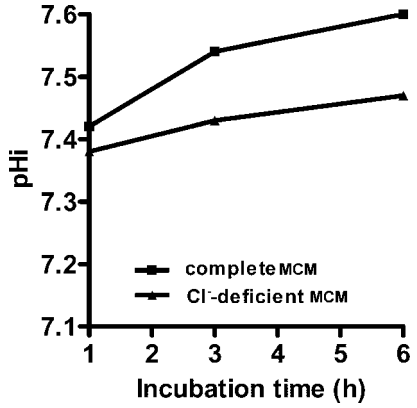

FIG. 3. The Cl^- dependence of sperm pHi. Washed spermatozoa were incubated in Cl^--deficient or complete MCM. Five micromolar BCECF was loaded for 30 min before determination of the pHi. The representative time course changes in pHi during capacitation showed suppressed increase in pHi when sperm were incubated in Cl^--deficient MCM compared with those incubated in complete MCM.

Höglund) at 4°C overnight. Sperm were washed with PBS three times and were incubated with secondary antibody (anti-mouse IgG+IgM-fluorescein isothiocyanate [FITC], ab47830, 1:1000; Abcam; and Alexa 488-conjugated goat anti-rabbit IgG, A11008, 1:1000; Invitrogen, San Diego, CA) in a dark room for 1 h at room temperature. Unbound antibody was removed by washing with PBS three times. The slides were then stored in a dark box before visualization.

Western Blotting

Proteins were resolved by SDS-PAGE on polyacrylamide gels, followed by transfer onto nitrocellulose membranes. Membranes were blocked with 4% milk in TBST for 1 h before being probed with primary mouse anti-human CFTR monoclonal antibody (CF3, ab2784, 1:2000; Abcam) or SLC26A3 antibody (sc34943, 1:500; Santa Cruz Biochemicals, Santa Cruz, CA) or its blocking peptide (sc34942p; Santa Cruz) at 4°C overnight. Membranes were washed three times in TBST, followed by incubation with 1:10 000 anti-mouse IgG-horseradish peroxidase (HRP) (ab6006, 1:10 000; Abcam) or 1:5000 donkey anti-goat IgG–HRP (sc2033; Santa Cruz) in TBST containing 1% BSA for 1 h at room temperature. Following three washes in TBST, proteins were detected using an enhanced chemiluminescence kit (ECL plus Western blotting detection reagents, RPN2132; GE HealthCare) according to the manufacturer's instructions.

Statistical Analysis

Results are given as means ± SEM. For statistical analysis, percentage data were transformed (arcsin $\sqrt{}$[percentage of cells/100]), and comparisons were made using one-way ANOVA, followed by Tukey or Dunnett post hoc tests. $P < 0.05$ was considered statistically significant.

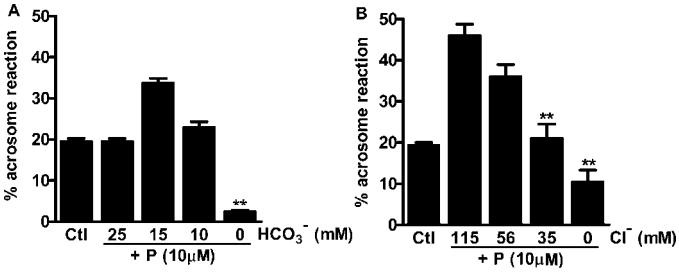

FIG. 2. Sperm capacitation requires both Cl^- and HCO_3^-. **A**) Effect of varying HCO_3^- concentrations on capacitation. Washed sperm were incubated in MCM with different concentrations of HCO_3^- but with constant Cl^- concentration (115 mM) for 6 h and then switched to Cl^-/HCO_3^- complete MCM for the AR induction (n = 5). **B**) Effect of varying Cl^- concentrations on capacitation. Sperm were incubated in MCM with different concentrations of Cl^- but with constant HCO_3^- concentration (25 mM) for 6 h and were then switched to Cl^-/HCO_3^- complete MCM for the AR induction (n = 3). Depletion of HCO_3^- or Cl^- prevented capacitation. Results are means ± SEM. **Compared with highest anion concentration, $P < 0.001$. The MCM incubation without progesterone (P, 10 μM) induction serves as a control (Ctl).

RESULTS

Dependence of Sperm Capacitation on Cl^- Concentrations

Using the sperm AR for assessing capacitation (two separate events), it was necessary to distinguish the effect of Cl^- on sperm capacitation from its effects on the AR. To do that, Cl^- replacement was made separately in deficient MCM or in complete MCM in which the AR was induced. When washed sperm were incubated initially in complete MCM for 6 h, followed by thorough washing and resuspension in Cl^--deficient MCM, there was a significant increase in the percentage of the AR compared with that in the control in response to stimulation with 10 μM progesterone or with ZP (1 ZP/μl) for 15 min (Fig. 1A). However, when sperm were incubated in Cl^--deficient MCM but resuspended in complete MCM during the AR induction, the percentage of AR was greatly reduced, with no significant difference between treatments of sperm regardless of the stimulus used (Fig. 1B), indicating that Cl^- has an essential role in sperm capacitation but not in the AR itself.

Sperm Capacitation Requires Both Cl^- and HCO_3^-

Because HCO_3^- had been shown to initiate sperm capacitation, we then tried to determine whether the effect of Cl^- on sperm capacitation could be independent of HCO_3^-. Two sets of experiments were performed. One set was conducted using MCM at different HCO_3^- concentrations with a normal Cl^- concentration (115 mM). As shown in Figure 2A, sperm capacitation was reduced as the concentration of HCO_3^- in the MCM decreased, with total abolishment when HCO_3^--deficient MCM was used for incubation, confirming an essential role of HCO_3^- in the process of capacitation.

The other set of experiments was performed using MCM of various Cl^- concentrations with a constant HCO_3^- concentration (25 mM). After incubation for 6 h, sperm were incubated in complete MCM and were stimulated with progesterone for 15 min before assessing the AR. The results showed that sperm capacitation decreased with decreasing Cl^- concentrations in the MCM, despite the presence of a sufficient amount of HCO_3^- (Fig. 2B), indicating that sperm capacitation requires

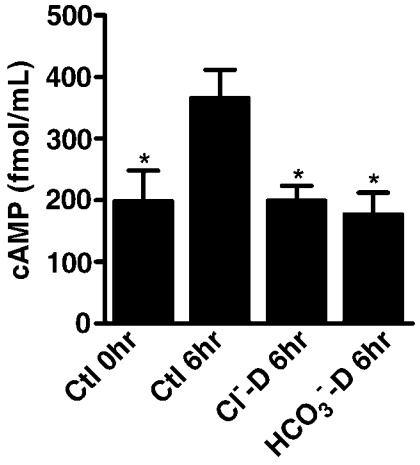

FIG. 4. Effect of Cl^- and HCO_3^- on capacitation-associated increases in sperm intracellular cAMP levels. Washed sperm were incubated separately in complete MCM (Ctl) for 0 h and 6 h or in Cl^--deficient (Cl^--D) and HCO_3^--deficient (HCO_3^--D) MCM for 6 h before intracellular cAMP measurement (n = 3). Results are means ± SEM. *Compared with control 6 h, $P < 0.05$.

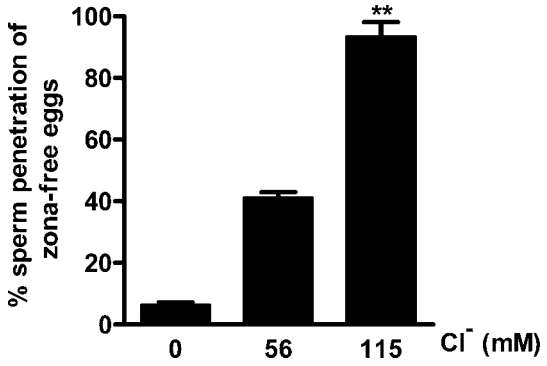

FIG. 6. Effect of Cl^- on sperm-egg fusion. Washed sperm were preincubated separately in complete MCM under capacitation conditions for 6 h and were then cocultured with 20–30 zona-free hamster eggs in complete BWW (final Cl^- concentration, ~115 mM) or in Cl^--deficient BWW (final Cl^- concentration, ~56 mM) for 3 h. In addition, sperm were incubated in Cl^--deficient MCM for 6 h, washed, and then cocultured with 20–30 zona-free hamster eggs in Cl^--deficient BWW (final Cl^- concentration, 0 mM) for 3 h. The percentage of spermatozoal penetration into eggs was assessed (n = 3). Results are means ± SEM. **Compared with ~56 mM Cl^- and with Cl^--deficient medium (0 mM Cl^-), $P < 0.001$.

Cl^- and HCO_3^-, with the possibility that Cl^- may be required for the transport of HCO_3^- into sperm.

Effect of Cl^- on Increased pHi During Capacitation

An increase in pHi of sperm due to the entry of HCO_3^- is an early hallmark event in the process of capacitation. Indeed, when sperm were incubated in complete MCM, the pHi increased with time (Fig. 3); however, it remained almost unchanged in Cl^--deficient MCM throughout the period of capacitation, consistent with the observed Cl^- dependence of sperm capacitation.

Effect of Cl^- and HCO_3^- on Sperm cAMP Levels

If Cl^- is required for transporting HCO_3^- into sperm (such as in the case of an anion exchanger) depletion of Cl^- should affect HCO_3^--dependent events that are known to be involved in sperm capacitation. We first examined whether cAMP production resulting from HCO_3^- activation of soluble adenylate cyclase could be affected by Cl^- depletion. Sperm were incubated separately in Cl^-/HCO_3^- complete MCM or in Cl^--deficient or HCO_3^--deficient MCM for 6 h. The intracellular cAMP levels in sperm measured by ELISA (Fig. 4) showed that the mean level of cAMP in sperm incubated in Cl^-/HCO_3^- complete MCM for 6 h was significantly elevated compared with that observed in the control (0 h). However, this elevation of intracellular cAMP was completely abolished when sperm were incubated in Cl^--deficient or HCO_3^--deficient MCM for the same amount of time ($P < 0.05$). The effect of Cl^- depletion on sperm cAMP levels was similar to that of HCO_3^- depletion, indicating the possible involvement of a similar mechanism in both cases, with the most likely candidate being an anion exchanger. Depletion of either ion ceases operation of the exchanger, resulting in the failure of HCO_3^- entry and cAMP production.

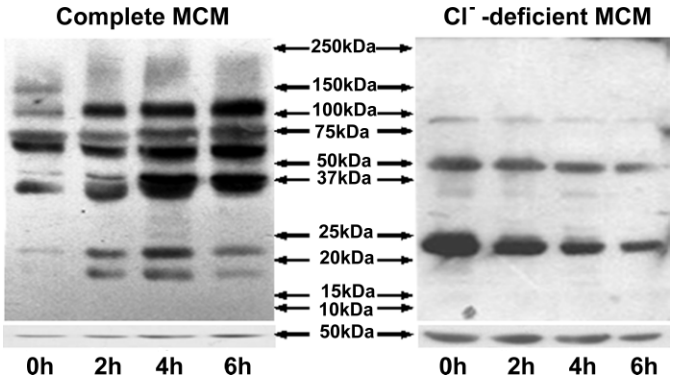

FIG. 5. Effect of depleted Cl^- on sperm protein tyrosine phosphorylation. Washed spermatozoa were incubated under capacitation conditions at 0, 2, 4, and 6 h, and then proteins were extracted for Western blot analysis. The primary antibody, a monoclonal mouse antibody against phosphotyrosine (p-Tyrosine, 4G10) 1:2000, and the second antibody, peroxidase-conjugated affinity-purified anti-mouse IgG 1:7500, were used. Left: Protein tyrosine phosphorylation in guinea pig sperm incubated in complete MCM. Right: Protein tyrosine phosphorylation in sperm incubated in Cl^--deficient MCM showing reduced phosphorylated protein with time.

Effect of Cl^- on Capacitation-Related Protein Tyrosine Phosphorylation

To further confirm whether Cl^- is involved in mediating capacitation-associated HCO_3^--dependent processes, its effect on protein tyrosine phosphorylation during sperm capacitation was examined. Washed sperm were separately incubated in Cl^-/HCO_3^- complete MCM or in Cl^--deficient MCM for 0, 2, 4, and 6 h. The results showed that sperm incubated in Cl^-/HCO_3^- complete MCM were accompanied by a time-dependent increase in protein tyrosine phosphorylation of a subset of proteins of 25–120 kDa normally observed during sperm capacitation. However, sperm incubated in Cl^--deficient MCM showed reduced protein tyrosine phosphorylation, with only two phosphorylated proteins at about 23 and 50 kDa observed (Fig. 5). These two protein bands were also attenuated with incubation time in the absence of Cl^- in MCM, suggesting that Cl^- is also required for protein tyrosine phosphorylation, a well-known event downstream of HCO_3^- activation of cAMP production.

Effect of Cl^- on Sperm-Fertilizing Capacity

In vitro fertilization may measure the actual physiological function of the sperm-capacitated status. To determine whether

sperm-fertilizing ability is affected by Cl^-, we used a zona-free hamster egg model as a test system for the assessment of fertilizing capacity in guinea pig spermatozoa [37, 38, 41]. Washed sperm were preincubated in complete or Cl^--deficient MCM for 6 h. Zona-free hamster eggs in complete or Cl^--deficient Biggers-Whitten-Whittingham medium (BWW) were introduced separately into sperm suspension (1:1, 50 μl each) in complete or Cl^--deficient MCM for coincubation for 3 h. Sperm were exposed to a constant concentration of complete Cl^- (~115 mM), a half concentration of Cl^- (~56 mM), or Cl^--deficient medium (both MCM and BWW Cl^- deficient). As shown in Figure 6, the penetration rate of sperm declined significantly with a decrease in concentration of Cl^- in the medium. Sperm-egg fusion almost ceased when spermatozoa and eggs were exposed in both types of Cl^--deficient media. To evaluate whether Cl^- directly affects the fertilizing ability of zona-free hamster eggs, we preincubated the eggs in one-third Cl^- BWW medium for 3 h, washed them in complete BWW medium, and then resuspended them in the same medium for coculture for 3 h. Similarly, sperm were preincubated in complete MCM for 6 h and were then mixed with eggs for 3 h as already described. Fertilizing capacity of the zona-free hamster eggs was not significantly affected and was similar to that in complete MCM (data not shown).

Effects of Inhibitors and Antibody of SLC26A3 and CFTR on Capacitation-Associated HCO_3^--Dependent Events

Although we had previously demonstrated an important role of CFTR in transporting HCO_3^- into sperm that is necessary for capacitation [14], the observed Cl^- dependence of capacitation-associated HCO_3^--dependent events suggested additional involvement of an exchanger for Cl^-/HCO_3^-. Because *SLC26A3* mutations in men have been associated with subfertility [42], the exchanger may be involved in sperm capacitation, perhaps working together with CFTR for the transport of HCO_3^-. We tested this hypothesis by examining the effects of inhibitors of CFTR and of the candidate anion exchanger, SLC26A3, as well as its antibody on several capacitation-associated events in the following experiments.

CFTRinh-172, niflumate, and SLC26A3 antibody inhibit sperm capacitation. CFTR inhibitor (CFTRinh-172) in various concentrations (5–100 nM) was added to sperm suspensions at the beginning of capacitation, incubated for 6 h, and then washed and resuspended in inhibitor-free MCM challenged with 10 μM progesterone for 15 min before measurement of the AR. As shown in Figure 7A, compared with the progesterone-induced AR, the percentage of AR decreased significantly with increased concentrations of CFTRinh-172 in the MCM ($P < 0.001$), indicating the involvement of CFTR in sperm capacitation. When sperm were capacitated in HCO_3^--deficient MCM, there was no significant difference between the progesterone-induced and CFTRinh-172 groups. Similarly, niflumate (an inhibitor more selective for SLC26A3) at various concentrations (1–100 μM) or 4,4′-di-isothiocyanatostilbene-2, 2′-disulfonic acid (DIDS, 1–100 μM) (an inhibitor of several anion exchangers that are sensitive to SLC26A6 and the SLC4 family) was added to the capacitating medium but not to the medium for inducing the AR. As shown in Figure 7B, a comparison of the concentration-dependent effects between niflumate and DIDS showed a more potent effect of niflumate over that of DIDS. There was no significant difference between the two treatments when sperm were incubated in HCO_3^--deficient MCM (Fig. 7C).

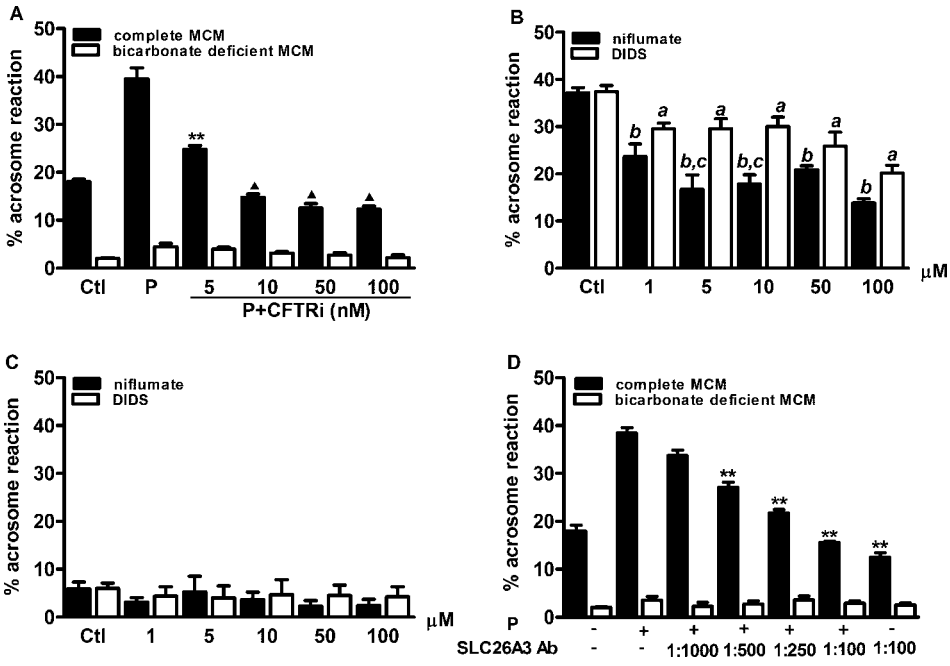

FIG. 7. Effects of CFTR inhibitor (CFTRi) and anion exchanger inhibitors, as well as SLC26A3 antibody, on capacitation with or without bicarbonate. Washed sperm were incubated in complete or HCO_3^--deficient MCM without (Ctl) or with CFTRinh-172 (CFTRi, 5–100 nM), niflumate (1–100 μM), DIDS (1–100 μM), or SLC26A3 antibody (1:1000 to 1:100) separately for capacitation and were then washed and challenged with progesterone (P, 10 μM) for 15 min before the AR assessment. Results are means ± SEM. **A**) Inhibitory effect of CFTRi on capacitation (n = 6–8). **Compared with the progesterone-treated group, $P < 0.001$; triangle, compared with the CFTRi 5 nM group, $P < 0.001$. **B**) Comparison of concentration-dependent inhibitory effects of niflumate and DIDS on capacitation in complete MCM (n = 3). *a*, Compared with DIDS control (Ctl), $P < 0.05$–0.001; *b*, compared with niflumate Ctl, $P < 0.001$; *c*, compared with corresponding DIDS treatment, $P < 0.01$. **C**) Comparison of concentration-dependent inhibitory effects of niflumate and DIDS on capacitation in HCO_3^--deficient MCM (n = 3). **D**) Effect of SLC26A3 antibody (Ab) on capacitation. (n = 3). **Compared with the progesterone-treated group, $P < 0.001$.

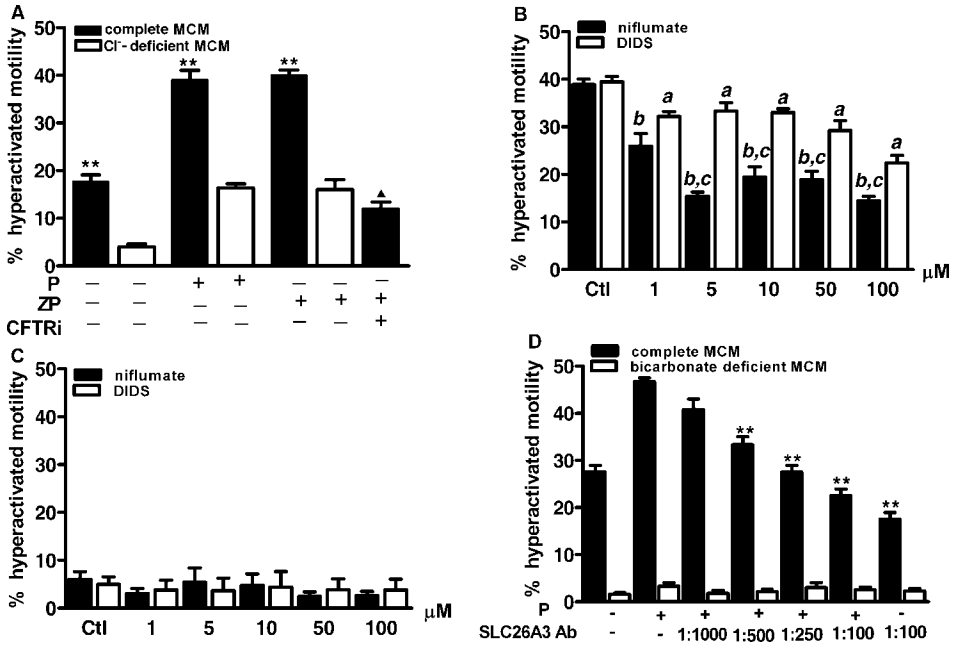

FIG. 8. Effects of Cl^- and transport inhibitors, as well as SLC26A3 antibody, on HAM with or without bicarbonate. Washed sperm were incubated in Cl^--deficient MCM, complete MCM, or HCO_3^--deficient MCM with or without CFTRinh-172 (CFTRi, 100 nM), niflumate (1–100 μM), DIDS (1–100 μM), or SLC26A3 antibody (1:1000 to 1:100) separately for capacitation and were then washed and challenged with progesterone (10 μM) or ZP (1 ZP/μl) for 15 min before HAM assessment. Results are means ± SEM. **A**) Depletion of Cl^- and CFTRi reduced sperm HAM (n = 3). **Compared with corresponding Cl^--deficient MCM, $P < 0.001$; triangle, compared with ZP treatment in complete MCM, $P < 0.001$. **B**) Comparison of concentration-dependent inhibitory effects of niflumate and DIDS on HAM in complete MCM (n = 3). *a*, Compared with DIDS control (Ctl), $P < 0.05$–0.001; *b*, compared with niflumate Ctl, $P < 0.001$; *c*, compared with corresponding DIDS treatment, $P < 0.05$–0.001. **C**) Comparison of concentration-dependent inhibitory effects of niflumate and DIDS on HAM in HCO_3^--deficient MCM (n = 3). **D**) Effect of SLC26A3 antibody (Ab) on sperm HAM (n = 3). **Compared with progesterone-treated group, $P < 0.001$.

Because the inhibitors used may not be exclusive for SLC26A3, its specific antibody was also used. Various concentrations of SLC26A3 antibody (1:100 to 1:1000) were added to sperm suspensions for capacitation for 6 h and then were washed, resuspended in antibody-free medium, and challenged with 10 μM progesterone for 15 min before assessment of the AR. As shown in Figure 7D, the AR was markedly reduced with increased concentrations of antibody in the medium compared with the progesterone-treated group ($P < 0.001$), indicating the involvement of SLC26A3. When capacitated in HCO_3^--deficient MCM, there were no significant differences between the various treatment groups.

Sperm-hyperactivated motility. HAM is a capacitation-associated event that may also serve as a potential assay for capacitation [39, 40]. First, it was necessary to confirm whether Cl^- would have a similar effect on HAM as it did on the other capacitation-associated events already described. Washed sperm were incubated separately in Cl^--deficient or complete MCM with or without CFTR inhibitor (CFTRinh-172) for 6 h and were then stimulated with progesterone (10 μM) or ZP (1 ZP/μl) for 15 min before assessment for sperm HAM. As shown in Figure 8A, sperm in complete MCM in response to progesterone or ZP exhibited significantly higher HAM than sperm in Cl^--deficient MCM. When CFTRinh-172 was added, the ZP-induced AR was inhibited. Similar to the results observed with the induced AR, the inhibitors of anion exchangers, as well as its antibody, also significantly inhibited sperm HAM (Fig. 8, B and D), further confirming the involvement of SLC26A3 in the process of sperm capacitation. When the concentration-dependent effects of niflumate vs. DIDS were compared, a more potent effect of niflumate was observed over that of DIDS ($P < 0.05$ to $P < 0.001$) (Fig. 8B). Similarly, there were no differences between niflumate and DIDS treatments or among different concentrations of SLC26A3 antibody when sperm were capacitated in HCO_3^--deficient MCM (Fig. 8, C and D).

Expression and Localization of CFTR and SLC26A3 in Sperm

The functional studies herein clearly demonstrated the involvement of CFTR and an anion exchanger, which is likely to be SLC26A3; however, their expression and localization have not been demonstrated in guinea pig sperm, to our knowledge. In fact, SLC26A3 has not been shown to be expressed in sperm of any species as far as we know. Using indirect immunofluorescence staining, we demonstrated that CFTR was localized to the sperm equatorial segment, as previously demonstrated in human and mouse sperm [14], whereas SLC26A3 was localized over the acrosomal anterior area of the sperm head (Fig. 9, A and B). The expression of CFTR and SLC26A3 was further demonstrated by Western blot analysis, which confirmed the presence of a 170-kDa protein of CFTR, as shown previously [14], and the presence of a 75-kDa protein of SLC26A3.

DISCUSSION

Although it had been established that HCO_3^- has an essential role in sperm capacitation [3–7], the present study demonstrated an equally important role of Cl^- in the process by mediating the entry of HCO_3^- that is required for capacitation. To initiate capacitation, HCO_3^- has to first enter into sperm and act on an HCo_3^- sensor, soluble adenylate cyclase, which in

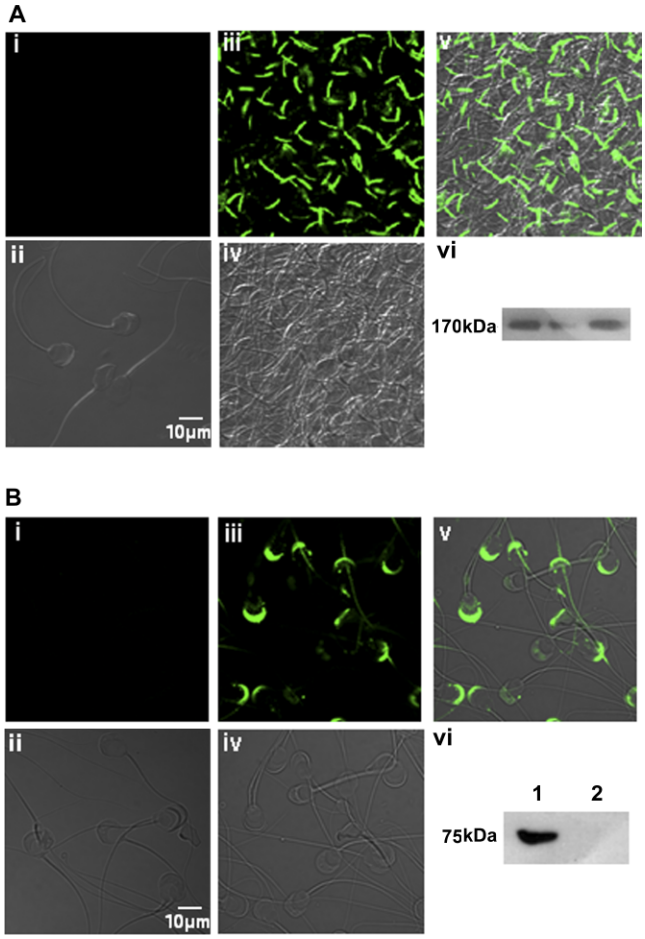

FIG. 9. Localization and expression of CFTR and SLC26A3 in guinea pig sperm. **A**) Immunostaining and Western blot analysis of CFTR in guinea pig sperm using a mouse monoclonal antibody (CF3) to CFTR (ab2784, 1:500 or 1:2000) and the secondary antibody anti-mouse IgG+IgM-FITC (ab47830, 1:1000) or anti-mouse-HRP (ab6006, 1:10 000). i) Negative immunostaining control of CFTR with the mouse isotype-specific IgM. ii) Corresponding differential interference contrast image. iii) Immunofluorescence of CFTR localized to the equatorial segment region of the sperm head. iv) Corresponding differential interference contrast image of guinea pig sperm. v) Superimposed image of CFTR staining and differential interference contrast image (magnification ×630). vi) Western blot analysis of CFTR in guinea pig sperm recognized a specific band of about 170 kDa. Left to right: protein loading of 30 μg, 10 μg, and 20 μg. **B**) Immunostaining and Western blot analysis of SLC26A3 in guinea pig sperm using an antiserum against SLC26A3 and Alexa 488-conjugated goat anti-rabbit IgG (A11008, 1:1000) or SLC26A3 antibody (sc34943, 1:500) and donkey anti-goat IgG-HRP (sc2020, 1:5000). i) Negative control without primary antibody. ii) Corresponding differential interference contrast image of guinea pig spermatozoa. iii) Immunofluorescence of SLC26A3 in guinea pig spermatozoa localized in the anterior acrosome region. iv) Corresponding differential interference contrast image. v) Superimposed image of SLC26A3 staining and differential interference contrast image (magnification ×630). vi) Western blot analysis of SLC26A3 in guinea pig spermatozoa. Lane 1: SLC26A3 antibody. Lane 2: Negative control with blocking peptide (sc34942p). Bar = 10 μm.

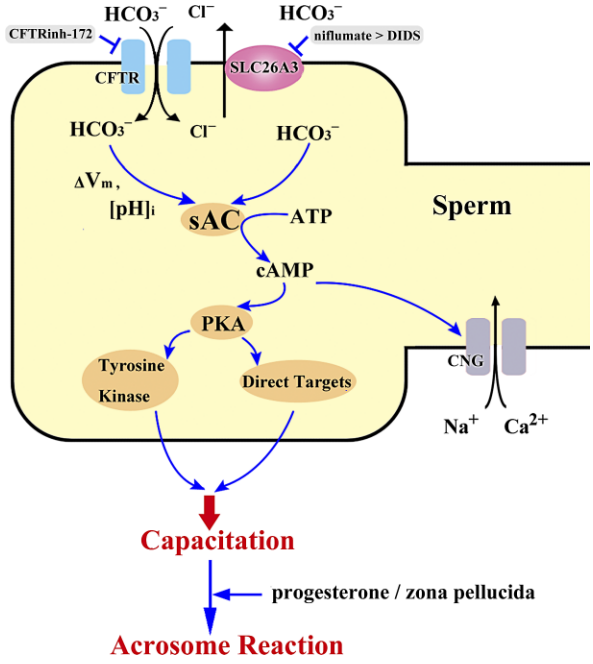

FIG. 10. Schematic illustration of involvement of SLC26A3 and CFTR in bicarbonate entry and in bicarbonate-dependent events involved in sperm capacitation. The HCO_3^- influx is mediated by SLC26A3 with an exchange of $2Cl^-/1HCO_3^-$. Apart from its reported role in conducting HCO_3^- directly, CFTR may act as a Cl^- channel to provide a recycling pathway for Cl^- that is required for SLC26A3 function. The sites of action for inhibitors CFTRinh-172, niflumate, and DIDS, as well as the intracellular HCO_3^--dependent events, are also shown. sAC, soluble adenylyl cyclase; Vm, membrane potential; PKA, protein kinase A; [pH]i, intracellular pH; CNG, cyclic nucleotidegated channel.

turn triggers the signaling cascade leading to capacitation [11]. Some of the key events in this cascade such as increased pHi, cAMP production, and protein tyrosine phosphorylation, which have been previously shown to be dependent on HCO_3^-, were also found to be dependent on Cl^- in MCM in the present study. The assessment of sperm capacitation by the induced AR in the present study also supports the Cl^- dependence of capacitation but not of the AR. Findings by other investigators have suggested a Cl^- dependence of the AR process. Replacing Cl^- with Br^- in sperm-suspending medium was shown to result in inhibition of the zona-initiated hamster sperm AR [43]. Others reported that Cl^- was required for the progesterone-initiated AR in human and porcine sperm [44]. Sperm capacitation and the AR are sequential but separate processes [22], and the other investigators did not differentiate these two processes. In the present study, Cl^- depletion in the capacitating medium, but not in the AR-inducing medium, reduced acrosome-reacted sperm, indicating that Cl^- is required for capacitation but not for the AR. Indeed, agonist-induced HAM, which is an event associated with sperm capacitation, was also found to be dependent on Cl^- in the present study. Together with the demonstrated Cl^- dependence of the capacitation-associated key events, the present results confirm that Cl^- is required for capacitation.

The dependence of the capacitation-associated events on Cl^-, in addition to HCO_3^-, suggests the involvement of a Cl^-/HCO_3^- exchanger in mediating the entry of HCO_3^-. Indeed, an inhibitor of the anion exchanger was shown to reduce the HCO_3^--dependent sperm capacitation assessed by the AR or by HAM in the present study. This is in agreement with previous studies [19–21] demonstrating the presence of an anion exchanger in sperm. Although it has been known for years that an exchanger for Cl^- and HCO_3^- is present in mature sperm, its exact role in sperm function and its molecular identity are unknown as far as we know. The present study not only confirms the involvement of an anion exchanger in mediating HCO_3^- entry that is necessary for capacitation but also provides evidence indicating that the exchanger could be SLC26A3, with its expression in sperm demonstrated by

immunofluorescence staining and by Western blot analysis. The involvement of SLC26A3 is further supported by pharmacological evidence demonstrating a more potent effect of niflumate, an inhibitor more selective for SLC26A3 [45, 46], over that of DIDS, an inhibitor known to inhibit a wide spectrum of anion exchangers and cotransporters, including SLC4 member SLC4A2 (also known as AE2) [47]. Furthermore, SLC4A2 is expressed in mature mammalian sperm [48] and has been shown to be essential in spermiogenesis [49]. However, the observed more potent effect of niflumate over DIDS suggests that SLC26A3 has a more primary role in sperm capacitation. This notion was further supported by the observed inhibitory effect of its specific antibody on sperm capacitation. The specificity of the antibody against SLC26A3 has been previously reported [50, 51]; therefore, its interference with sperm capacitation observed in the present study confirms the involvement of SLC26A3 in the process. A sodium bicarbonate cotransporter has also been proposed to be involved in mouse sperm capacitation [52]. Although this mechanism may be a possible pathway for bicarbonate entry into sperm during capacitation, it cannot explain the Cl^- dependence of capacitation that was observed in the present study. Taken together, the present results suggest that SLC26A3 has a primary role in transporting bicarbonate into sperm, on which capacitation depends.

Patients with congenital chloride diarrhea with mutations in *SLC26A3* exhibit reduced fertility [42, 50], although the exact cause is unknown as far as we know. In the present study, we found that the capacity of spermatozoal penetration of oocytes was greatly reduced with decreased Cl^- concentrations. Fertilizing ability was almost abolished in Cl^--deficient MCM compared with complete MCM, indicating that Cl^- is required for fertilization. Based on the present findings, it is highly possible that defective SLC26A3 impairs HCO_3^- entry into sperm and affects the capacitation process, leading to reduced fertilizing ability and decreasing the fertility rate, as seen in patients with congenital chloride diarrhea. Therefore, SLC26A3 could be a potential molecular target for diagnosis of male infertility.

The present results also indicate that operation of the anion exchanger requires the involvement of CFTR as a Cl^- channel to provide a recycling pathway for Cl^-. Without proper CFTR function, the entry of HCO_3^- would be disrupted due to lack of Cl^- in exchange, resulting in capacitation failure, as demonstrated in the present study using CFTR inhibitor. The importance of CFTR in the process of sperm capacitation and in male fertility has been demonstrated in our previous investigations using CF heterozygote sperm, although it was unclear whether it had a direct or an indirect role [14]. The present study not only confirms the importance of CFTR in capacitation but also demonstrates an indirect role of CFTR in transporting HCO_3^- into sperm by providing a recycling pathway for Cl^- necessitated by the anion exchanger. The parallel working of CFTR with a Cl^-/HCO_3^- exchanger has been established for pancreatic HCO_3^- secretion [17, 18]. Although localized in sperm head, CFTR and SLC26A3 are not localized to the same region, suggesting that CFTR protein in sperm does not directly interact with the anion exchanger protein as it does in the pancreas [45, 53]. Nevertheless, the two sites in the sperm head where CFTR and SLC26A3 are localized are close enough to allow ion flux exchange, and the present study provides clear evidence for a functional interaction between CFTR and the SLC26A3 exchanger that leads to sperm capacitation, which is illustrated in a working model shown in Figure 10. The demonstrated involvement of SLC26A3 in sperm capacitation, in addition to CFTR, may shed new light on our understanding of molecular mechanisms regulating male fertility and may explain some cases of male infertility.

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