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Bicarbonate Is Required for Migration of Sperm Epididymal Protein DE (CRISP-1) to the Equatorial Segment and Expression of Rat Sperm Fusion Ability[1]

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ABSTRACT

Numerous studies have demonstrated that sperm capacitation is a bicarbonate-dependent process. In the rat, capacitation has not been studied as much as in other species, mainly because of the difficulties in carrying out functional assays with this animal model. In the present study, we have examined the influence of bicarbonate in the overall rat sperm capacitation process by analyzing involvement of the anion in 1) protein tyrosine phosphorylation, 2) migration of epididymal protein DE (also known as CRISP-1) from the dorsal region to the equatorial segment of the sperm head that occurs during capacitation, and 3) ability of sperm to fuse with the egg. Incubation of sperm under capacitating conditions produced a time-dependent increase in protein tyrosine phosphorylation. This phosphorylation did not occur in the absence of HCO_3^- and rapidly increased by either exposure of sperm to HCO_3^- or replacement of the anion by a cAMP analog (dibutyryl-cAMP) and a phosphodiesterase inhibitor (pentoxifylline). The absence of HCO_3^- also produced a significant decrease in the percentage of cells showing migration of DE to the equatorial segment. This parameter was completely restored by addition of the anion, but dibutyryl-cAMP and pentoxifylline were not sufficient to overcome the decrease in DE migration. Sperm capacitated in the absence of HCO_3^- were unable to penetrate zona-free eggs independent of the presence of the anion during gamete coincubation. Exposure of these sperm to bicarbonate, or replacement of the anion by dibutyryl-cAMP and pentoxifylline, only partially restored the sperm fusion ability. Altogether, these results indicate that, in addition to its influence on protein tyrosine phosphorylation, bicarbonate is required to support other rat sperm capacitation-associated events, such as migration of DE to the equatorial segment, and expression of the ability of sperm to fuse with the egg.

epididymis, fertilization, gamete biology, sperm capacitation

INTRODUCTION

On ejaculation, mammalian sperm are not able to fertilize an egg; they become fertilization competent only after a series of molecular, biochemical, and physiological changes, collectively referred to as capacitation, that occur in the female tract [1]. During capacitation, changes in membrane dynamics and properties render spermatozoa able to undergo the acrosome reaction (AR) and hyperactivated motility required for fertilization. These changes are regulated by the activation of cell-signaling cascades during residence of sperm 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. These events include the removal of cholesterol by cholesterol acceptors, such as BSA, from the sperm plasma membrane, modifications in plasma membrane phospholipids, increased tyrosine phosphorylation of proteins, and changes in intracellular ion concentrations (for review, see [2]).

Numerous studies have demonstrated that capacitation is a bicarbonate-dependent process [3–7]. The transmembrane movement of bicarbonate has been associated with the increase in intracellular pH observed during capacitation [8, 9] and with the regulation of cAMP metabolism [10]. Adenylyl cyclase, the enzyme responsible for cAMP synthesis, is markedly stimulated by physiologically relevant HCO_3^- concentrations [11–13], and it likely is soluble testicular bicarbonate-dependent adenylyl cyclase [14–16]. In several species, the HCO_3^--dependent increase in cAMP that occurs during capacitation is implicated in the regulation of a phosphorylation cascade leading to the increase in protein tyrosine phosphorylation [17–20].

Because of the importance of bicarbonate for sperm capacitation, we investigated the influence of this anion in the overall capacitation process in the rat. Although protein tyrosine phosphorylation is a key event that occurs during capacitation, it is not sufficient to consider sperm to be capacitated [18, 21]. Completion of capacitation is usually evaluated by two other parameters considered to be benchmark endpoints of this process, such as the ability of sperm to undergo the AR and to fertilize the egg. In the rat, however, the evaluation of these two parameters is more complicated than in other species, because methods for detecting sperm acrosomal status are hindered by the thinness of the rat acrosome and in vitro fertilization assays are very difficult to achieve in most laboratories working with this animal model.

For many years, our laboratory has been working with the molecular mechanisms that render rat sperm able to fertilize the egg. More specifically, we have studied the role of rat epididymal protein DE (also known as CRISP-1) in sperm function. First described by our group, this member of the cysteine-rich secretory protein (CRISP) family (32 kDa) [22, 23] is synthesized in an androgen-dependent manner by the proximal segments of the epididymis and associates with the sperm surface during maturation [24,

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[2]Correspondence: Vanina G. Da Ros, Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, Buenos Aires 1428, Argentina. FAX: 54 11 4 786 2564; e-mail: vgdaros@dna.uba.ar

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25]. Originally localized on the dorsal region of the sperm head, DE migrates to the equatorial segment (ES) during capacitation [26]. Several observations under both in vitro and in vivo capacitating conditions support the idea that this redistribution takes place concomitantly with occurrence of the AR [26] and, therefore, could be used as an indicator for the capacitation status of the cell.

The relocalization of DE to the ES, the region through which the sperm fuses with the egg, led us to explore the participation of DE in this specific event of fertilization. For that purpose, our laboratory successfully developed an in vitro sperm-egg fusion assay using rat zona-free eggs that confirmed the involvement of DE in gamete membrane fusion [27, 28]. Because only capacitated and acrosome-reacted sperm can fuse with the egg plasma membrane, the sperm-egg fusion assay used in our laboratory becomes a tool for evaluating the completion of capacitation in the rat.

In view of this, in the present study we have examined the influence of bicarbonate in the overall rat sperm capacitation process by analyzing involvement of the anion in protein tyrosine phosphorylation, migration of DE to the ES, and the sperm ability to fuse with the egg.

MATERIALS AND METHODS

Culture Media

The basic culture medium used throughout the present study contains 25 mM $NaHCO_3$ (Sigma Chemical Co., St. Louis, MO) [26]. The HCO_3^--free medium was prepared by omitting the addition of the anion, and the pH was maintained at 7.2–7.4 by the presence of 25 mM Hepes (Sigma).

Animals

Adult (age, 90–120 days) male and immature (age, 26–29 days) female Sprague-Dawley rats were maintained at 23°C with a 12L:12D photoperiod. Experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH).

Preparation of Sperm

Rat sperm were recovered from cauda epididymides, placed in a conical tube, covered with 2 ml of HCO_3^--free medium, and allowed to swim up at 37°C. After 10 min, aliquots of the upper sperm layer were added to 400 μl of media with or without HCO_3^- previously placed in tissue culture wells (16 mm; Costar, Cambridge, MA) to give a final concentration of 0.5–1 $\times$ 10^6 sperm/ml. Sperm suspensions were then incubated under paraffin oil at 37°C for different periods of time. For those cases in which capacitation was carried out in a HCO_3^--containing medium, incubation was performed in an atmosphere of 5% CO_2 in air.

For viability assessment, 40 μl of sperm suspensions were stained with prewarmed, 0.5% (v/v) eosin (yellowish; Sigma) in saline solution, and the incorporation of the dye was evaluated by light microscopy. The percentage of viability was calculated as the number of sperm that did not incorporate the dye over the total number of sperm counted. For evaluation of motility, 10 μl of sperm suspension were placed on prewarmed slides, and the percentage of motility was determined subjectively under a light microscope.

To determine the reversibility of the effect produced by the absence of bicarbonate during capacitation, sperm incubated in media without the anion for 3 h were exposed to 25 mM HCO_3^- for 15, 30, or 60 min. To evaluate whether the bicarbonate pathway is dependent on cAMP, spermatozoa were incubated in HCO_3^--free medium containing 5 mM N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP; Sigma) and 3 mM pentoxifylline (PTX; Sigma) for 4 or 5 h at 37°C.

In all cases, sperm were recovered on completion of the incubation period, and protein tyrosine phosphorylation, DE migration to the ES, and sperm fusion ability were analyzed.

To analyze the effect of bicarbonate on the Ca^{2+} ionophore-induced migration of DE, sperm previously incubated in media with or without HCO_3^- for 3 h were or were not exposed to 10 μM calcium ionophore A23187 (Sigma) for an additional hour and then subjected to indirect immunofluorescence (IIF) to analyze the localization of DE.

Sperm-Egg Penetration Assay

Recovery and treatment of oocytes. Female rats were superovulated by an injection (s.c.) of eCG (20 UI; Syntex, Buenos Aires, Argentina), followed by an administration (s.c.) of hCG (25 IU; Sigma) 48 h later. Metaphase II-arrested oocytes were collected from the oviducts of superovulated animals 12–15 h after hCG administration. Cumulus cells were removed by incubating the oocyte-cumulus complexes for 3 min in HCO_3^--free medium containing 0.1% (w/v) hyaluronidase (type IV; Sigma). After washing in fresh medium, the zonae pellucidae were dissolved by treating the oocytes with acid Tyrode solution (pH 2.5) for 10–20 sec. Finally, zona-free oocytes were thoroughly washed in fresh medium and distributed among treatment groups.

Gamete coincubation. Sperm prepared as described above were added to zona-free eggs at a final concentration of 0.5–1.5 $\times$ 10^5 cells/ml. Gametes were coincubated in the presence or absence of HCO_3^- for 2–3 h under paraffin oil at 37°C. Eggs were then washed in fresh medium to remove loosely adherent sperm, mounted on slides, and analyzed for evidence of sperm penetration under a phase-contrast microscope (400×). Eggs were considered to have been penetrated if a decondensing sperm head or two pronuclei and a sperm tail were present in the ooplasm.

Indirect Immunofluorescence

Sperm were fixed in 2% (w/v) paraformaldehyde in PBS for 10 min at room temperature. After extensive washing with PBS, sperm were air-dried on poly-L-lysine (0.01 mg/ml; Sigma)-coated slides, incubated with normal goat serum (NGS; 5% [v/v] in PBS) for 30 min at 37°C, and then exposed overnight at 4°C to a 1:100 dilution in 1% (v/v) NGS in PBS of rabbit polyclonal anti-DE [29] or monoclonal anti-phosphotyrosine antibodies (clone 4G10; Upstate Biotechnology, Lake Placid, NY). After washing three times in PBS, sperm exposed to anti-DE or anti-phosphotyrosine were incubated at 37°C with a 1:100 dilution in PBS of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Ig) G (Sigma) or FITC-conjugated goat anti-mouse IgG (Sigma), respectively. After 30 min, slides were washed and mounted in 90% (v/v) glycerol in PBS. For each preparation, 200 cells were examined with a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics.

Determination of Protein Tyrosine Phosphorylation

Sperm were washed twice with PBS and resuspended in Laemmli sample buffer [30]. After a 5-min incubation, samples were boiled for 5 min and centrifuged at 4000 $\times$ *g* for 3 min. The supernatants were recovered, boiled in the presence of 70 mM 2-β-mercaptoethanol (Sigma) for 5 min, and then centrifuged again at 4000 $\times$ *g* for 3 min. Supernatants were stored at −20°C until used. Solubilized proteins (corresponding to 0.75 $\times$ 10^6 spermatozoa/lane) were separated on 7.5% (w/v) polyacrylamide gels with 0.1% (w/v) SDS and then transferred onto nitrocellulose membranes according to the method described by Towbin et al. [31]. Nonspecific protein-binding sites on the membrane were blocked with 2% (w/v) dry skimmed milk in PBS (blocking solution). The membrane was then probed with the monoclonal anti-phosphotyrosine antibody (1:10 000) in blocking solution for 1 h. After washing with PBS and 0.1% (v/v) Tween 20, the membrane was incubated with anti-mouse peroxidase-conjugated IgG (1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in blocking solution for 1 h. The membrane was extensively washed, and reactive bands were detected by enhanced chemiluminescence (ECL kit; Amersham Life Science, Inc., Oakville, ON, Canada) according to the manufacturer's instructions. Negative controls involved preabsorption of the anti-phosphotyrosine antibody with 40 mM *O*-phosphotyrosine (Sigma) for 1 h. All incubations were performed at room temperature.

Calculations and Statistical Analysis

The percentages of sperm showing spontaneous and ionophore-induced migration of DE were analyzed by the Wilcoxon test and the chi-square test, respectively. Sperm motility and viability values and the percentages of penetrated eggs were analyzed by the chi-square test. Calculations were performed using Prism 3.0 software (GraphPad Software, San Diego, CA). Results were considered to be significantly different at $P < 0.05$.

RESULTS

Effect of Bicarbonate on Protein Tyrosine Phosphorylation

Considering that protein tyrosine phosphorylation is an important event during capacitation, we first examined the

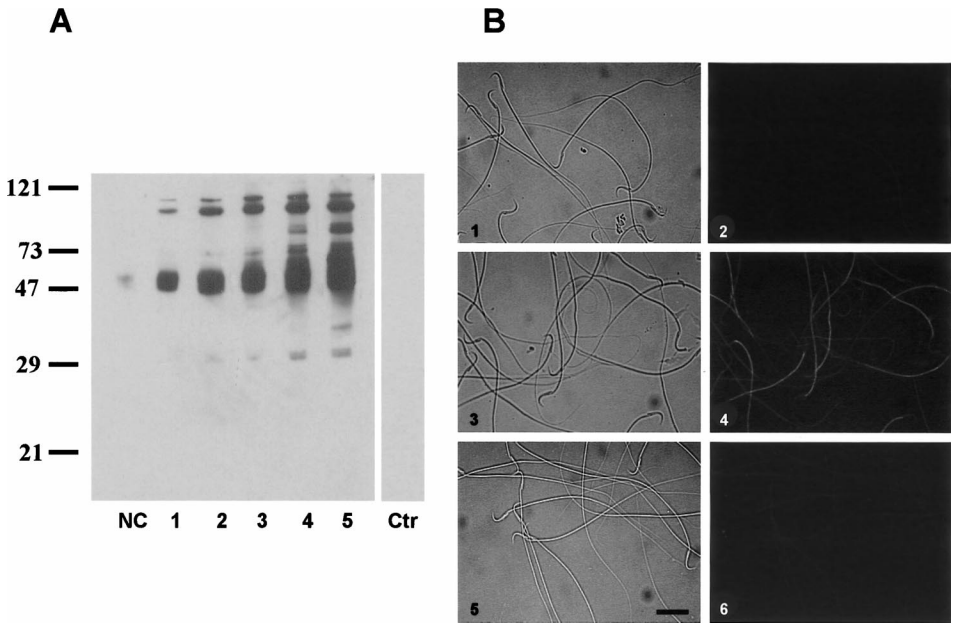

FIG. 1. Protein tyrosine phosphorylation during rat sperm capacitation. **A**) Western blot analysis of tyrosine-phosphorylated proteins. Rat epididymal sperm were incubated under capacitating conditions for 5 h. Aliquots were removed hourly, and sperm proteins were analyzed by Western blot using a monoclonal anti-phosphotyrosine antibody. The reactive bands were detected by enhanced chemiluminescence. Molecular mass standards (kDa) are shown on the left. Ctr, Protein extract from 5 h-incubated sperm revealed without primary antibody; NC, noncapacitated sperm. **B**) Localization of tyrosine-phosphorylated proteins on spermatozoa. Phase-contrast (left) and IIF (right) images of noncapacitated sperm (1 and 2) and of sperm incubated in capacitating medium for 5 h (3–6). The IIF was performed using a monoclonal anti-phosphotyrosine antibody (4) or without primary antibody as a control (6). Bar = 20 μm.

influence of bicarbonate in this signal transduction pathway. For this purpose, sperm were incubated for 5 h in a medium known to support capacitation as judged by the ability of sperm incubated in this medium to fuse with zona-free eggs [27]. Sperm aliquots were then recovered at various time intervals, and protein extracts were analyzed for tyrosine phosphorylation by Western blot using an anti-phosphotyrosine antibody. Results shown in Figure 1A revealed a time-dependent increase in tyrosine phosphorylation of proteins in the range of 30–120 kDa. When sperm were fixed and subjected to IIF using the same antibody, a complete negative reaction was observed for noncapacitated cells, but a clear fluorescent labeling on the midpiece and principal piece of the tail was detected in 80% of sperm incubated for 5 h (Fig. 1B). A negative reaction was observed in both Western blot and IIF experiments when proteins were revealed without primary antibody (Fig. 1).

To investigate whether bicarbonate is required for the capacitation-associated increase in protein tyrosine phosphorylation, sperm were incubated for either 3 or 5 h in a medium lacking bicarbonate. No effects on viability (64% ± 11% [mean ± SEM] vs. 60% ± 5%, n = 5; not significant [NS]) or motility (63% ± 2% vs. 68% ± 1%, n = 3; NS) were observed at the end of the incubation, but sperm incubated in the absence of bicarbonate did not display the pattern of phosphotyrosine-containing proteins present in

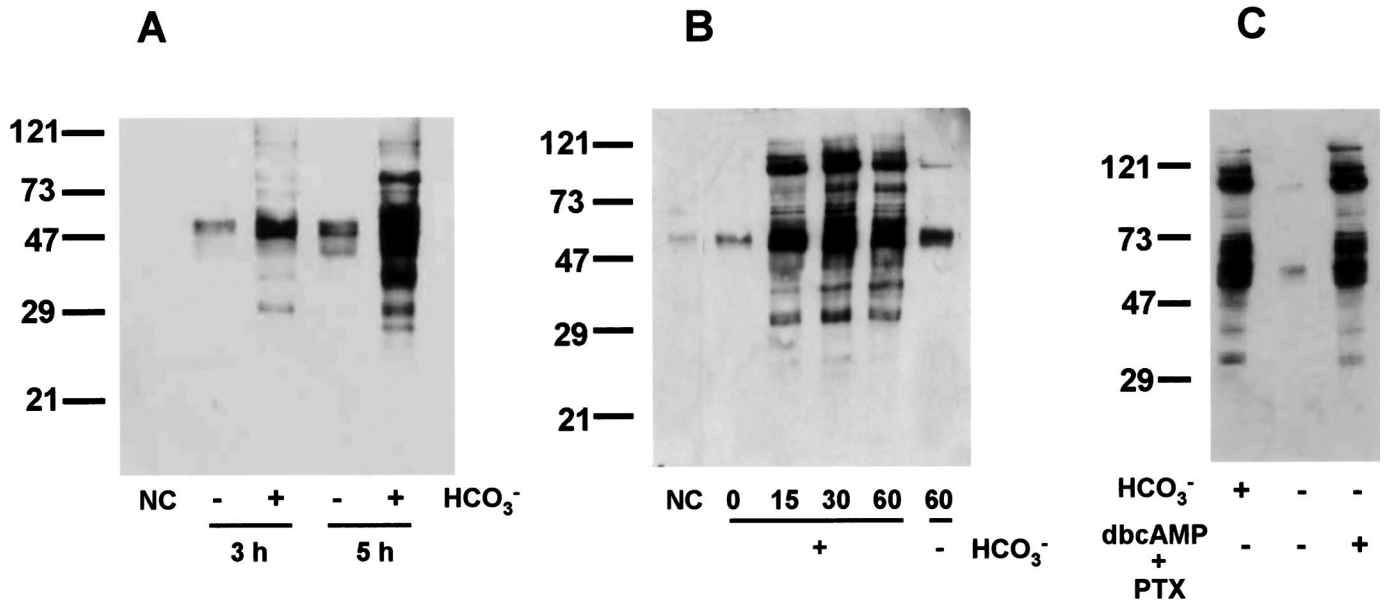

FIG. 2. Influence of HCO_3^- on sperm protein tyrosine phosphorylation. **A**) Effect of the absence of HCO_3^- in the medium. Sperm were incubated in media with or without HCO_3^- for 3 or 5 h. Aliquots were then removed, and sperm proteins were analyzed for tyrosine phosphorylation by Western blot using a monoclonal anti-phosphotyrosine antibody. **B**) Reversibility of the effect observed in the absence of HCO_3^-. Sperm were incubated in the absence of HCO_3^- for 3 h, and protein tyrosine phosphorylation was evaluated at different times (0, 15, 30, and 60 min) after the addition of bicarbonate to the medium. **C**) Effect of replacement of HCO_3^- by dbcAMP and PTX. Sperm were incubated in media with or without bicarbonate or in a medium devoid of HCO_3^- but containing 5 mM dbcAMP and 3 mM PTX. Samples were recovered 5 h later and analyzed for phosphorylation in tyrosine residues. Molecular mass standards (kDa) are shown on the left. NC, Noncapacitated sperm.

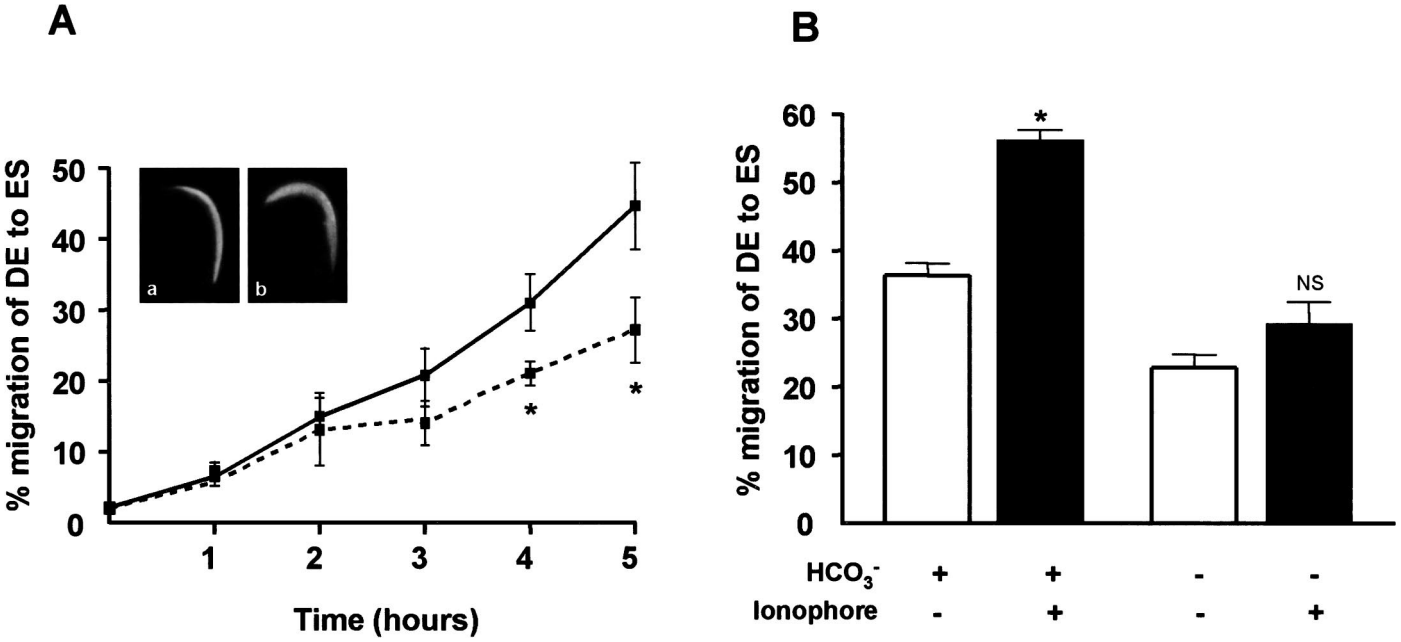

FIG. 3. Influence of HCO_3^- on spontaneous and ionophore-induced migration of DE. **A**) Sperm were incubated in media with (solid line) or without (dotted line) HCO_3^- for 5 h. Aliquots were removed hourly and analyzed for redistribution of protein DE by IIF using anti-DE as primary antibody. Results represent the mean ± SEM of four independent experiments. *$P < 0.02$ vs. solid line. The IIF image shows protein DE on the dorsal region (a) and equatorial segment (b) of the rat sperm head. Original magnification ×625. **B**) Sperm previously incubated in media with or without HCO_3^- for 3 h were or were not exposed to 10 μM calcium ionophore for an additional hour and then analyzed for migration of protein DE by IIF. Results represent the mean ± SEM of five independent experiments. *$P < 0.001$ vs. HCO_3^- (+)/ionophore (−), not significant (NS) vs. HCO_3^- (−)/ionophore (−).

controls (Fig. 2A) and were completely unlabeled when subjected to IIF (data not shown). However, a protein of approximately 50 kDa presented some tyrosine phosphorylation even in the absence of bicarbonate (Fig. 2A). A complete negative reaction (identical to that previously shown in Fig. 1A) was observed when the primary antibody was omitted (data not shown).

To examine the reversibility of the effects observed, the anion was added to sperm incubated for 3 h in a HCO_3^--free medium, and samples recovered 15, 30, and 60 min later were analyzed for protein phosphorylation in tyrosine residues. As shown in Figure 2B, at 15 min after the addition of the anion, the phosphorylation pattern was already similar to that observed when sperm were incubated for 5 h in a bicarbonate-containing medium.

To examine whether the addition of a membrane-permeable cAMP analog could replace bicarbonate in inducing protein tyrosine phosphorylation, sperm were incubated for 5 h in a medium devoid of HCO_3^- but containing 5 mM dbcAMP and the phosphodiesterase-inhibitor PTX at 3 mM. The exposure to these cAMP-elevating reagents did not cause a detrimental effect on spermatozoa, because neither sperm viability (67% ± 5% vs. 60% ± 4%, n = 4; NS) nor motility (67% ± 4% vs. 63% ± 3%, n = 4; NS) were affected by the treatment. Under these conditions, sperm displayed a protein tyrosine phosphorylation pattern similar to that observed for sperm continuously incubated in the presence of the anion (Fig. 2C), and the percentage of cells showing labeling in the tail was not different from that in controls (69% ± 4% vs. 83% ± 3%; NS). These data suggest that, similar to other mammalian species, tyrosine phosphorylation in rat sperm is regulated by a cAMP-dependent pathway.

Effect of Bicarbonate on Migration of Protein DE

Considering that DE migrates from the dorsal region to the ES of the sperm head during capacitation (see the immunofluorescence images in Fig. 3A), we examined the influence of bicarbonate on this parameter at different times during a 5-h incubation period. Those sperm incubated in the presence of the anion exhibited a time-dependent increase in DE migration, reaching a maximum value at 5 h. Sperm incubated in the absence of HCO_3^- behaved similarly during the first 3 h but exhibited levels of redistribution significantly lower than those in controls thereafter (Fig. 3A).

As another approach to the same question, we examined the influence of HCO_3^- on the reported increase in DE migration induced by Ca^{2+}-ionophore [26]. For this purpose, sperm were incubated 4 h in the presence or absence of HCO_3^-, being exposed or not exposed to 10 μM ionophore A23187 during the last hour. As shown in Figure 3B, the lack of HCO_3^- in the medium prevented the ionophore-induced migration of DE observed under control conditions.

In the following experiment, sperm incubated in HCO_3^--free medium for 3 h were then exposed to the anion. As shown in Figure 4A, migration of DE was completely restored only 30 min after addition of bicarbonate to the medium. However, the decrease in redistribution of DE observed in the absence of HCO_3^- could not be overcome by incubation of sperm in a medium containing dbcAMP and PTX (Fig. 4B).

Effect of Bicarbonate on Sperm Fusion Ability

Considering that only sperm that have undergone capacitation and AR are able to fuse with the oolemma, the sperm fusion ability represents a good indicator for the occurrence of a complete capacitation process. In view of this, the next step was to examine the influence of HCO_3^- in rat sperm fusion ability. For this purpose, cauda epididymal sperm were incubated in the presence or absence of HCO_3^- for 4 h and then coincubated with zona-free eggs for an additional 2–3 h. Sperm incubated in the absence of

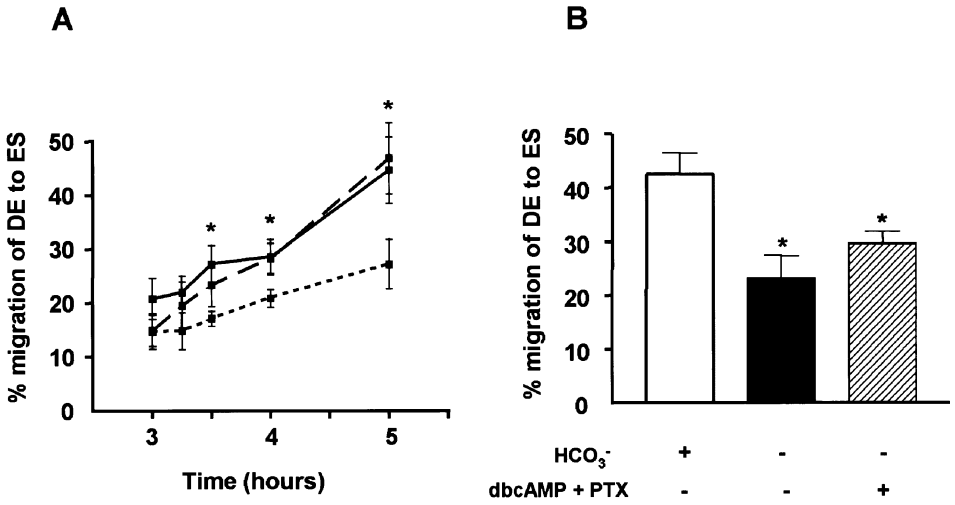

FIG. 4. **A**) Reversibility of the effect produced by the absence of HCO_3^- on DE migration. Sperm were incubated in the presence (solid line) or absence (dotted line) of HCO_3^- for 5 h, and redistribution of protein DE was evaluated by IIF at different times during incubation. In parallel, bicarbonate was added to sperm incubated for 3 h in a HCO_3^--free medium, and sperm were evaluated for migration of DE at different times after addition of the anion (dashed line). Results represent the mean ± SEM of four independent experiments. $^*P < 0.05$, dashed line vs. dotted line. **B**) Effect of replacement of HCO_3^- by dbcAMP and PTX on DE migration. Sperm were incubated in media with or without bicarbonate or in a medium devoid of HCO_3^- but containing 5 mM dbcAMP and 3 mM PTX. After 5 h, sperm were recovered and analyzed for migration of DE by IIF. Results represent the mean ± SEM of seven independent experiments. $^*P < 0.05$ vs. HCO_3^- (+).

bicarbonate were unable to penetrate zona-free eggs (1 penetrated egg out of 61 eggs) (Fig. 5). To discriminate whether this reduction was caused by absence of the anion during capacitation or gamete fusion, sperm either were first incubated in HCO_3^--free medium and then added to zona-free eggs in the presence of the anion or were first capacitated in the presence of HCO_3^- and then coincubated with the eggs in HCO_3^--free medium. Sperm incubated under the first experimental design were also unable to fertilize the eggs (2 penetrated eggs out of 67 eggs) independent of the presence of HCO_3^- during fertilization. By contrast, in the absence of HCO_3^- during gamete fusion, high levels of penetration were observed for sperm previously incubated in HCO_3^--containing medium (Fig. 5), indicating that bicarbonate is necessary for expression of the sperm fusion ability during capacitation but not for the sperm-egg fusion event itself.

To investigate the reversibility of this effect, sperm incubated for 3 h in the absence of HCO_3^- were exposed to the anion for different periods of time and then coincubated with zona-free eggs for 2–3 h to evaluate their fusion ability. As shown in Figure 6A, sperm incubated in the absence of HCO_3^- penetrated only 1 of 77 eggs, but exposure of sperm to bicarbonate for 15–30 min produced a significant, although low, increase in the percentage of fertilization, which was not modified by further incubation (data not shown). In addition, replacement of HCO_3^- by dbcAMP and PTX during capacitation only partially restored the sperm fusion ability observed for controls (Fig. 6B).

DISCUSSION

Because of the importance of HCO_3^- for sperm capacitation, in the present study we have examined the influence of the anion in different parameters associated with this process in the rat. To explore the relevance of HCO_3^- for tyrosine phosphorylation, we first examined the pattern of proteins phosphorylated in tyrosine at different times during capacitation. Similar to what has been reported for other species, rat sperm incubated under capacitating conditions showed a time-dependent increase in tyrosine-phosphorylated proteins. In agreement with this, the localization of fluorescent labeling in the midpiece and principal piece of the tail was coincident with that previously reported for mouse and human capacitated spermatozoa [32, 33]. Although the significance of protein tyrosine phosphorylation in the flagella remains to be determined, evidence suggests that it might be linked to the development of sperm hyperactivated motility [34, 35], required for penetration of the cumulus and zona pellucida, or to an eventual fusion process between the flagellum and the oolemma [36].

The Western blot pattern of phosphorylated proteins observed in our study at the end of capacitation is similar to that recently described by Roberts et al. [37], but it differed from that reported by Lewis and Aitken [38], who observed very low levels of protein tyrosine phosphorylation in rat cauda epididymal sperm incubated in Biggers Whitten Whittingham medium for 3 h. Moreover, in the latter study, phosphorylated proteins were confined to the posterior mar-

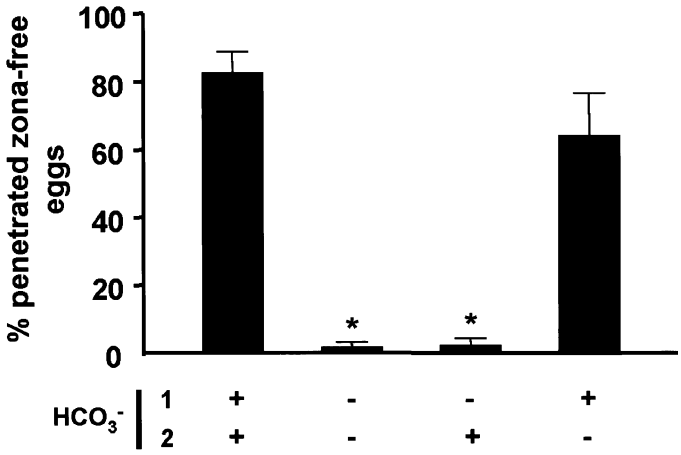

FIG. 5. Influence of HCO_3^- on sperm fusion ability. Sperm were incubated in media with or without HCO_3^- for 4 h (1), then coincubated with zona-free eggs in media with or without the anion for 2–3 h (2). The percentage of penetrated eggs was evaluated. Results represent the mean ± SEM of six independent experiments. $^*P < 0.001$ vs. HCO_3^- (+) in 1.

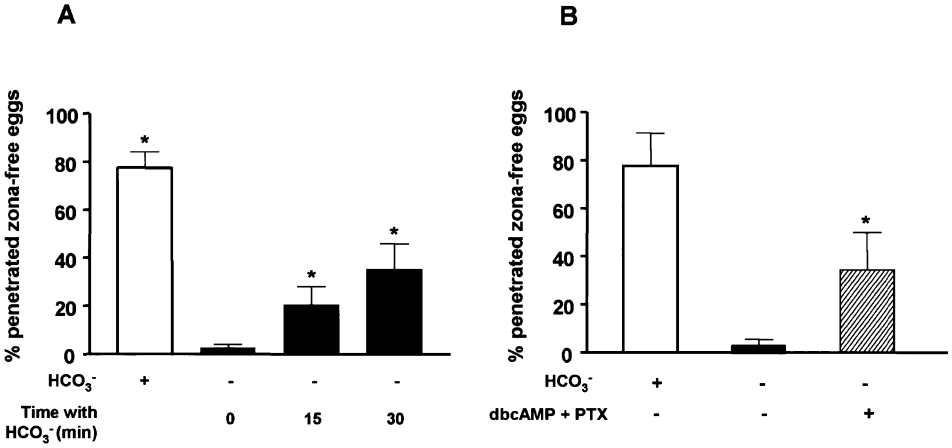

FIG. 6. **A**) Reversibility of the effect produced by the absence of HCO_3^- on sperm fusion ability. Sperm incubated in the absence of HCO_3^- for 3 h (−) were exposed to the anion for different times (0, 15, or 30 min) and then coincubated with zona-free eggs for the evaluation of egg penetration. Sperm incubated for 3 h in the presence of bicarbonate (+) were used as a control. Results represent the mean ± SEM of six independent experiments. $^*P < 0.05$ vs. HCO_3^- (−) at time 0. **B**) Effect of replacement of HCO_3^- by dbcAMP and PTX on sperm fusion ability. Sperm were incubated for 4 h in media with or without bicarbonate or in a medium devoid of HCO_3^- but containing 5 mM dbcAMP and 3 mM PTX and then coincubated with zona-free eggs for evaluation of their sperm fusion ability. Results represent the mean ± SEM of five independent experiments. $^*P < 0.001$ vs. remaining groups.

gin of the acrosome. Interestingly, these investigators only observed both the phosphorylation pattern of proteins and the tail labeling described in our studies when sperm were incubated for 3 h in the presence of dbcAMP and PTX. These results could be attributed to the different incubation conditions used by the investigators, which might not allow complete sperm capacitation. The spermatozoa used in our studies, on the other hand, attained a full state of capacitation within 3–4 h as judged by their ability to penetrate zona-free eggs.

The importance of HCO_3^- for protein tyrosine phosphorylation was indicated by the absence of most of the bands in extracts from sperm incubated in a medium devoid of the anion. This conclusion was confirmed by the complete recovery of the protein phosphorylation pattern soon after the addition of bicarbonate to the medium. The observation that sperm require only 15 min of exposure to HCO_3^- to reach the phosphorylation pattern commonly observed after 4 or 5 h of incubation in a complete medium indicates that HCO_3^--independent events are also required for expression of tyrosine-phosphorylated proteins during capacitation.

In agreement with previous reports [17–19], cAMP agonists, such as dbcAMP, together with a phosphodiesterase inhibitor (PTX) were able to fully stimulate protein tyrosine phosphorylation in HCO_3^--free media. This result differs from that of Baker et al. [20], who found that, in the absence of HCO_3^-, protein tyrosine phosphorylation in the rat did not return to control levels even after exposure of spermatozoa to a maximal dbcAMP-plus-PTX stimulus. Thus, although these authors proposed that the factor responsible for this failure is low intracellular pH, our results support the notion that, in the rat, like in the mouse, the effect of HCO_3^- on protein tyrosine phosphorylation likely results from the ability of the anion to regulate the sperm adenylyl cyclase [17].

Although protein tyrosine phosphorylation represents a necessary intracellular event during capacitation, its occurrence does not allow the evaluation of the overall capacitation process [18, 21]. For this reason, in the present study we examined the influence of HCO_3^- on other capacitation-associated parameters, such as migration of epididymal protein DE from the dorsal region to the ES of the sperm head and the ability of rat sperm to fuse with the egg. Regarding DE migration, it is interesting that previous observations, such as the significant decrease in the percentage of cells showing redistribution of DE in a Ca^{2+}-free medium and the stimulatory effect of Ca^{2+}-ionophore on this phenomenon [26], strongly favor an association between migration of DE and occurrence of the AR. This idea is further supported by in vivo observations showing that, although no more than 20% of free-swimming or cumulus-associated sperm recovered from the oviducts exhibited DE redistribution, all perivitelline acrosome-reacted sperm presented DE over the ES [26]. The significant decrease in spontaneous and ionophore-induced DE migration observed in the absence of HCO_3^-, together with the complete restoration of this parameter after addition of the anion, indicates that HCO_3^- also modulates the relocalization of the protein in the sperm head. However, contrary to what was observed for protein tyrosine phosphorylation, dbcAMP and PTX failed to overcome the decrease in DE migration observed in the absence of HCO_3^-, suggesting that this anion probably regulates DE migration through cAMP-independent processes that are part of the capacitation process.

Our results provide evidence that HCO_3^- also influences rat sperm fusion ability, because sperm incubated in a HCO_3^--free medium were unable to penetrate zona-free eggs regardless of the presence of the anion during gamete coincubation. Considering that capacitation and AR are necessary for gamete fusion [1], our results clearly indicate that HCO_3^- is essential for completion of the capacitation process in the rat. The presence of HCO_3^- during capacitation is also required for fusion ability in mouse [3] and human sperm [39], but it is only beneficial in hamster [5] and guinea pig sperm [40]. Thus, the requirement of bicarbonate for the expression of sperm fusion ability during capacitation seems to differ depending on the species.

The high fertilization rates observed when sperm capacitated in the presence of HCO_3^- were coincubated with the eggs in the absence of the anion indicated that HCO_3^- is not required for the sperm-egg fusion event. This result is in accordance with that of Bhattacharyya and Yanagimachi [40], who showed that acrosome-reacted guinea pig sperm are able to fertilize zona-free eggs in a Hepes-buffered, bicarbonate-free medium. Therefore, the requirements of HCO_3^- for sperm function differ from those of Ca^{2+}, be-

cause this cation is required for both capacitation and gamete fusion [41–43].

The finding that addition of HCO_3^- to sperm incubated in a bicarbonate-free medium produced a significant, although incomplete, restoration of the sperm fusion ability indicates the irreversibility of some bicarbonate-dependent events relevant for expression of the sperm fusion ability. The bicarbonate-dependent suppression of sperm fusion ability could not be overcome by replacement of HCO_3^- by dbcAMP and PTX during capacitation, suggesting that the impact of HCO_3^- on this parameter would be the result of more than a cAMP deficiency.

Taken together, the results of the present work suggest that, in addition to its influence on protein tyrosine phosphorylation, bicarbonate is essential to support other rat sperm capacitation-associated events, such as redistribution of protein DE to the ES and expression of sperm fusion ability. The bicarbonate regulation of protein tyrosine phosphorylation is mediated by a cAMP pathway, as in other species, but migration of DE and expression of the sperm fusion ability seem to require additional bicarbonate properties. One possibility is that the influence of HCO_3^- on intracellular pH plays an important role in these two functional events. In addition, HCO_3^- might be able to interact and modulate the activity of other enzymes or molecules important for the destabilization of the sperm plasma membrane that occurs during capacitation in preparation for subsequent events, such as migration of proteins, AR, and sperm-egg fusion.

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