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Roles of Bicarbonate, cAMP, and Protein Tyrosine Phosphorylation on Capacitation and the Spontaneous Acrosome Reaction of Hamster Sperm[1]

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ABSTRACT

Capacitation is a prerequisite for successful fertilization by mammalian spermatozoa. This process is generally observed in vitro in defined $NaHCO_3$-buffered media and has been shown to be associated with changes in cAMP metabolism and protein tyrosine phosphorylation. In this study, we observed that when $NaHCO_3$ was replaced by 4-(2-hydroxyethyl)1-piperazine ethanesulfonic acid (HEPES), hamster sperm capacitation, measured as the ability of the sperm to undergo a spontaneous acrosome reaction, did not take place. Addition of 25 mM $NaHCO_3$ to $NaHCO_3$-free medium in which spermatozoa had been preincubated for 3.5 h, increased the percentage of spontaneous acrosome reactions from 0% to 80% in the following 4 h. Addition of anion transport blockers such as 4,4′-diiso thiocyano-2,2′-stilbenedisulfonate (DIDS) or 4-acetomido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) to the $NaHCO_3$-containing medium inhibited the acrosome reaction, with maximal inhibition at 600 μM, and with an EC_{50} of 100 μM. Increasing either extracellular or intracellular pH did not induce the acrosome reaction in $NaHCO_3$-free medium. In contrast, addition of 500 μM dibutyryl cAMP (dbcAMP), alone or together with 100 μM 1-methyl-3-isobutylxanthine (IBMX), induced the acrosome reaction in spermatozoa incubated in $NaHCO_3$-free medium. These compounds also partially reversed the inhibition of the acrosome reaction caused by the DIDS or SITS in complete medium. In contrast to these results, IBMX or dbcAMP did not induce acrosome reactions in cells incubated in Ca^{2+}-free medium. When hamster sperm were incubated in the absence of $NaHCO_3$ or in the presence of $NaHCO_3$ and DIDS, cAMP concentrations were significantly lower than the values obtained from sperm incubated in complete medium. Protein tyrosine phosphorylation has also been shown to be highly correlated with the onset of capacitation in many species. During the first hour of capacitation, an increase in protein tyrosine phosphorylation was observed in complete medium. In the absence of $NaHCO_3$, the increase in protein tyrosine phosphorylation was delayed for 45 min, and this delay was overcome by the addition of dbcAMP and IBMX. The induction of the acrosome reaction by calcium ionophore A23187 in $NaHCO_3$-free medium was delayed 2 h, as compared with control medium. This delay was not observed in the presence of dbcAMP and IBMX. Taken together, these results suggest that a cAMP pathway may mediate the role of $NaHCO_3$ in the capacitation of hamster spermatozoa and that protein tyrosine phosphorylation is necessary but not sufficient for complete capacitation.

INTRODUCTION

Sperm capacitation is a poorly understood maturational process that occurs in vivo in the female reproductive tract and can also be accomplished in defined media in vitro, the endpoint of which confers upon the sperm the ability to undergo the acrosome reaction and to fertilize an egg [1]. Capacitation has been demonstrated to be correlated with changes in sperm intracellular ion concentrations, plasma membrane fluidity, metabolism, and motility ([1] and references therein). This process can be achieved in vitro in balanced salt solutions containing appropriate concentrations of electrolytes, metabolic energy sources, and serum albumin (as the primary protein source). It appears that specific components of these media play an important role in promoting the capacitation process. Work in a variety of species has suggested that $NaHCO_3$ is required for capacitation [2–6], although the possibility that $NaHCO_3$ is also necessary for the acrosome reaction [7, 8] cannot be eliminated. It has been suggested that an anion transporter, capable of transporting HCO_3^- and similar to that described in somatic cells [9], exists in guinea pig [10], porcine [11], hamster [12], bovine [8], and human sperm [13]. Such transporters, therefore, may mediate the $NaHCO_3$ effects on sperm capacitation and the acrosome reaction.

The roles of $NaHCO_3$ in sperm capacitation or subsequently in the induction of the acrosome reaction are not well understood at the molecular level. Of particular relevance to the requirement of extracellular $NaHCO_3$ for these events is the observation that HCO_3^- has been demonstrated to be involved in the regulation of mammalian sperm cAMP metabolism [5, 12, 14–16] and that changes in the concentration of cAMP have been linked to both capacitation [17–20] and the acrosome reaction [21]. Recently, we demonstrated that there is an increase in protein tyrosine phosphorylation associated with capacitation and that both processes were dependent on the presence of $NaHCO_3$ in the capacitation medium [4]. Interestingly, the increase in protein tyrosine phosphorylation is mediated by a cAMP pathway through the activation of protein kinase A (PKA) [5, 22, 23].

In this report we have investigated the relationship between cAMP and protein tyrosine phosphorylation during hamster sperm capacitation. The spontaneous acrosome reaction in actively motile spermatozoa can be used as a criterion for the completion of capacitation [1]. Using this criterion, we report that $NaHCO_3$ is necessary for capacitation and that the absence of $NaHCO_3$ in the capacitation

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medium could be overcome by the addition of cAMP agonists. 4,4′-Diiso thiocyano-2,2′-stilbenedisulfonate (DIDS) and/or 4-acetomido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), compounds that inhibit Cl^-/HCO_3^- antiporters, also inhibit the spontaneous acrosome reaction, and this inhibition was partially overcome by cAMP agonists. As expected, an increase in protein tyrosine phosphorylation followed the incubation of hamster sperm in media that support capacitation. The data are consistent with the idea that protein tyrosine phosphorylation, which is up-regulated by cAMP, is necessary but not sufficient for complete capacitation.

MATERIALS AND METHODS

Culture Media

All of the modified Tyrode's media used for sperm incubations contained 1 mg/ml polyvinyl alcohol (PVA) and 10 μM hypotaurine. TL medium (designated as TAL in [24]) contained 25 mM $NaHCO_3$ and 9 mg/ml BSA (fraction V; Sigma Chemical Co., St. Louis, MO). HEPES-buffered medium (HL) was derived from TL by replacing the $NaHCO_3$ with 25 mM 4-(2-hydroxyethyl) 1-piperazine ethanesulfonic acid (HEPES; pH 7.6). In some experiments, the pH of HL was adjusted to different values (pH 6.8–8.2) using NaOH. Addition of 25 mM $NaHCO_3$ to HL yielded medium HLB. The variations in ionic strength in all cases were compensated for by adjusting the NaCl concentration. The osmolarity in each of these media was measured using a Wescor 5500 osmometer (Wescor Inc., Logan, UT) and adjusted to 295 mOsm by addition of NaCl. Before sperm addition, media were covered with paraffin oil and equilibrated for 2 h in 5% CO_2 in air at 37°C. To ensure that there was no HCO_3^- formed in HL medium in experiments using this medium, the incubations were performed in a water bath equilibrated at 37°C. DIDS was dissolved in HL as a 10-strength stock solution and added to the medium just before sperm addition. In some experiments, 500 μl of 50 mM $NaHCO_3$ or NaCl in HL medium (pH 7.5) was added to a 500-μl sperm suspension preincubated in HL medium for 3.5 h; after this solution was added, the sperm were incubated under 5% CO_2 in air. The pH of this medium was measured and did not vary during the incubation period. All chemicals, unless indicated, were purchased from Sigma Chemical Co.

Determination of the Acrosome Reaction

Epididymal spermatozoa from mature Syrian golden hamsters were washed with sucrose-phosphate-buffered saline medium and purified through a glass bead column [25] to remove immotile cells, and then 0.5 ml of 4×10^5 sperm/ml were incubated in the proper media in Cluster 24 multiwell dishes (Costar, Cambridge, MA) for 1–8 h at 37°C. Sperm motility was examined using an inverted microscope with phase contrast optics, and, in all cases studied, it was found that 90% of the cells were motile at the beginning of the incubation period. The percentage of motile cells remained close to 90% during 5 h of incubation and declined to 60% in the following 3 h of incubation.

Aliquots of the sperm suspensions were taken at different times over a total period of 8 h and observed under phase contrast using a Nikon Labophot microscope (×400; Nikon Instruments, Garden City, NY). At least 100 motile cells were examined by a ''blind'' observer per individual incubation time in triplicate determinations. The acrosome reaction was classified according to the methods of Talbot et al. [26] and Yanagimachi and Phillips [27]. Those cells displaying modified or lost acrosomes were counted together as acrosome-reacted spermatozoa. Motility was evaluated as the percentage of motile cells regardless of the pattern of motility. The results depicted in the figures represent the mean ± SD of at least three experiments with triplicate determination.

Statistical differences between groups were determined using Student's *t*-test, comparing the treated group with the respective control after arcsin transformation [28].

Measurement of cAMP

cAMP was extracted from cells with cold 5% trichloroacetic acid and purified by ion exchange on DOWEX 50W × 8 resin as described previously [29]. RIA of 2′-*O*-acetyl cAMP was carried out using ^{125}I-cAMP according to the method of Steiner et al. [30] with minor modifications [29]. A specific antiserum against cAMP was purchased from Chemicon International (Temecula, CA).

Determination of Protein Tyrosine Phosphorylation

Epididymal sperm were collected under paraffin oil and diluted (1:9) into sperm extender. The hamster sperm extender was a modification of the Beltsville poultry extender [31] and contained 70 mM KCl, 60 mM NaCl, 0.2 mM $CaCl_2$, 2.0 mM $MgCl_2$, 5 mM glucose, 20 mM HEPES, 0.1 mM hypotaurine, 1 mg/ml PVA, and 100 U/ml penicillin, pH 6.8. After the determination of sperm concentration, the cells were diluted to 2.5×10^6 in 3 ml of TL or HL. In some experiments, 1 mM dibutyryl cAMP (dbcAMP) and 200 μM 1-methyl-3-isobutylxanthine (IBMX; final concentration) were added to HL before sperm addition. Aliquots of sperm were taken at different times and processed for the determination of protein tyrosine phosphorylation as described previously [4]. SDS-PAGE [32] was performed in 10% gels. Electrophoretic transfer of proteins to Immobilon P (Millipore Corp., Bedford, MA) in all experiments was carried out according to the method of Towbin et al. [33] at 70 V (constant) for 2 h at 4°C. Immunodetection of proteins was performed at room temperature as described previously [34] using a monoclonal antibody against phosphotyrosine (clone 4G10; Upstate Biotechnology Inc., Lake Placid, NY) and enhanced chemiluminescence detection using an ECL kit (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

RESULTS

Bicarbonate Was Necessary for the Spontaneous Acrosome Reaction of Hamster Sperm, an Index of Capacitation.

To investigate the question whether $NaHCO_3$ is necessary for the capacitation of hamster sperm, we incubated sperm in complete medium (HLB) or in medium devoid of $NaHCO_3$ (HL) and then followed the percentage of spontaneous acrosome reactions in the motile sperm population. The spontaneous acrosome reaction can be considered as a marker of capacitation since uncapacitated sperm are not able to undergo this exocytotic process spontaneously [1]. The percentage of motile sperm in media containing or devoid of $NaHCO_3$ was close to 90% following 5 h of incubation and declined at the same rate in both media to 60% at 8 h of incubation. When the sperm were incubated in HLB (which contains 25 mM HEPES and 25 mM

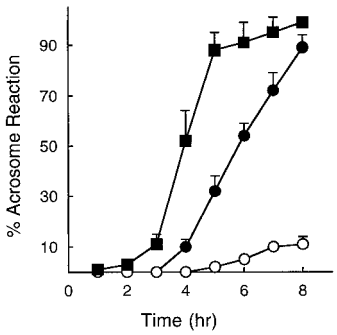

FIG. 1. Time course of the capacitation-dependent spontaneous acrosome reaction in caudal hamster sperm incubated in various media. Suspensions of cauda epididymal sperm were incubated in HLB (squares) or in HL medium (open circles) for the times indicated at the bottom of the figure and analyzed for the percentages of motile cells with modified or lost acrosomes by phase contrast microscopy. In a parallel experiment, sperm were incubated in HL medium, and after 3.5 h $NaHCO_3$ was added to a final concentration of 25 mM (HL to HLB; closed circles) and the percentage of motile cells with modified or lost acrosomes was determined by phase contrast microscopy. The results depicted represent the mean ± SD (n = 12) of at least three independent experiments.

$NaHCO_3$) acrosome reactions were initiated 3 h after the beginning of the incubation period, and the percentage of acrosome-reacted spermatozoa increased with time (Fig. 1). Similar results were seen in medium TL (with 25 mM $NaHCO_3$; data not shown): after 4 h of incubation, 50% of the cells underwent the acrosome reaction and 90% underwent this exocytotic event by 5 h. In medium HL (containing 25 mM HEPES and no $NaHCO_3$), the acrosome reaction was detected only at low levels (7%) even after 6 h of incubation (Fig. 1).

When $NaHCO_3$ (25 mM final concentration) was added to sperm in HL medium after 3.5 h of incubation in this medium (Fig. 1, closed circles), many spermatozoa underwent the acrosome reaction within next 1.5 h (Fig. 1). Four hours after the addition of $NaHCO_3$, nearly 80% of the cells underwent the acrosome reaction, similar to the level of acrosome reactions that had occurred in HLB medium (Fig. 1). Addition of 25 mM NaCl (final concentration) to HL medium (to replace $NaHCO_3$) did not change the time course of the acrosome reaction in this medium (data not shown). The kinetics of the spontaneous acrosome reaction following the addition of $NaHCO_3$ suggests that $NaHCO_3$ was required for capacitation and not for the acrosome reaction, since we would expect a faster spontaneous acrosome reaction response if $NaHCO_3$ had a direct effect on the induction of this exocytotic process.

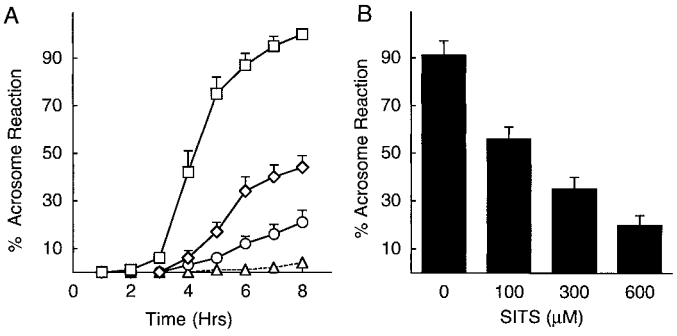

FIG. 2. Effect of inhibitors of anion transport on the percentage of sperm undergoing the capacitation-dependent spontaneous acrosome reaction. **A**) Concentration-dependent effects of DIDS. Sperm suspensions were incubated in control medium TL (squares) or in the same medium containing DIDS at levels of 100 μM (diamonds), 300 μM (circles), and 600 μM (triangles). The percentages of motile cells with modified or lost acrosomes were determined at different time periods. The results depicted represent the mean ± SD (n = 9) of at least three independent experiments. **B**) Concentration-dependent effects of SITS. Sperm suspensions were incubated in control medium TL or in the same medium containing different concentrations of SITS as shown. After 8 h of incubation, the percentages of motile cells with modified or lost acrosomes were determined. The results depicted represent the mean ± SD (n = 9) of three independent experiments.

To investigate whether $NaHCO_3$ stimulates capacitation by increasing intracellular pH, spermatozoa were incubated in HL or TL medium containing different concentrations of NH_4Cl (5, 10, 20, or 30 mM), which has been shown to elevate intracellular pH [35]. Under these conditions, the incidence of spontaneous acrosome reactions was not significantly different from that of controls at any time of incubation (data not shown). To further investigate this possibility, sperm were incubated in $NaHCO_3$-free media (HL) at pH values from 6.8 to 8.2. At 5 h, the percentage of acrosome-reacted cells was low (0–5%) under all conditions tested (data not shown), demonstrating that increasing the extracellular pH did not compensate for the absence of $NaHCO_3$. Taken together, these data suggest that changes in intracellular pH are not the mechanism by which $NaHCO_3$ functions to initiate capacitation.

Spontaneous Acrosome Reaction in Sperm Was Inhibited by Anion Transporter Inhibitors

DIDS and SITS are blockers of anion transport in red cells [9], and DIDS has been shown to be an inhibitor of HCO_3^- and SO_4^{2-} uptake in porcine spermatozoa [11]. Therefore, the effects of these anion transport inhibitors on $NaHCO_3$-stimulated capacitation and acrosome reaction were examined. Spermatozoa were incubated in TL medium containing various concentrations of DIDS; 100 μM DIDS was able to inhibit the acrosome reaction by 50% after 8 h of incubation, reaching maximum inhibition with 600 μM (Fig. 2A). SITS, a weaker stilbene analogue of DIDS, was also able to block the spontaneous acrosome reaction with a similar IC_{50}; the results at 8 h are depicted in Figure 2B. These compounds, even at the highest concentration tested (600 μM), did not affect the percentage of motile cells during the incubation period (data not shown). These experiments suggest that $NaHCO_3$ functions to regulate the capacitation-dependent spontaneous acrosome reaction of hamster sperm by a mechanism involving a stilbene-sensitive anion transport system.

Sperm cAMP Concentrations Were Elevated in Complete Media As Compared to $NaHCO_3$-Deficient Media

Since HCO_3^- has been shown to activate sperm adenylyl cyclase [5, 12, 15, 16], we determined whether the presence of extracellular $NaHCO_3$ in the medium altered intracellular sperm cAMP concentrations. Fresh sperm obtained as described in *Materials and Methods* were incubated in HL or in HLB medium in the presence or in the absence of 100 μM IBMX to inhibit cyclic nucleotide phosphodiesterase activities. When compared with those of sperm incubated in complete medium containing $NaHCO_3$ (HLB), the cAMP levels were significantly lower in sperm incubated in a medium either devoid of $NaHCO_3$ (HL medium) or in complete medium (HLB) supplemented with 600 μM DIDS (Fig. 3); this effect was seen in either the presence or in the absence of the phosphodiesterase inhibitor. Results of these experiments suggest that $NaHCO_3$ modulates sperm

cAMP metabolism and that this occurs through a DIDS-sensitive mechanism.

cAMP Agonists Stimulated the Capacitation-Dependent Spontaneous Acrosome Reaction in the Absence of $NaHCO_3$

The inhibition of cAMP accumulation in the absence of $NaHCO_3$ or by DIDS provides additional evidence connecting HCO_3^-, an HCO_3^- transporter, the stimulation of adenylyl cyclase, and the subsequent increase in intracellular cAMP levels. These data are consistent with previous work from our laboratory demonstrating that mouse sperm PKA activity increases during capacitation, but not when the sperm are incubated in media devoid of $NaHCO_3$ [20].

To investigate whether $NaHCO_3$ stimulates the spontaneous acrosome reaction by raising intracellular cAMP concentrations, spermatozoa were incubated in HL medium in the absence or presence of 500 μM dbcAMP, alone or in combination with IBMX (100 μM). The acrosome reaction was then scored over a period of 8 h (Fig. 4). During the first 5 h, no significant increases in the percentage of acrosome reactions were observed with the different treatments. However, when 500 μM dbcAMP or 100 μM IBMX were present in the medium at the beginning of incubation, a 7-fold (35%) and 9-fold (45%) increase in the percentage of acrosome reactions, respectively, was observed after 8 h of incubation, when compared to sperm incubated in control medium lacking $NaHCO_3$. When dbcAMP and IBMX were added together, nearly 90% of the motile spermatozoa underwent the acrosome reaction at 7 h after the onset of incubation, and these values approximated those seen in complete media (Fig. 4). These results demonstrate that the action of HCO_3^- on the spontaneous acrosome could be mimicked by cAMP agonists, suggesting that a cAMP pathway functioned downstream from the effect of this anion. The slow kinetics of induction of the acrosome reaction suggests that cAMP cannot overcome other steps of capacitation (e.g., cholesterol removal) and that HCO_3^- may also be involved in cAMP-independent processes that are or are not a part of the capacitation process. This is consistent with the fact that the addition of dbcAMP and IBMX did not change the kinetics of the spontaneous acrosome reaction of sperm incubated in complete medium (data not shown). Taken together, these data suggest that cAMP does not directly stimulate the acrosome reaction but that this second messenger stimulates capacitation.

cAMP Agonists Stimulated the Capacitation-Dependent Spontaneous Acrosome Reaction in the Presence of the Anion Antiporter Inhibitors DIDS and SITS

In TL medium, the acrosome reaction was blocked by DIDS and SITS, known inhibitors of Cl^-/HCO_3^- antiporters (Fig. 2, A and B). Since cAMP agonists could stimulate spontaneous acrosome reactions in media devoid of $NaHCO_3$, we investigated whether these cAMP agonists were able to reverse the inhibitory effects of DIDS on the acrosome reaction. Spermatozoa were incubated in TL media containing DIDS, DIDS plus dbcAMP, DIDS plus IBMX, or DIDS plus dbcAMP and IBMX; the results are shown in Figure 5A. Eight hours after the onset of incubation, only 5% of spermatozoa in DIDS-containing medium were acrosome-reacted. In contrast, an increase to 3-fold (15%) and to 4-fold (20%) of acrosome reactions were observed in DIDS-containing medium supplemented with dbcAMP or IBMX, respectively. The combination of dbcAMP and IBMX increased the percentage of acrosome-reacted spermatozoa to 35% in the presence of DIDS, a 7-fold stimulation. Since DIDS inhibits the uptake of HCO_3^-, this experiment also suggests that HCO_3^- is acting through a cAMP pathway and that the cAMP response is downstream from the DIDS effect. Similar results were observed when the spontaneous acrosome reaction was inhibited by SITS. The combined use of dbcAMP and IBMX overcame the inhibition by SITS from 25% to 75% (Fig. 5B) after 8 h incubation.

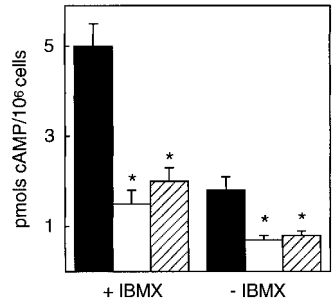

FIG. 3. cAMP concentrations of sperm incubated in the absence or presence of $NaHCO_3$ or in the absence or presence of DIDS. Sperm suspensions were incubated with or without IBMX in HLB medium in the absence (closed bars) or presence of 600 μM of DIDS (striped bars), or in HL medium (open bars). After 30 min, the sperm suspensions were extracted with 5% trichloroacetic acid, and cAMP was purified as described in *Materials and Methods.* cAMP concentration of each sample was determined by RIA as described in *Materials and Methods.* Data represent the mean ± SD (n = 5); $*p < 0.005$.

Capacitation-Associated Increase in Protein Tyrosine Phosphorylation Occurred Independently of cAMP and $NaHCO_3$ in Hamster Sperm

Recently, we and others have demonstrated that there is an increase in protein tyrosine phosphorylation associated with capacitation in sperm from several species [4, 22, 23, 36, 37]. The capacitation-associated increase in protein tyrosine phosphorylation in mouse sperm was dependent on the presence of BSA, Ca^{2+}, and $NaHCO_3$, and phosphorylation could be recovered in the absence of each of these

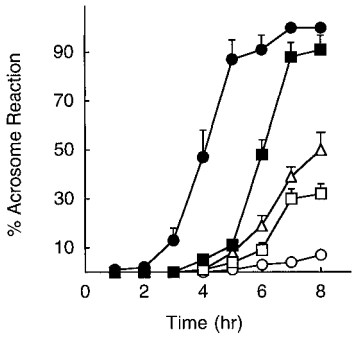

FIG. 4. Effects of dbcAMP and IBMX on the percentage of caudal epididymal hamster sperm undergoing spontaneous acrosome reactions in medium devoid of $NaHCO_3$. Sperm suspensions were incubated in HL medium with no additions (open circles) or with the addition of 500 μM dbcAMP (open squares), 100 μM IBMX (triangles), or both compounds (closed squares). A parallel incubation was made in medium HLB (25 mM (closed circles). The incubations were performed for the times indicated, and the percentages of motile cells with modified or lost acrosomes were determined by phase contrast microscopy. The results depicted represent the mean ± SD (n = 9) of at least three independent experiments.

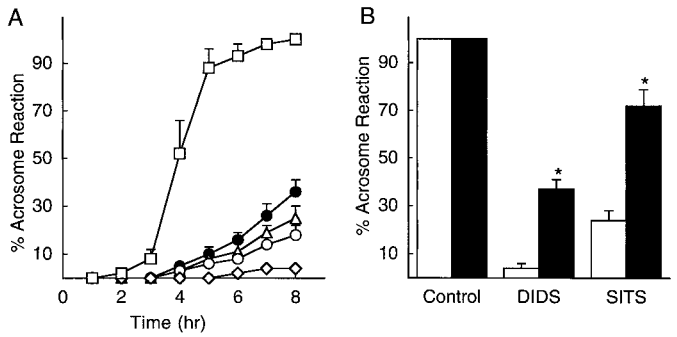

FIG. 5. Effect of dbcAMP and IBMX on the percentage of caudal epididymal hamster sperm undergoing spontaneous acrosome reactions incubated in medium containing DIDS or SITS. **A**) Sperm suspensions were incubated in medium TL in the absence (squares) or in the presence of 600 μM of DIDS and the following additions: none (diamonds), 500 μM dbcAMP (open circles), 100 μM IBMX (triangles), or 500 μM dbcAMP plus 100 μM IBMX (closed circles). The incubations were performed for the times indicated, and the percentages of motile cells with modified or lost acrosomes were determined by phase contrast microscopy. The results depicted represent the mean ± SD (n = 9) of at least three independent experiments. **B**) Sperm suspensions were incubated for 8 h in the absence (open bars) or in the presence (closed bars) of 1 mM dbcAMP and 100 μM IBMX in HLB media with the addition or not of 600 μM DIDS or SITS as shown. The results depicted represent the mean ± SD (n = 9) of at least three independent experiments. *$p < 0.005$ when comparing the values from dbcAMP- and IBMX-treated sperm with the respective control.

medium components by the addition of cAMP agonists [5]. Since HCO_3^- and cAMP were also associated with the capacitation and the subsequent induction of the spontaneous acrosome reaction of hamster sperm, we analyzed whether protein tyrosine phosphorylation was also associated with the capacitation process of hamster sperm. Similar to the case in mouse sperm, there was a time-dependent increase in protein tyrosine phosphorylation in complete medium supporting capacitation (Fig. 6A). When hamster sperm were incubated for the same period of time in sperm extender [31], medium that does not permit sperm capacitation (unpublished results), the increase in protein tyrosine phosphorylation was not observed (data not shown). Incubation of sperm in medium devoid of $NaHCO_3$ delayed the onset of protein tyrosine phosphorylation for 45 min when compared to incubation in complete medium containing $NaHCO_3$ (Fig. 6B), and the presence of 1 mM dbcAMP and 200 μM IBMX accelerated the increase in protein tyrosine phosphorylation in the absence of $NaHCO_3$ (Fig. 6C). Since these experiments were conducted in a water bath at 37°C in the absence of a CO_2 environment, the delay in the onset of the protein tyrosine phosphorylation in medium devoid of $NaHCO_3$ was probably not due to the slow formation of HCO_3^-. Although there was a more rapid tyrosine phosphorylation response in the presence of $NaHCO_3$, the increase in protein tyrosine phosphorylation also occurred in the absence of this anion. These results suggest that although there is a capacitation-associated increase in protein tyrosine phosphorylation in hamster sperm, this increase is not sufficient to allow the sperm to be fully capacitated, since there is an absolute requirement for $NaHCO_3$ for this to occur (Fig. 1). Moreover, in contrast to the situation in mouse sperm, it is likely that a cAMP-independent pathway is also able to induce protein tyrosine phosphorylation in hamster sperm.

A23187-Induced Acrosome Reaction Was Delayed in the Absence of Bicarbonate

The divalent cation ionophore A23187 has been shown to accelerate the spontaneous [26] and zona pellucida-induced acrosome reaction [7]. In order to investigate wheth-

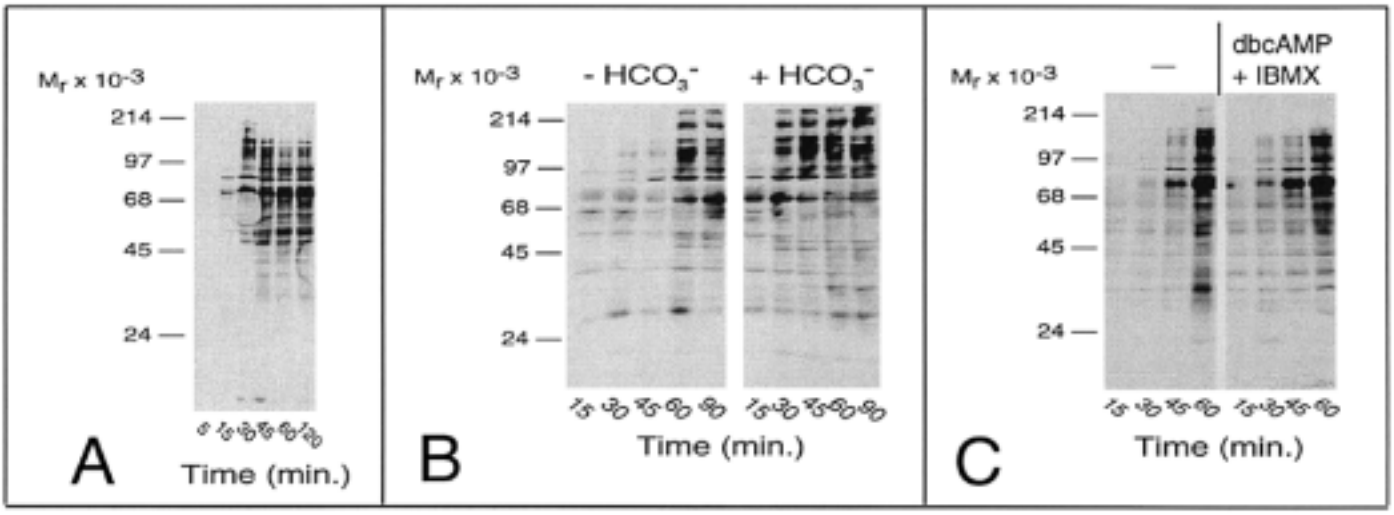

FIG. 6. Protein tyrosine phosphorylation in caudal epididymal hamster sperm under incubation conditions that support capacitation. **A**) Time course of protein tyrosine phosphorylation in complete medium. Sperm were incubated under conditions conducive to capacitation in complete medium. At the times indicated, an aliquot of the sperm suspension (2×10^6 cells) was removed and extracted as described in *Materials and Methods,* analyzed by PAGE, immunoblotted, and then probed using a monoclonal antibody against phosphotyrosine. **B**) Time course of protein tyrosine phosphorylation in hamster sperm incubated in the absence or presence of $NaHCO_3$. Sperm were incubated in the absence or in the presence of $NaHCO_3$. At the time indicated, an aliquot of the sperm suspension was removed (2×10^6 cells) and extracted as described in *Materials and Methods,* analyzed by PAGE, immunoblotted, and then probed using a monoclonal antibody against phosphotyrosine. **C**) Effect of cAMP agonists on the time course of protein tyrosine phosphorylation in the absence of $NaHCO_3$. Sperm were incubated in the absence of $NaHCO_3$, and in the absence or in the presence of 1 mM dbcAMP and 100 μM IBMX. At the times indicated, an aliquot of the sperm suspension was removed (2×10^6 cells) and extracted as described in *Materials and Methods,* analyzed by PAGE, immunoblotted, and then probed using a monoclonal antibody against phosphotyrosine. Experiments shown in **A–C** were performed at least three times with similar results. Shown are representative experiments. The y-axis of each gel indicates molecular weight standards.

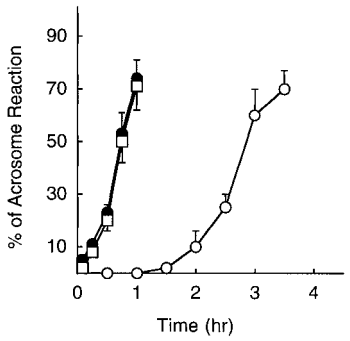

FIG. 7. Effects of dbcAMP and IBMX on the calcium ionophore A23187-induced acrosome reaction. Sperm suspensions were incubated in the absence of $NaHCO_3$ (open symbols) and in the presence of 1 μM A23187 with (squares) or without (open circles) 500 μM dbcAMP and 100 μM of IBMX. A parallel incubation was made in complete medium HLB in the presence of 1 μM A23187 (closed circles). The incubations were performed for the times indicated at the bottom of the figure, and the percentages of motile cells with modified or lost acrosomes were determined by phase contrast microscopy as described in *Materials and Methods*. The results depicted represent the mean ± SD (n = 9) of at least three independent experiments.

er A23187 can induce the acrosome reaction in $NaHCO_3$-free medium, spermatozoa were incubated with A23187. In control HLB medium, in which acrosome reactions are observed normally after 3 h, the addition of 1 μM A23187 accelerated this process (Fig. 7). After 30 min, the acrosome reaction was detected in 25% of living cells, reaching a maximal level (70%) at 1 h. It is important to stress that when the sperm were incubated in the presence of A23187, the percentage of motile sperm was reduced significantly to less than 10% of motile cells at 1-h incubation (data not shown). When the effects of A23187 were tested in HL medium devoid of $NaHCO_3$, there was a marked delay in the onset of the acrosome reaction (Fig. 7), with sperm beginning to react only after 2 h of incubation. Once initiated, acrosome reactions reached maximal levels within the next 90 min (Fig. 7). The percentage of motile cells in this case was maintained at about 90% until the sperm began to acrosome-react, and motility then started to decrease to less than 10% in about 1.5 h, similar to that observed in the complete medium (data not shown). The addition of 500 μM dbcAMP and 100 μM IBMX to sperm in media devoid of $NaHCO_3$ at the beginning of the incubation period caused an elevation in the percentage of A23187-induced acrosome reactions that followed the kinetics of the ionophore-induced acrosome reactions in medium containing $NaHCO_3$ (Fig. 7). The effect of these cAMP agonists was also observed in the percentage of motile sperm that decreased in medium devoid of $NaHCO_3$ to less than 10% in 1 h (data not shown). These results suggest that cAMP can substitute for $NaHCO_3$ to support A23187-induced acrosome reactions as well as A23187-induced inhibition of the percentage of motile cells. When spermatozoa were incubated in Ca^{2+}-free medium, the rate of spontaneous acrosome reactions was low (10%) after a 3-h incubation in either the presence or absence of A23187, and no effect was observed in the percentage of motile sperm (data not shown), suggesting that A23187 actions are mediated by Ca^{2+}. The addition of 500 μM dbcAMP and 100 μM IBMX did not increase the incidence of acrosome-reacted cells in any of these cases above control levels (data not shown). These data suggest that an elevation of cAMP is necessary but not sufficient to induce the acrosome reaction and are consistent with a role of an HCO_3^-/cAMP pathway in the capacitation of hamster sperm. An additional conclusion of these experiments is that A23187 will induce the acrosome reaction in hamster sperm only after these cells are capacitated. On the basis of the time course of protein tyrosine phosphorylation during capacitation (Fig. 6, A–C), it is interesting to note that these phosphorylations must occur temporally before the time that A23187 will induce an acrosome reaction. This further supports the role for protein tyrosine phosphorylation in preparing the sperm to undergo an acrosome reaction.

DISCUSSION

Spermatozoa are exposed progressively to increasing concentrations of HCO_3^- during epididymal maturation [38], and higher HCO_3^- levels are found in semen because of secretions of the accessory glands [15, 39]. In the female tract, HCO_3^- is secreted by the tubal epithelium, reaching concentrations between 35 and 90 mM in the oviductal fluid; moreover, the concentration of this anion increases after ovulation [40, 41]. HCO_3^- is also known to stimulate the motility and respiration of spermatozoa [40, 42, 43] and influences the appearance of hyperactivated movement and capacitation [2, 3]. Thus, this anion plays a key role in sperm function.

Our previous studies [12, 29] indicated that activators of protein kinase C such as oleyl acetyl glycerol (OAG) and 12-*O*-tetradecanoyl-phorbol-13-acetate (PMA) increase cAMP concentrations in hamster spermatozoa during capacitation. This stimulation is $NaHCO_3$-dependent and is inhibited by DIDS and SITS, suggesting that a HCO_3^-/Cl^- antiporter is involved in these aforementioned effects. In our present work, we have shown that in the presence of $NaHCO_3$, DIDS and SITS lowered the incidence of the spontaneous acrosome-reaction after 5 h, with an EC_{50} of 100 μM. This EC_{50} is consistent with the inhibition of the HCO_3^-/Cl^- antiporter activity in other cell types, such as erythrocytes [9]. Such a DIDS-sensitive anion exchanger has been described in porcine sperm [18], and its presence was proposed in guinea pig sperm on the basis of studies in which DIDS blocked the bicarbonate-dependent acrosome reaction induced by nigericin [10]. Moreover, Spira and Breibart [8] found that DIDS binds covalently to the bovine sperm plasma membrane, and a molecule immunologically related to the erythrocyte band 3 was recently described in human sperm [13]. All of these results are consistent with the presence and function of an anion antiporter in mammalian sperm.

The effects of $NaHCO_3$ observed in the present studies could be due to intracellular alkalinization, to the stimulation of adenylyl cyclase, or to both. In guinea pig sperm, high extracellular pH causes the acrosome reaction, even in the absence of $NaHCO_3$ [44, 45]. We did not observe an effect of media buffered at more alkaline pHs on the acrosome reaction in an $NaHCO_3$-free medium. In addition, intracellular alkalinization plays important roles in the motility activation of bull spermatozoa [46] and in the acrosome reaction of hamster spermatozoa [47]. Although we did not measure pH_i in response to external $NaHCO_3$, sperm incubated with NH_4Cl, an agent known to elevate pH_i [35], show no effect on the percentage of acrosome-reacted spermatozoa in media devoid of $NaHCO_3$, indicating that is not possible to overcome the absence of $NaHCO_3$ by increasing pH_i.

Sperm adenylyl cyclase has been shown to be stimulated by HCO_3^- in a variety of species, including the mouse, pig,

bull, and hamster [5, 12, 15, 16]. Although the idea that cAMP is involved in sperm physiology has been in the literature for many years [48], only its role in sperm motility is well established [49, 50]. The role of cAMP in the capacitation process as well as in the acrosome reaction is still controversial [1]. Previous reports from our group and others have suggested a role for cAMP during capacitation. On the basis of this work, it would be predicted that cAMP concentrations should rise during capacitation. Although some authors have observed elevations in cAMP concentrations when spermatozoa were incubated under conditions that support capacitation [17–19], others have reported decreasing concentrations of cAMP under these conditions [51, 52]. Recently, we measured PKA activity in mouse sperm and demonstrated that the activity of this enzyme increases under conditions that support capacitation, but not when the sperm are incubated in the absence of $NaHCO_3$, which does not support capacitation [20]. Regarding the possible participation of cAMP in the $NaHCO_3$-dependent acrosome reaction, we observed that cAMP accumulation in $NaHCO_3$-containing media was significantly higher than in $NaHCO_3$-free medium. When dbcAMP, IBMX, or the combination of the two were added to $NaHCO_3$-free media, the percentage of acrosome-reacted spermatozoa rose significantly, although with a slower kinetics, in comparison with the acrosome reaction in the $NaHCO_3$-containing medium (Fig. 3). These results suggest that the ability of HCO_3^- to stimulate the acrosome reaction is mediated by a cAMP pathway. Using a biochemical approach for measuring the acrosome reaction, Spira and Breitbart [8] reached similar conclusions in bovine spermatozoa, observing that HCO_3^- is no longer required when the cAMP levels are artificially elevated by the use of dbcAMP. The addition of dbcAMP and IBMX partially, but significantly, reversed the effect of DIDS on the acrosome reaction. It is possible that DIDS exerts other effects on sperm function through its covalent binding to the sperm membrane, independent of the inhibition of an anion antiporter.

Recently, we reported that a cAMP-dependent increase in protein tyrosine phosphorylation was correlated with the capacitation of mouse sperm under a variety of conditions [4, 5], and similar results were obtained in other species [22, 23, 36, 37]. In the present report, we found that there is also a time-dependent increase in protein tyrosine phosphorylation in hamster sperm. This increase in protein tyrosine phosphorylation was delayed in media lacking $NaHCO_3$, and cAMP agonists such as dbcAMP and IBMX were able to accelerate the increase in protein tyrosine phosphorylation in $NaHCO_3$-free media. However, in contrast with our findings in mouse sperm, $NaHCO_3$ was not absolutely required for the increase in protein tyrosine phosphorylation, suggesting that hamster sperm use more than one regulatory mechanism to regulate the increase in protein tyrosine phosphorylation. Nevertheless, this increase was not observed when the sperm were maintained for several hours in sperm extender, a medium that is based on the ionic composition of epididymal fluid and that prevents hamster sperm motility and capacitation (unpublished results). These results, added to what is known about mouse, bovine, pig, and human sperm capacitation, suggest that although protein tyrosine phosphorylation is necessary for this process, other processes should occur before completion of capacitation at least in the case of hamster sperm.

Although hyperactivation of motility in mammalian sperm accompanies capacitation in many species [1, 53], it is possible to separate these two processes in vitro [2]. In the case of hamster sperm, we cannot completely rule out that tyrosine phosphorylation of some proteins could mediate changes in the motility pattern of the sperm. However, hypermotility occurs in hamster sperm at least 2 h after the onset of protein tyrosine phosphorylation, suggesting that these two processes are independent.

A23187 has been shown to induce the acrosome reaction in the sperm of almost all species tested, suggesting that Ca^{2+} is a primary mediator of this exocytotic reaction. In this report, we have shown, at least for the hamster, that when the spermatozoa are made fully permeable to Ca^{2+}, they still require $NaHCO_3$ to undergo the acrosome reaction. These results are in agreement with those published by Lee and Storey [7], who showed that A23187 failed to accelerate the zona pellucida-mediated acrosome reaction when mouse sperm capacitation was carried out in $NaHCO_3$-free medium, and with those of Spira and Breitbart [8], who showed that A23187 was 50% less potent in inducing the acrosome reaction of bovine sperm in the absence of $NaHCO_3$. Our results show that dbcAMP and IBMX can bypass the HCO_3^- requirement for the A23187-induced acrosome reaction when this anion is absent from the media. The ability of dbcAMP to substitute for the $NaHCO_3$ requirement in the A23187-induced acrosome reaction demonstrates that the most probable mechanism for the HCO_3^- effect is through the activation of the adenylyl cyclase and the accumulation of intracellular cAMP. Since A23187 catalyzes the exchange between Ca^{2+} ions and H^+ ions, this experiment also supports the conclusion that alkalinization of the intracellular milieu is not sufficient to overcome the lack of HCO_3^- in the capacitation medium. This conclusion does not rule out the possibility that HCO_3^--driven intracellular alkalinization could act synergistically with its ability to stimulate cAMP synthesis.

Monitoring the acrosome reaction only in live spermatozoa permitted us to differentiate spontaneous versus false acrosome reactions that can occur after sperm death [1]. However, there is not a clear step to differentiate the capacitation process from the spontaneous acrosome reaction in the hamster. In fact, there is no marker to define the capacitation process other than the acrosome reaction itself or the ability to fertilize an egg [54, 55]. Sabeur and Meizel [56] reported that after capacitation of human sperm, progesterone is able to induce the acrosome reaction in media with low HCO_3^- (1 mM). However the values were lower by 60% when compared with the progesterone effect in sperm incubated in media with high $NaHCO_3$ (25 mM). This work suggests that HCO_3^-, through activation of cAMP synthesis, could also be necessary for the acrosome reaction. These results contrast with experiments from Shi and Roldan [6], who demonstrated that although HCO_3^- was necessary for capacitation, it was not necessary for the zona pellucida-induced acrosome reaction. The results from Sabeur and Meizel [56] could reflect some differences between species, or alternatively, it is possible that during the washing steps used by these authors, human sperm displayed a reversal of the capacitation process. It is generally expected that a given factor would induce the capacitation process when it needs a specific incubation time to trigger the acrosome reaction. On the other hand, a substance would be considered to act specifically on the acrosome reaction if, when added to capacitated spermatozoa, it triggered exocytosis almost immediately. In the present work, when $NaHCO_3$ was added 3.5 h after the start of incubation, the kinetics of the acrosome reaction were the same as, if not slower than, that in control medium (Fig. 1). If capac-

itation had been completed in the absence of $NaHCO_3$, its addition would have induced the acrosome reaction fully, within minutes. These results indicate that $NaHCO_3$ is acting on capacitation and that the effect on the acrosome reaction is secondary, as postulated by Boatman and Robbins [3] and by Shi and Roldan [6].

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