Tyrosine Phosphorylation of the A Kinase Anchoring Protein 3 (AKAP3) and Soluble Adenylate Cyclase Are Involved in the Increase of Human Sperm Motility by Bicarbonate[1]

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ABSTRACT

Mammalian testicular spermatozoa are immotile, thus, to reach the oocyte, they need to acquire swimming ability under the control of different factors acting during the sperm transit through the epididymis and the female genital tract. Although bicarbonate is known to physiologically increase motility by stimulating soluble adenylate cyclase (sAC) activity of mammalian spermatozoa, no extensive studies in human sperm have been performed yet to elucidate the additional molecular mechanisms involved. In this light, we investigated the effect of in vitro addition of bicarbonate to human spermatozoa on the main intracellular signaling pathways involved in regulation of motility, namely, intracellular cAMP production and protein tyrosine phosphorylation. Bicarbonate effects were compared with those of the phosphatidyl-inositol-3 kinase inhibitor, LY294002, previously demonstrated to be a pharmacological stimulus for sperm motility. Bicarbonate addition to spermatozoa results in a significant increase in sperm motility as well as in several hyperactivation parameters. This stimulatory effect of bicarbonate and LY294002 is mediated by an increase in cAMP production and tyrosine phosphorylation of the A kinase anchoring protein, AKAP3. The specificity of bicarbonate effects was confirmed by inhibition with 4,4′-di-isothiocyanostilbene-2,2′-disulfonic acid. We remark that, in human spermatozoa, bicarbonate acts primarily through activation of sAC to stimulate tyrosine phosphorylation of AKAP3 and sperm motility because both effects are blunted by the sAC inhibitor 2OH-estradiol. In conclusion, our data provide the first evidence that bicarbonate stimulates human sperm motility and hyperactivation through activation of sAC and tyrosine phosphorylation of AKAP3, finally leading to an increased recruitment of PKA to AKAP3.

cyclic adenosine monophosphate, kinases, signal transduction, sperm motility and transport

INTRODUCTION

Mammalian spermatozoa released from the testis are immotile and unable to fertilize. To reach the oocyte and acquire the competence to fertilize, they need to undergo a sequential series of complex processes of activation consisting of maturation and acquisition of motility in the epididymis as well as capacitation and development of the hyperactivated motility in the female reproductive tract. All these processes are under the control of different factors acting during the sperm transit through the epididymis and the female uterus and oviduct. Among these factors, bicarbonate (HCO_3^-) has been demonstrated to play a pivotal role in regulating sperm capacitation and acrosome reaction in mammalian spermatozoa [1–14]. Moreover, a recent finding indicates the importance of HCO_3^- for a correct spermatogenesis in germ-cell differentiation in the mouse [15]. Although it is known that HCO_3^- increases motility by stimulating adenylate cyclase activity and cAMP production in mammalian spermatozoa, the majority of the data have been obtained in the mouse and in the boar (for review, see [16]) and only few studies have been performed in human sperm [1, 4, 10, 17, 18]. Indeed, in the human, a comprehensive study investigating the molecular mechanisms by which HCO_3^- stimulates sperm motility is lacking.

Recently, we have demonstrated that pharmacological inhibition of phosphatidyl inositol 3 kinase (PI3K) activity by LY294002 stimulates human sperm motility by increasing intracellular cAMP level and tyrosine phosphorylation of AKAP3, an A kinase anchoring protein (AKAP) of the fibrous sheath in sperm tails [19]. This effect finally leads to the recruitment of PKA in this sperm compartment associated with an increase in sperm motility [20], pointing out the relevance of such a mechanism in regulation of human sperm motility.

In the present paper, for the first time, we perform a complete analysis of the effect of HCO_3^- on sperm motility and hyperactivation in swim-up-selected human spermatozoa in comparison with the effect of LY294002 by using computer-assisted sperm analysis (CASA). Moreover, we investigate the concomitant increase in cAMP levels, tyrosine phosphorylation of AKAP3 and AKAP3-PKA interaction, underlying HCO_3^- stimulation of sperm motility. Similar to the action of LY294002, we report here that HCO_3^- induces an increase in cAMP production and tyrosine phosphorylation of AKAP3 and other related proteins, but unlike LY294002, without affecting PI3K activity.

Conflicting data exist about the nature of the mammalian sperm adenylate cyclase. In fact, despite some studies describing the presence of the classical membrane adenylate cyclase (mAC) [16, 21, 22], recent findings demonstrate the prevalent expression in these cells of a distinct soluble adenylate cyclase (sAC) [19, 23–25], which does not possess a transmembrane domain, is insensitive to forskolin and G-protein regulation and is selectively activated by HCO_3^- in a pH-independent mechanism [18, 23–25]. The precise compartmentalization of sAC in distinct subcellular micro-

domains [26, 27] provides the mechanism for localized cAMP rise to specifically activate PKA in different cellular compartments. In fact, unlike mAC, cytosolic sAC associated with different cell organelles, could diffuse and generate cAMP at the site where its target enzyme PKA is localized [28].

However, the coexistence of both mAC and sAC in spermatozoa could not be excluded and a functional interplay between the two types of enzymes could be hypothesized in regulating the timing of sperm acquisition of the maximal fertilization potential [29]. In this light, we decided to further investigate the involvement of sAC in mediating the effects of HCO_3^- in human sperm using the specific sAC inhibitor 2OH-estradiol [30]. Our findings demonstrate that sAC plays a major role in HCO_3^- stimulation of tyrosine phosphorylation of AKAP3 in sperm tails, finally leading to stimulation of sperm motility and hyperactivation.

MATERIALS AND METHODS

Chemicals

All reagents for human sperm preparation were from Irvine (Santa Ana, CA). All reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Amersham Pharmacia Biotech Italia (Cologno Monzese, Italy). The conjugated secondary antibody, 2OH-estradiol, 17β-estradiol, and 4,4′-di-isothiocyanostilbene-2,2′-disulfonic acid (DIDS) as well as the other general reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Peroxidase-conjugated PY20 antibody, LY294002, and H89 were obtained from Calbiochem (La Jolla, CA). [γ-^{32}P]ATP and the RIA kit for cAMP measurement were from NEN Life Science (Boston, MA). Anti-AKAP3 FSP95 rat antibody was kindly provided by Prof. John Herr (Charlottesville, VA) and LY303511 supported by Ely Lilly (Indianapolis, IN). The BM enhanced-chemiluminescence system was purchased from Roche Diagnostic (Milan, Italy). The protein measurement kit was from Bio-Rad Laboratories, Inc. (Hercules, CA).

Preparation of Spermatozoa

All the experiments were performed using spermatozoa from semen samples obtained from men who met all the World Health Organization (WHO) criteria for normozoospermia [31], after the approval of the Hospital Committee for Investigations in Humans and after informed patient consent. Samples with leukocytes and/or immature germ cell concentration greater than 10^6/ml were not included in the study. Semen samples were processed by swim-up technique as previously described [32]. Briefly, for swim-up selection, 1-ml aliquots of semen were gently layered with 1 ml of 1% human serum albumin (HSA)-Hepes-buffered human tubal fluid (HTF) medium not containing HCO_3^-. According to Holt and Harrison [33], HCO_3^-/CO_2 was added in the form of suitable aliquots of a 750 mM aqueous solution of NaHCO$_3$ saturated with 100% CO_2 (a ratio of HCO_3^-: CO_2, yielding, after dilution, pH 7.4 at 37°C); thus, such addition did not disturb the pH of the medium. To prevent loss of CO_2 during subsequent incubation, the HCO_3^--containing samples were maintained under 5% CO_2 in air. After 1 h, 800 μl of the upper medium phase were collected and checked for sperm count and motility. After washing, sperm samples containing about 5×10^6 cells/ml were incubated in 1% HSA-Hepes-buffered HTF medium not containing HCO_3^- for the indicated times with the stimuli.

In some experiments, unselected ejaculated spermatozoa treated with the different stimuli were subjected to motility evaluation according to the WHO manual [31].

SDS-PAGE and Western Blot Analysis

After the different treatments, sperm samples were processed for SDS-electrophoresis (SDS-PAGE) as previously described [20]. Briefly, sperm samples were washed and resuspended in lysis buffer [20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% NP-40, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After protein measurement (Coomassie kit; Bio-Rad Laboratories), the sperm extracts, containing approximately 30 μg of protein, were diluted in equal volume of 2× Laemmli reducing sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 2.5% py-

ronin, and 200 mM dithiothreitol), incubated at 95°C for 5 min, and loaded onto 8% or 10% polyacrylamide-bisacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose. In some experiments, equivalent protein loading was verified by staining parallel gels with Coomassie R. After a 2-h incubation in 1% BM blocking (Roche) in TTBS solution (Tris-buffered saline containing 0.1% Tween 20, pH 7.4), nitrocellulose membranes were washed and then immunostained with peroxidase-conjugated PY20-HRP or anti-AKAP3 FSP95 antibody followed by peroxidase-conjugated secondary antibody. The antibody-reacted proteins were revealed by an enhanced-chemiluminescence system (BM; Roche). For reprobing with different antibodies, nitrocellulose membranes were washed for 30 min at 50°C in stripping buffer (10 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) and reprobed with the specific primary antibodies.

Immunoprecipitation Analysis

Because AKAP3 is detergent resistant due to its strong association to the fibrous sheath compartment, we used a SDS extraction method to lyse spermatozoa before performing immunoprecipitation [20]. Briefly, 50 million sperm were extracted in SDS-lysis buffer (20 mM Tris-HCl, pH 7.4, 0.2% SDS, 1 mM PMSF) for 30 min. After 5 min of boiling at 95°C, samples were kept for 10 min on ice and centrifuged at 127 000 rpm for 5 min at room temperature. Extracted supernatants were then subjected to AKAP3 immunoprecipitation by first incubating for 1 h with 30 μl of protein G-Sepharose for preclearing. Precleared lysates were then incubated for 1 h using 3 μg of anti-AKAP3 antibody followed by overnight incubation at 4°C with 50 μl of protein G-Sepharose. The immunobeads, obtained by centrifugation at 6000 rpm at 4°C for 5 min, were washed three times in lysis buffer and then resuspended in 10 μl of 2× reducing sample buffer and subjected to SDS-PAGE followed by Western blot analysis.

PI3 Kinase Assay

PI3K activity was evaluated in an in vitro assay as previously described [20]. Sperm samples treated or not for 15 min with 15 mM HCO_3^-, 10 μM LY294002, or 10 μM LY303511 were extracted in lysis buffer A (20 mM Tris, pH 7.4, 137 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1% NP-40, 1 mM Na$_3$VO$_4$, 1 mM PMSF). After protein measurement, aliquots of sperm extracts containing equivalent amounts of proteins (300 μg) were incubated for 1 h with 50 μl of protein G-Sepharose for preclearing. Precleared lysates were then incubated for 1 h using 3 μg of rabbit anti-p85 PI3K (06-195; Upstate Biotechnology, Lake Placid, NY) on ice followed by overnight incubation at 4°C with 50 μl of protein A-Sepharose. Sepharose beads were washed twice in lysis buffer and twice with a 10 mM Tris-HCl (pH 7.4) containing 0.1 mM EGTA and 5 mM LiCl. Beads were then suspended in kinase buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA) containing 20 μg of L-α-phosphatidylinositol (Sigma Chemical Co.), 25 mM MgCl$_2$ and 10 μCi of [γ^{32}P]ATP and incubated for 20 min at room temperature. Reaction was stopped by addition of 60 μl of 6 M HCl and then 160 μl of chloroform:methanol (1:1) were added. Lipids were then resolved by thin-layer chromatography plates (TLC silica gel 60) (Merck Laborchimica, Florence, Italy) in chloroform, methanol, water, and ammonium hydroxide (60:47:11.3:2). Dried TLC sheets were developed by autoradiography. Band intensity quantification was performed using a Kodak image-analysis system. Aliquots (1:4) of the p85 immunobeads were subjected to Western blot analysis with the same antibody to ensure that protein G pulled down the same amount of enzyme in both samples (not shown).

Intracellular cAMP Levels

Sperm samples (5×10^6 cells) treated with 15 mM HCO_3^- for 1–15 min, were washed twice in PBS and overnight extracted in absolute ethanol at −20°C. Intracellular cAMP levels were evaluated by a RIA kit (NEN Life Science, Boston, MA) in supernatants after centrifugation (1000 g, 10 min), lyophilization, and reconstitution in 0.05 M sodium acetate buffer, pH 6.2 [20]. Experiments were carried out in duplicate and results expressed as percentage of stimulation over the control, taken as 100%.

Evaluation of Sperm Motility and Hyperactivation

Motility and hyperactivation parameters such as sort fraction (SF), average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), beat-cross frequency (BCF), linearity (LIN = VSL/VCL) and
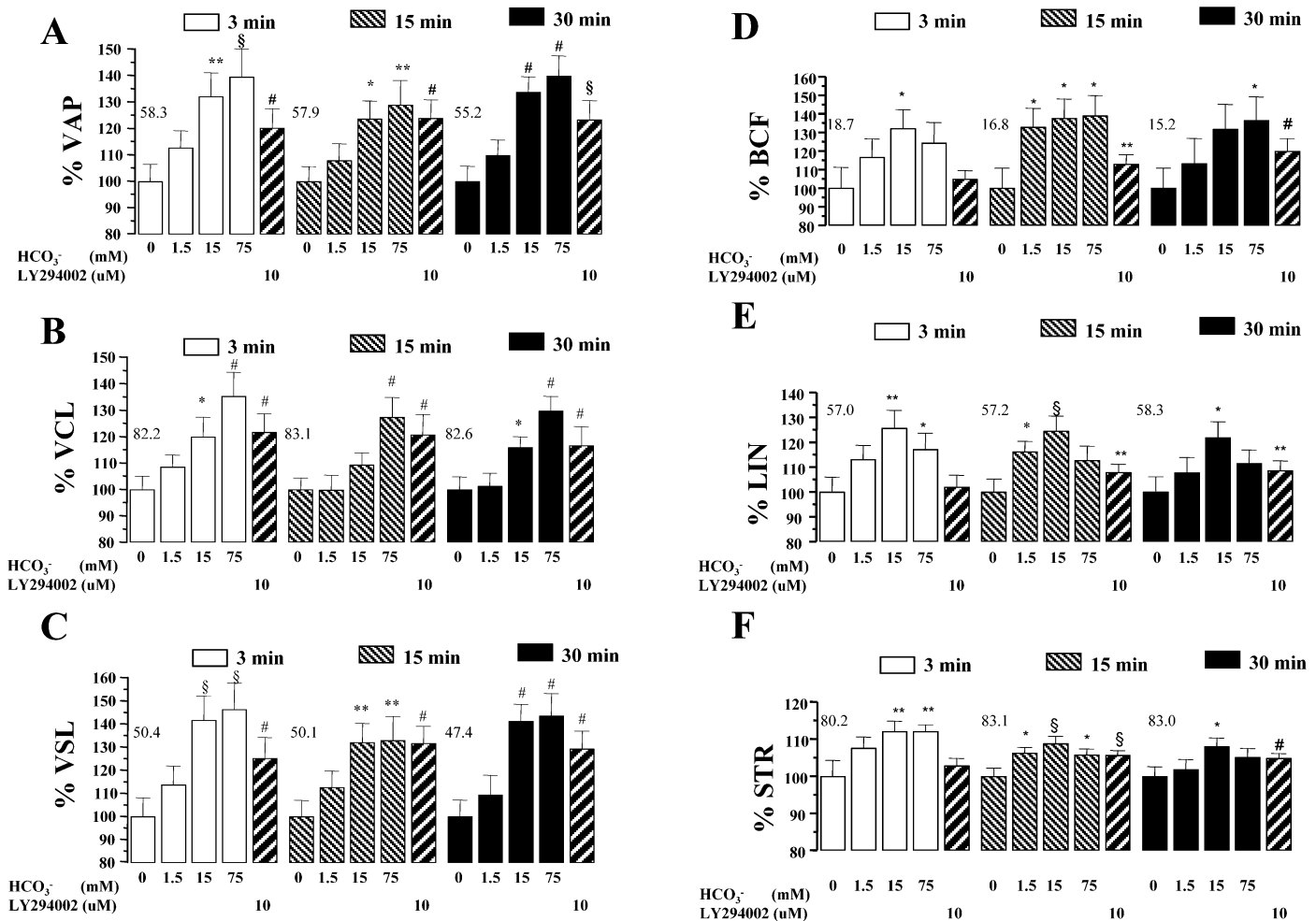
Swim up-selected Sperm

FIG. 1. HCO_3^- and LY294002 stimulate hyperactivation parameters VAP, VCL, VSL, BCF, LIN, and STR in swim-up-selected human spermatozoa. Swim-up-selected sperm samples were incubated for the indicated times in the presence of increasing concentrations of HCO_3^- (1.5, 15, and 75 mM; n = 10) or LY294002 (10 µM, n = 13) and average path velocity (VAP, **A**), curvilinear velocity (VCL, **B**), straight-line velocity (VSL, **C**), beat-cross frequency (BCF, **D**), linearity (LIN, **E**), and straightness (STR, **F**) were evaluated by CASA. Data represent mean ± SEM over the respective controls (0) taken as 100%. Mean raw values are indicated for all the controls (VAP, VCL, VSL: µm/sec; BCF: Hz; LIN and STR: %). Dunnet test: *, $P < 0.05$; **, $P < 0.01$; §, $P < 0.005$; #, $P < 0.001$ versus the respective controls (0).

straightness (STR = VSL/VAP) were evaluated by CASA (Hamilton Thorn Research, Beverly, MA). The settings used during CASA procedures were analysis duration of 1 sec (30 frames); minimum contrast, 80; minimum size, 3; low size and high size gates, 0.7 and 2.6; low intensity and high intensity gates, 0.34 and 1.40 [20]. A minimum of 100 cells and four fields were analyzed for each aliquot. All analyses were performed at 37°C. Motility was expressed as forward and rapid motility (type a + b and type a motility, respectively, according to the WHO manual [31]).

Evaluation of Sperm Viability

Sperm viability was evaluated under phase-contrast light microscope by the eosin technique or the hyposmotic swelling test, according to the WHO manual [31].

Statistical Analysis

Data are expressed as mean ± SEM for n experiments as indicated in figure legends. In graphs showing data obtained by CASA, mean ± SEM were calculated by mediating data from the indicated number of experiments, where every measurement was obtained by analyzing a minimum of 100 cells and four fields for each sample, as indicated in the *Evaluation of Sperm Motility and Hyperactivation* section.

Statistical analysis was performed with a Dunnet test when comparing

more than two classes of samples or with a Student *t*-test for paired or unpaired data, when comparing two classes.

RESULTS

HCO_3^- Stimulates Motility and Hyperactivation Parameters in Human Spermatozoa

Addition of sodium HCO_3^- to swim-up-selected spermatozoa results in a significant stimulation of rapid and forward sperm motility, as evaluated by CASA (not shown). The effect of HCO_3^- on sperm motility is similar to that exerted by the PI3K inhibitor LY294002 (10 µM; not shown), which has been previously demonstrated to be an in vitro pharmacological stimulus of sperm motility [20, 32, 34]. To investigate whether the increase in the percentage of forward and rapid motility of spermatozoa induced by HCO_3^- is associated with a change in sperm hyperactivation, we measured the effect of the anion on those parameters associated with sperm hyperactivation using CASA. Again, the effect of HCO_3^- was compared with that of LY294002, previously shown to induce a significant in-
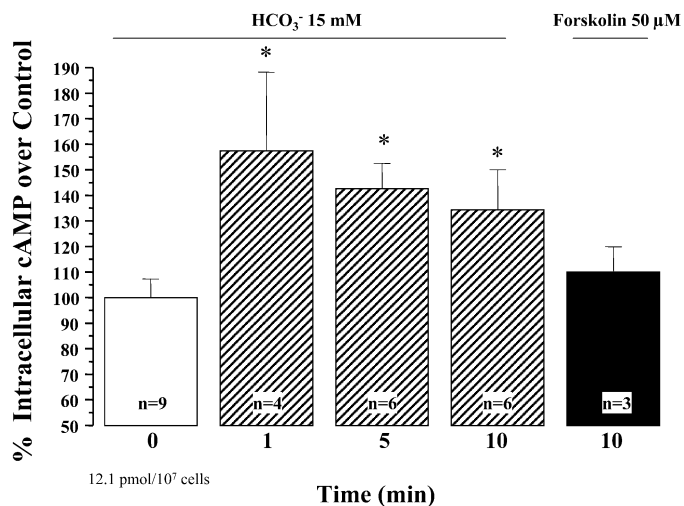
FIG. 2. HCO_3^- but not forskolin increases intracellular cAMP levels in swim-up-selected human spermatozoa. After different times of incubation with HCO_3^- (15 mM) or with forskolin (50 μM, 10 min), swim-up-selected sperm samples were washed and extracted in ethanol. Intracellular cAMP concentrations were evaluated by a RIA kit. Mean raw values are indicated for the control (time 0). Data represent means ± SEM over the control (time 0) taken as 100% from the indicated number of experiments. Dunnet test: *, $P < 0.05$ versus 0.

FIG. 3. HCO_3^- stimulates tyrosine phosphorylation of sperm proteins in a dose-dependent manner. Western blot analysis of sperm lysates from swim-up-selected spermatozoa stimulated for 30 min with increasing concentrations of HCO_3^- (1.5, 15, and 75 mM). Tyrosine phosphorylated proteins were revealed with PY20-HRP antibody (**A**) and after stripping with anti-AKAP3 antibody (**B**). Molecular weight markers ($M_r \times 10^{-3}$) are indicated to the left of the blots. Results are representative of three similar experiments.

crease in VAP, VSL, and VCL in swim-up-selected spermatozoa [20, 32, 34]. HCO_3^- (Fig. 1, A–F) induces a significant dose-dependent increase in VAP, VCL, VSL, BCF, LIN, and STR at all time points in swim-up-selected spermatozoa, similar to that exerted by LY294002 (10 μM). Moreover, the ability of HCO_3^- to stimulate sperm hyperactivation was further confirmed by the dose-response increase in the sort fraction (SF), indicating the percentage of hyperactivated spermatozoa in the sample (C: 9.2% ± 1.2%; 15 mM HCO_3^-: 22.4% ± 2.9%; 75 mM HCO_3^-: 33.3% ± 3.0%; n = 30; Dunnet test: $P < 0.001$ versus control for both concentrations). All the other hyperactivation parameters seem to be only slightly affected at the different time points and concentrations tested (not shown).
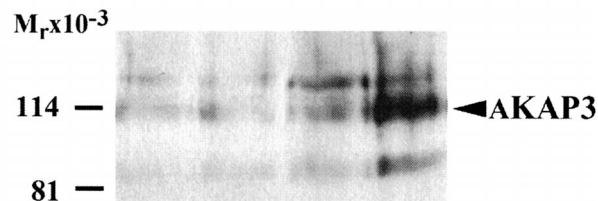
HCO_3^- Stimulates cAMP Production and Tyrosine Phosphorylation of Sperm AKAP3: Involvement of a Soluble Adenylate Cyclase

To investigate the molecular mechanisms underlying the stimulatory effect of HCO_3^- on sperm motility, we measured the intracellular levels of cAMP in swim-up-selected spermatozoa treated for different times with 15 mM HCO_3^-. As shown in Figure 2, HCO_3^- stimulates a rapid and sustained increase in intracellular cAMP levels. In contrast, forskolin, a well-known pharmacological stimulus of classic membrane adenylate cyclase, is ineffective, suggesting the involvement of the soluble form of this enzyme, which has been previously described to be present and active in spermatozoa [18, 23, 25].
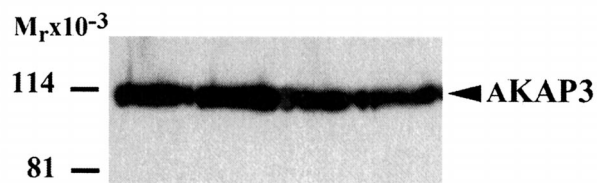
Because we have previously demonstrated that LY294002 stimulates sperm motility by inducing a concomitant increase in cAMP production and tyrosine phosphorylation of AKAP3, a scaffolding protein mainly associated with the fibrous sheath in sperm tail [20, 35–37], we next studied the ability of HCO_3^- to influence the tyrosine phosphorylation pattern of sperm proteins and, in particular, of AKAP3.

Western blot analysis of sperm lysates using PY20HRP antibody shows that sperm treatment with increasing doses

of HCO_3^- for 15 min induces a dose-dependent increase in tyrosine phosphorylation of at least three major sperm proteins of about 82, 86, and 110 kDa (Fig. 3A). Stripping and reprobing the same membrane with anti-AKAP3 FSP95 antibody reveals that the 110-kDa band comigrates with AKAP3 (Fig. 3B). The identity of this 110-kDa band,
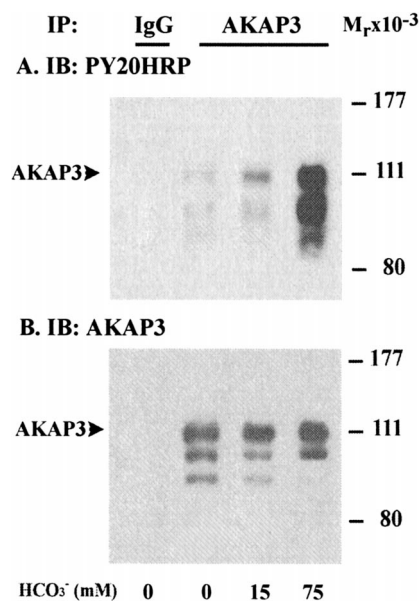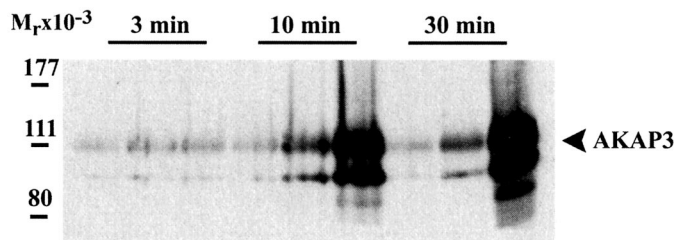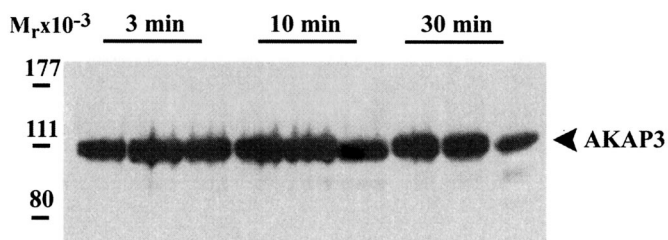

FIG. 4. HCO_3^- stimulates tyrosine phosphorylation of AKAP3. After a 15-min treatment, sperm samples were extracted in SDS-buffer and immunoprecipitated using anti-AKAP3 antibody (AKAP3) or with nonrelated IgG. Western blot analysis of the immunoprecipitated beads using anti-phosphotyrosine antibody (**A**, IB: PY20-HRP) or after stripping and reprobing with anti-AKAP3 antibody (**B**, IB: AKAP3), is shown. Molecular weight markers ($M_r \times 10^{-3}$) are indicated to the right of the blots. Results are representative of three similar experiments.

A. PY20HRP

B. AKAP3

FIG. 5. Tyrosine phosphorylation of sperm proteins is stimulated by HCO_3^- in a time-dependent manner. Western blot analysis of sperm lysates from swim-up-selected spermatozoa stimulated for different times (0, 3, 10, 30 min) with HCO_3^- (HCO_3^- 15 and 75 mM) using the PY20-HRP antibody (**A**) or, after stripping and reprobing with anti-AKAP3 antibody (**B**), is shown. Molecular weight markers ($M_r \times 10^{-3}$) are indicated to the left of the blots. Results are representative of three similar experiments.
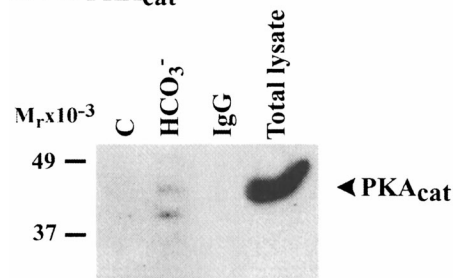
A. IB: PKA$_{cat}$

B. IB: AKAP3

FIG. 6. HCO_3^- increases the interaction of PKA with AKAP3 in human spermatozoa. Swim-up-selected spermatozoa treated or not for 15 min with 75 mM HCO_3^- were extracted in an immunoprecipitation buffer containing 0.1% SDS and immunoprecipitated with anti-AKAP3 antibody. Western blot analysis of the immunobeads using anti-PKA catalytic subunit (**A**) or anti-AKAP3 (**B**) antibodies is shown. A total sperm lysate and immunoprecipitated beads using nonrelated IgG were run as positive and negative controls, respectively. Molecular weight markers ($M_r \times 10^{-3}$) are indicated to the left of the blot. Results are representative of two similar experiments.

which is tyrosine phosphorylated in response to HCO_3^-, is confirmed by immunoprecipitation experiments from HCO_3^--treated sperm samples using the anti-AKAP3 antibody (Fig. 4). Western blot analysis using PY20HRP antibody as probe shows a marked increase in tyrosine phosphorylation of AKAP3 in HCO_3^--treated samples compared with the control (Fig. 4A). Stripping and reprobing the same membrane with anti-AKAP3 antibody shows that this antibody pulls down the same amount of proteins in the different samples (Fig. 4B). Moreover, it suggests that the two additional proteins of 82 and 86 kDa, which are tyrosine phosphorylated in response to HCO_3^- (Fig. 4A), might be related to the AKAP family because they are immunoprecipitated and revealed by anti-AKAP3 antibody (Fig. 4B). The same two additional bands are also revealed in Western blot analysis of total lysates at higher film exposure (not shown). The increase in tyrosine phosphorylation of AKAP3 and of the other two sperm proteins is already present after 3 min of stimulation with 15 and 75

mM HCO_3^- and is long lasting (Fig. 5), as it occurs for the cAMP production induced by HCO_3^- (Fig. 2).

Because tyrosine phosphorylation of AKAP3 by LY294002 treatment of human spermatozoa has been demonstrated to result in an increased interaction of PKA with this scaffolding protein [20], we investigated if this is the case also for tyrosine phosphorylation of AKAP3 induced by HCO_3^-. Coimmunoprecipitation experiments in which sperm protein extracts immunoprecipitated for AKAP3 were revealed in Western blot analysis with an antibody against the PKA catalytic subunit to detect the amount of this protein bound through its regulatory subunit to AKAP3, show that 75 mM HCO_3^- increases the amount of PKA catalytic subunit coimmunoprecipitated with AKAP3 compared with the control (Fig. 6A). Figure 6B shows the stripping and reprobing of the same membrane as in Figure 6A with anti-AKAP3.

To demonstrate that the effects on sperm motility and protein tyrosine phosphorylation are due to the entry of HCO_3^- in the cells, we evaluated the effect of inhibition of the HCO_3^- transport through plasma membrane using DIDS [6,9]. As shown in Figure 7, addition of DIDs (18 and 54 μM) to swim-up-selected spermatozoa significantly reduces the stimulatory effect of two different concentrations of HCO_3^- (15 mM and 75 mM) on sperm rapid (Fig. 7A) and forward (not shown) motility, hyperactivation pa-

TABLE 1. Dibutyryl cAMP reverts 2OH-estradiol inhibition of sperm motility. Swim-up-selected spermatozoa incubated for 15 min in the presence or absence of 2OH-estradiol (2OH-E$_2$, 20 μM) were added with 1mM dbcAMP, and motility parameters were evaluated by CASA. Data represent means $\pm$ SEM obtained in five different experiments.

	Control	2OH-E$_2$ (20 μM)	dbcAMP (1 mM)	dbcAMP + 2OH-E$_2$
Motile (%)	43.5 ± 3.12	21.5 ± 6.35*	64.5 ± 5.85†	71.5 ± 5.56*
Rapid (%)	17.5 ± 3.12	5.0 ± 1.48*	36.4 ± 7.81†	35.8 ± 9.27*
Progressive (%)	15.2 ± 3.17	2.5 ± 0.64*	32.7 ± 5.79†	32.7 ± 5.79*
Path velocity VAP (μm/sec)	59.8 ± 2.62	43.8 ± 3.33*	84.5 ± 2.06†	71.3 ± 4.37*

* $P < 0.05$, Dunnett test, versus control.
† $P < 0.001$, Dunnett test, versus control.

rameters (not shown), and tyrosine phosphorylation of AKAP3 (Fig. 7B). Because washing away added DIDS rescues sperm motility (not shown), the inhibitory effect exerted by this molecule seems to be due to a block of bicarbonate transporters rather than to an unspecific covalent binding to sperm plasma membrane [38].

An increase in intracellular cAMP as the one stimulated by HCO_3^- (Fig. 2) is followed by PKA activation, which has been suggested to be involved in tyrosine phosphorylation of proteins and stimulation of sperm motility [7–9, 39, 40]. Thus, we investigated the relationship between PKA activation and tyrosine phosphorylation of proteins in mediating sperm motility by evaluating the effect of the pharmacological inhibitor of PKA activity, H89, together with the one of the tyrosine kinases, erbstatin A, on sperm motility and tyrosine phosphorylation of proteins. As shown in Figure 8, both H89 (50 μM) and erbstatin (12.5 μg/ml) significantly inhibit sperm rapid (A) and forward (not shown) motility as well as VAP (B), VCL (C) and VSL (D) in basal conditions and in the presence of HCO_3^-. However, while erbstatin only heavily affected tyrosine phosphorylation of AKAP3, H89 slightly inhibited phosphorylation only at the highest dose of HCO_3^- (Fig. 9A). However, this slight inhibition is neither statistically significant nor comparable with the significant decrease in sperm motility and hyperactivated parameters observed when H89 is added in the presence of the 75 mM HCO_3^- (Fig. 9C). These data strongly indicate that tyrosine phosphorylation of AKAP3 is not downstream to PKA activation by HCO_3^-, although both processes finally converge on stimulation of sperm motility.

The involvement of sAC in mediating HCO_3^- stimulatory effects is further confirmed by the ability of the selective inhibitor of sAC, 2OH-estradiol (2OH-E2, 20 μM) [30], to significantly blunt the effect of two doses of HCO_3^- (15 and 75 mM) on rapid motility (Fig. 10A), hyperactivation parameters (Fig. 10, B–D) including sort fraction (Fig. 10E), as well as on tyrosine phosphorylation of AKAP3 (Fig. 10, F and G). 2OH-E2 exerts its inhibitory effects also in basal conditions, in particular, on tyrosine phosphorylation of AKAP3 (Fig. 10F), suggesting that sAC is involved in the stimulatory effects of HCO_3^-. 17β-estradiol (20 μM) used as negative control was ineffective both on sperm motility and hyperactivation parameters (not shown) as well as on tyrosine phosphorylation of proteins (Fig. 10F). As shown in Figure 10, A–E, 2OH-E2 is able to decrease motility parameters of swim-up-selected spermatozoa also in the absence of HCO_3^-. This inhibitory effect is rescued by the subsequent addition of the cAMP-stable analogue dibutyryl cAMP (dbcAMP; Table 1), further confirming that this inhibitor actually blocks cAMP production by sAC. In addition, the inhibitor reduces sperm motility and hyperactivation parameters (VAP, VCL, VSL, and BCF) also when added to ejaculated unselected spermatozoa, whereas 17β-estradiol (20 μM) is ineffective (Table 2). 2OH-E2 toxicity was excluded by checking sperm viability at different times of incubation (not shown). Overall, these findings indicate that sAC is constitutively active in human spermatozoa and that such activity supports sperm motility.

All the data presented suggest that HCO_3^- and LY294002 increase sperm motility by converging on the same signaling pathway involving stimulation of cAMP production by sAC and tyrosine phosphorylation of AKAP3 in sperm tails. Indeed, when added together, the two compounds show no synergic effect on stimulation of
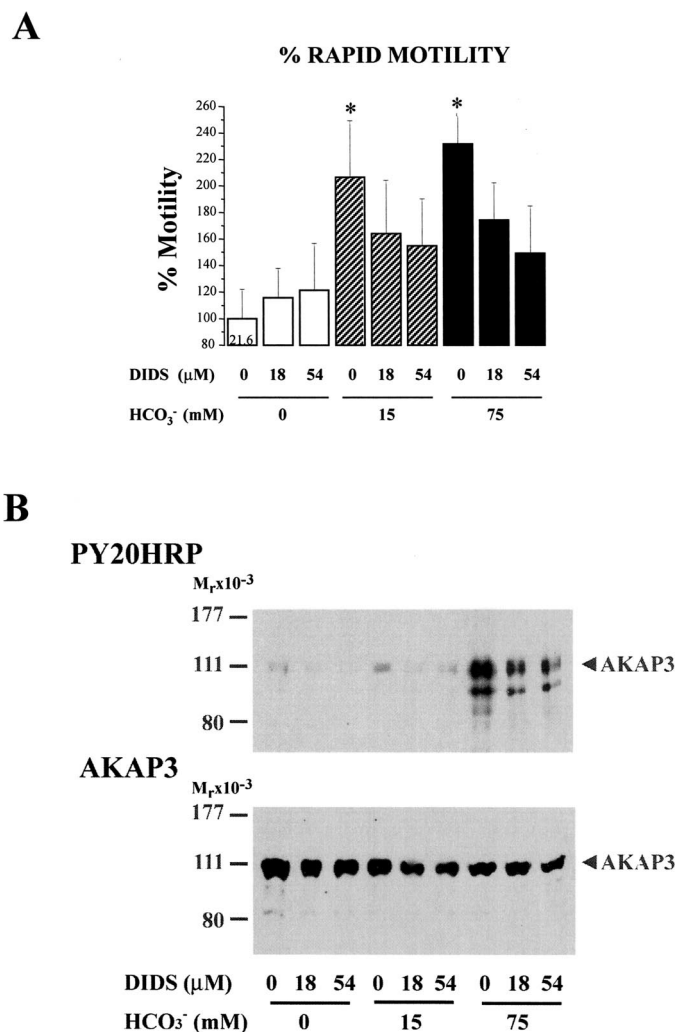

A

% RAPID MOTILITY

B

PY20HRP

AKAP3

FIG. 7. Inhibition of HCO_3^- transport by 4,4′-di-isothiocyanostilbene-2,2′-disulfonic acid (DIDS) reverses the stimulatory effects of HCO_3^- on both sperm motility and tyrosine phosphorylation of AKAP3. Swim-up-selected spermatozoa were incubated for 15 min in the presence of increasing concentrations of HCO_3^- (15 and 75 mM) with or without the HCO_3^- transport inhibitor DIDS (18 and 54 μM), and both sperm motility (**A**) and tyrosine phosphorylation of AKAP3 (**B**) were evaluated. Rapid sperm motility (**A**) was evaluated by CASA and is expressed as percentage of the control, taken as 100%. Mean raw value is indicated (%) for the control. Data represent means ± SEM obtained from five separate experiments. Dunnet test: *, $P < 0.05$ versus controls (DIDS 0). **B**) Tyrosine phosphorylation of sperm proteins was evaluated by Western blot analysis using antiphosphotyrosine PY20HRP antibody (**B**, upper panel). After the stripping procedure, AKAP3 was revealed by anti-AKAP3 antibody (**B**, lower panel). Molecular weight markers ($M_r \times 10^{-3}$) are indicated to the left of the blots. Results are representative of three similar experiments.

sperm motility and hyperactivation, again suggesting that they both act on the same molecular mechanisms (not shown).

Because LY294002 effects on motility are associated with the inhibition of sperm PI3K [20], we also evaluated PI3K activity in the presence of HCO_3^-. As shown in Figure 11A, 15 min of incubation with 15 and 75 mM HCO_3^- does not affect PI3K activity (upper panel), whereas in the same experiment, it stimulates sperm rapid and forward motility as well as hyperactivation parameters (VAP, VCL, VSL, lower panel) in a dose-dependent manner. For comparison, Figure 11B reports the inhibitory effects of sperm pretreatment (15 min) with 10 μM LY294002 but not with its inactive analogue, LY303511 [20, 41].

FIG. 8. Both inhibition of PKA by H89 and of tyrosine kinases by erbstatin blocks HCO_3^--induced increase in sperm motility. Swim-up-selected spermatozoa were incubated for 15 min in the presence of increasing concentrations of HCO_3^- (15 and 75 mM) with or without H89 (50 μM) or erbstatin (12.5 μg/ml). Rapid sperm motility (**A**), VAP (**B**), VCL (**C**), and VSL (**D**) were evaluated by CASA. Data represent mean ± SEM over the respective controls (0) taken as 100% from eight different experiments. Mean raw values are indicated for all the controls (rapid motility: %; VAP, VCL, VSL: μm/sec). Dunnet test: #, $P < 0.05$; 15 and 75 mM HCO_3^- versus controls without treatments. Student t-test: statistical significance between the two classes of treated and untreated samples is indicated in the figure; *, $P < 0.05$, erbstatin versus control without treatments.
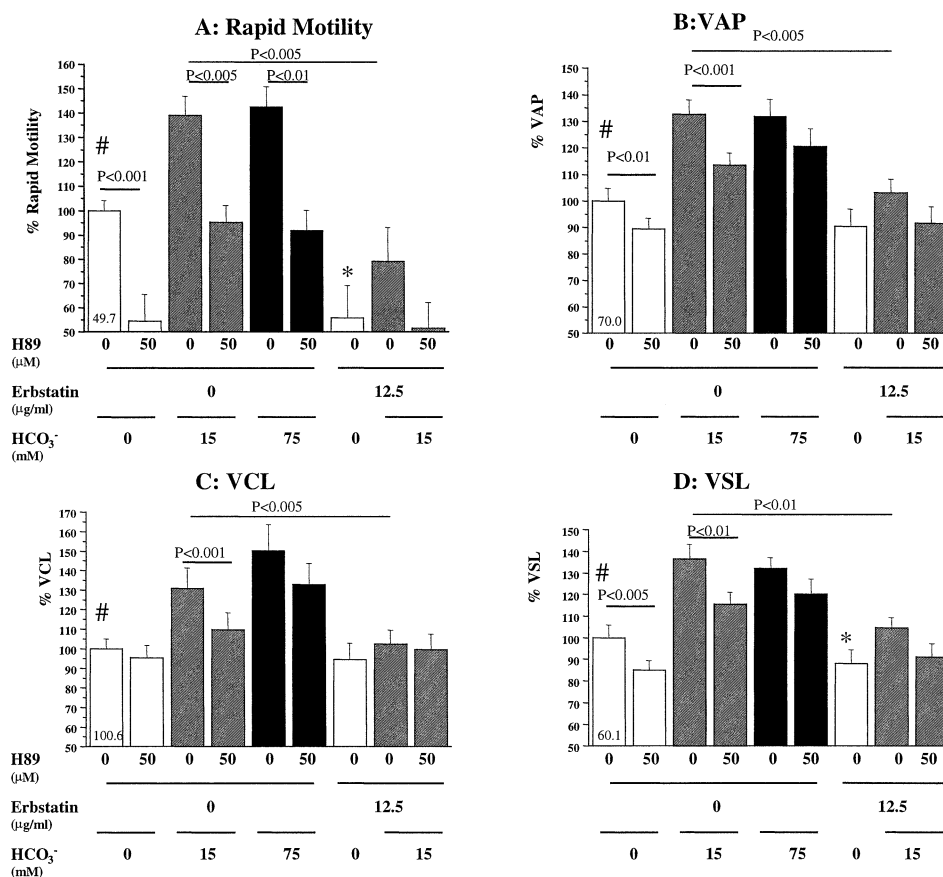
DISCUSSION

Several studies report the stimulatory effect exerted in vitro by HCO_3^- on different mammalian sperm functions such as capacitation, acrosome reaction, and motility. The importance of this factor in regulating sperm physiological activation also in vivo is suggested by exposure of spermatozoa to increasing concentrations of HCO_3^- during their journey from the testis to the site of fertilization. Indeed, spermatozoa pass through an increasing gradient of HCO_3^- from the proximal (2 mM) to the distal epididymal fluid (5–7 mM) and the seminal secretion (10–15 mM), finally culminating in the uterus and oviductal fluid (35–90 mM) [42–44], with an additional increase during ovulation [45].

A recent report demonstrates that coculture of sperm with endometrial cells not expressing the cystic fibrosis transmembrane conductance regulator (CFTR), a cotransporter of HCO_3^- and chloride, results in lower sperm capacitation and fertilizing ability [44]. Moreover, an increased expression of CFTR in the apical region of epithelial cells has been reported in the cauda compared with the initial segment and head of the epididymis [46], further suggesting an increased secretion of HCO_3^- in the lumen of the distal regions. The increased level of HCO_3^- in seminal plasma compared with epididymal fluids should allow motility to develop in the distal male genital tract. Okamura et al. [44] showed a positive correlation between lower levels of

TABLE 2. Inhibition of sAC by 2OH-estradiol affects sperm motility of unselected ejaculated human spermatozoa. Unslected ejaculated spermatozoa were incubated for 15 min in the presence or absence of 2OH-estradiol (2OH-E$_2$, 20 μM) or 17β-estradiol (17βE$_2$, 20 μM) and motility parameters were evaluated by CASA. Data represent means ± SEM increase over the respective controls, taken as 100% in 12 different experiments. Mean raw data are indicated in parentheses for the controls.

	Control	2OH-E$_2$ (20 μM)	17βE$_2$ (20 μM)
Motile	100.0 ± 18.1 (43.8%)	56.6 ± 15.6*	93.6 ± 23.3
Rapid	100.0 ± 19.1 (21.0%)	49.3 ± 14.6*	83.0 ± 23.0
Path velocity, VAP	100.0 ± 4.0 (61.1 μm/sec)	71.6 ± 14.1*	97.5 ± 5.5
Track speed, VCL	100.0 ± 4.2 (76.0 μm/sec)	72.8 ± 14.4*	98.3 ± 6.1
Progressive velocity VSL	100.0 ± 4.1 (52.5 μm/sec)	49.6 ± 9.7†	97.0 ± 5.4
Beat frequency, BCF	100.0 ± 17.3 (8.0 Hz)	69.3 ± 17.7	105.3 ± 23.7

* $P < 0.05$, Dunnett test, versus control.
† $P < 0.001$, Dunnett test, versus control.

HCO_3^- in the semen of infertile men and poor sperm motility. Yet, in the male reproductive fluids, HCO_3^- levels are kept low to prevent spermatozoa from undergoing premature activation and developing hyperactivated motility, processes that are stimulated by the 3–4 higher HCO_3^- concentrations present in the female reproductive tract [47]. In agreement with data previously obtained in porcine and mouse spermatozoa [2, 33], we demonstrate here that physiological concentrations of HCO_3^-, similar to those found in the female genital fluids and in the media used for in vitro fertilization techniques, significantly stimulate human sperm motility. The effect of HCO_3^- is similar to that exerted by LY294002, a pharmacological inhibitor of PI3K, previously demonstrated to stimulate human sperm motility [20, 32, 34].

We also applied CASA to study modifications of the different sperm motion parameters defining hyperactivation under the effect of HCO_3^-. This peculiar pattern of motility, associated with capacitation and characterized by a more vigorous and less symmetric beat of the sperm flagellum [48, 49], is essential for penetration of the cumulus oophorus and the zona pellucida surrounding the oocyte. HCO_3^- significantly stimulates hyperactivation in swim-up-selected spermatozoa, enhancing not only VAP, VCL, VSL, LIN, and STR, but also BCF and SF, which are the main features characterizing hyperactivated motility [48, 49]. Similar to our data, a rapid stimulation of flagellar beat frequency (occurring in seconds) exerted by HCO_3^- has been recently described in mouse spermatozoa by using stop-motion imaging and flagellar-waveform analysis [13].

The molecular mechanisms by which HCO_3^- stimulates sperm motility and capacitation have not yet been fully elucidated, in particular, in human spermatozoa. It has been demonstrated that this ion is able to directly stimulate sAC, with this effect being independent of an increase in intracellular pH [1, 23, 50]. We show here that HCO_3^- rapidly stimulates both an increase in cAMP and tyrosine phosphorylation of sperm AKAP3. Both effects are sustained, lasting over 15 min for cAMP, even in the absence of phosphodiesterase inhibitors and over 1 h for protein phosphorylation. That the effect of 15 mM HCO_3^- on AKAP3 phosphorylation is mediated by a specific activation of sAC is demonstrated by the ability of 2OH-estradiol to reverse it, whereas 17β-estradiol, used as negative control, is ineffective. Moreover, addition of dbcAMP to sperm sample is able to rescue 2OH-estradiol inhibition, indicating that this inhibitor actually blocks sAC-mediated cAMP production in human spermatozoa rather than interferes with Na^+/K^+ ATPase or mitochondrial ATPase [51]. The importance of sAC in regulating sperm motility has been recently stressed by targeted disruption of the sAC gene in the mouse, which dramatically impairs sperm forward motility and in vitro fertilizing ability, without affecting spermatogenesis [52]. Interestingly, motility can be restored in sAC KO mice by cAMP administration, suggesting the importance of cAMP in switching on the biochemical pathway leading to sperm motility rather then a direct influence of sAC in the structural function of the flagellum [52]. Although, in our hands, forskolin was not able to stimulate a significant increase in cAMP production in human spermatozoa, we cannot exclude the presence of mAC isoforms that could cooperate with sAC in regulating sperm functions.

A strict relationship between the AC/cAMP/PKA pathway and tyrosine phosphorylation of sperm proteins in regulating sperm motility and capacitation has been established in different species [12]. In particular, tyrosine phos-
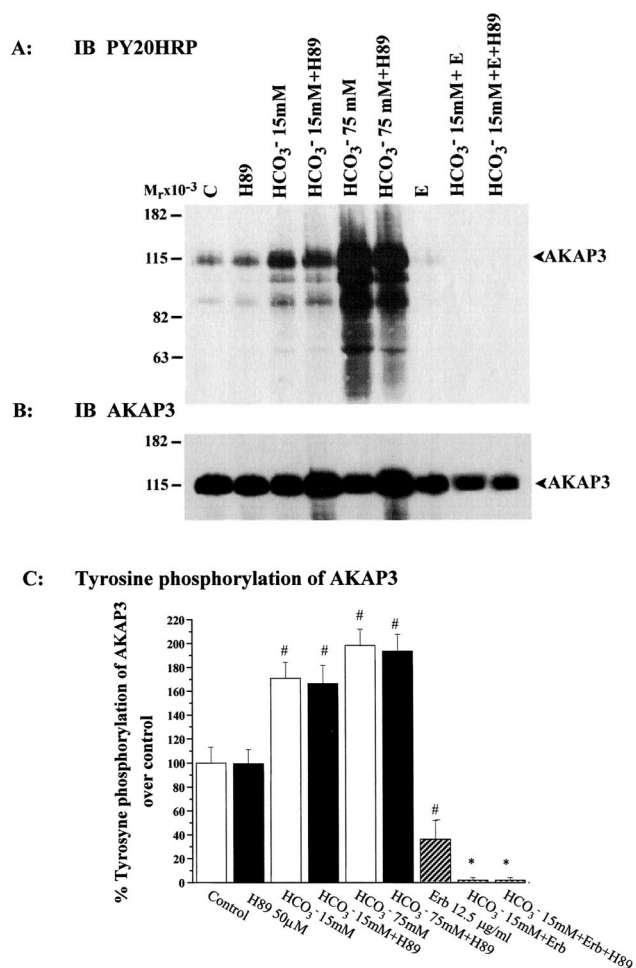

FIG. 9. Inhibition of tyrosine kinases but not of PKA blocks the HCO_3^--induced increase in sperm AKAP3 tyrosine phosphorylation. After evaluation of sperm motility, at 15-min stimulation with HCO_3^- (15 and 75 mM) with or without H89 (50 μM) or erbstatin (E, 12.5 μg/ml), the same sperm samples used in Figure 9 were processed for measurement of protein tyrosine phosphorylation. Western blot analysis of aliquots of sperm lysates using antiphosphotyrosine PY20HRP antibody (**A**) and, after the stripping procedure, with anti-AKAP3 antibody (**B**) is shown. Molecular weight markers ($M_r \times 10^{-3}$) are indicated to the left of the blots. **C**) densitometric evaluation of tyrosine phosphorylation of AKAP3 is calculated in percentage of band intensity over the control, taken as 100%. Representative of three similar experiments. Dunnet test: #, $P < 0.001$ versus control. Student t-test: *, $P < 0.001$ versus 15 mM HCO_3^-.

phorylation of flagellar proteins has been associated with increased sperm motility and hyperactivation [20, 53]. Here, we show that AKAP3 is the main phosphorylated protein in response to HCO_3^-. The importance of AKAP scaffolding proteins in regulating sperm motility has been remarked very recently, as KO mice for the *Akap4* gene, closely related to *Akap3,* show defects in sperm flagellum and motility [54]. Tyrosine phosphorylation of AKAP3 during capacitation [35–37] and in response to LY294002 treatment [20] has been demonstrated to play a key role in PKA recruitment and activation at the level of sperm tails [20, 55]. In fact, tyrosine phosphorylation of AKAP3 is associated with an increased binding of the protein to PKA, resulting in the recruitment and activation of the enzyme in this specific cell compartment, finally leading to an increase in sperm motility [20]. We demonstrate here that HCO_3^- stimulates an increased interaction of PKA catalytic subunit with AKAP3. Thus, because both HCO_3^- and LY294002 stimulate cAMP production, tyrosine phosphorylation of

FIG. 10. The sAC inhibitor 2OH-estradiol blocks the HCO_3^--induced increase in motility and tyrosine phosphorylation of proteins in swim-up-selected spermatozoa. Swim-up-selected spermatozoa were incubated for 15 min with increasing concentrations of HCO_3^- (15 and 75 mM) after 10 min treatment with 2OH-estradiol (2OH-E_2 20 μM) or 17β-estradiol (17βE_2 20 μM). Rapid motility (A) and hyperactivation parameters VAP (B), VCL (C), VSL (D), and sort fraction (SF; E) were evaluated by CASA. Data represent means $\pm$ SEM over the controls (untreated samples), taken as 100% obtained from 13 separate experiments. Mean raw values are indicated for all the controls (rapid motility: %; VAP, VCL, VSL: μm/sec; SF: %). Dunnet test: #, $P < 0.001$, 15 and 75 mM HCO_3^- versus 0. Student t-test: statistical significance between 2OH-E_2-treated and untreated samples is indicated in the different panels. After motion measurements, Western blot analysis of aliquots of treated samples using antiphosphotyrosine PY20HRP antibody (F) and, after stripping and reprobing, anti-AKAP3 antibody (G), confirms the identity of the protein band at 110 kDa with AKAP3. Molecular weight markers ($M_r \times 10^{-3}$) are indicated to the right of the blots. Results are representative of two similar experiments.
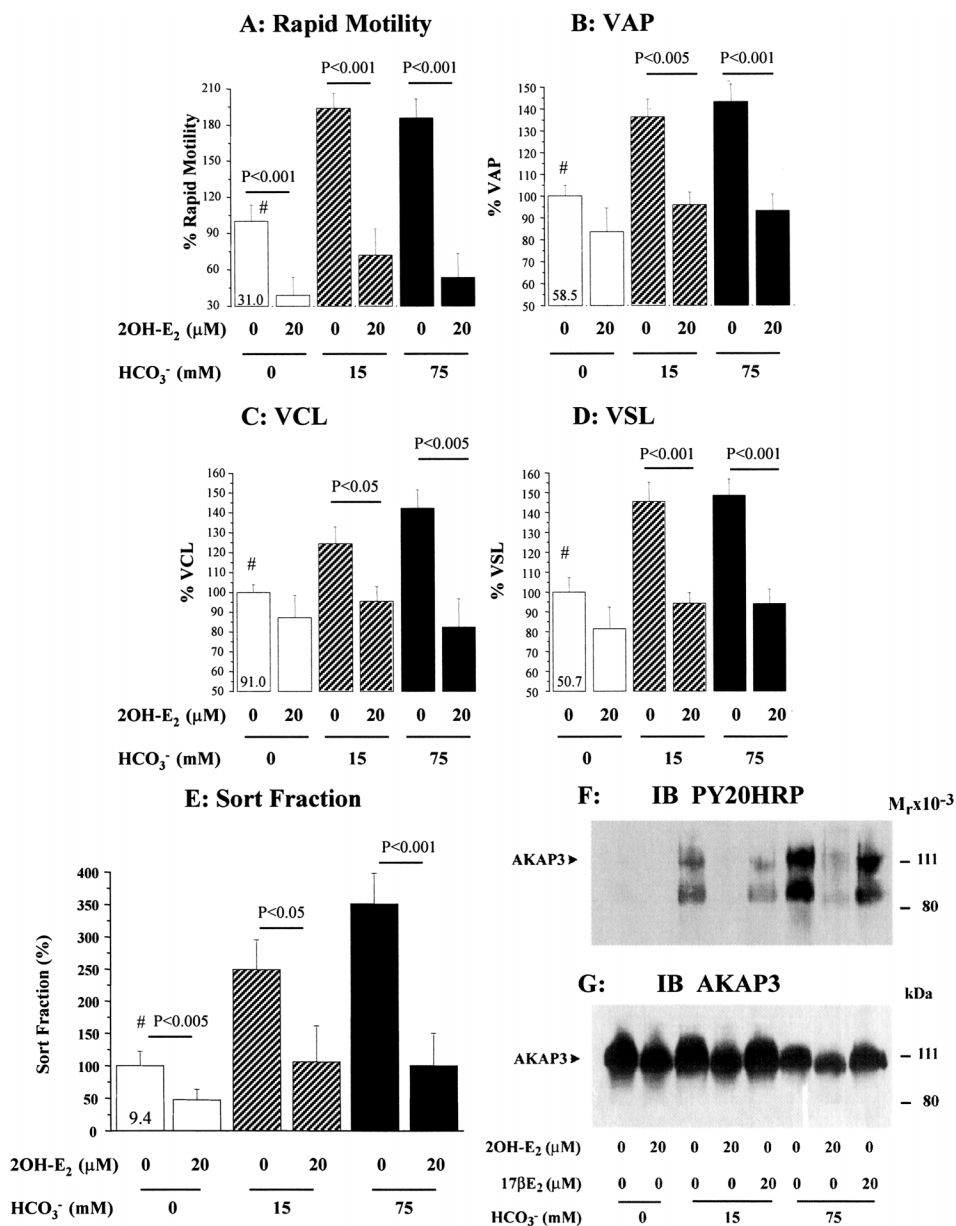
AKAP3 and an increased interaction of PKA with AKAP3, resulting in stimulation of sperm motility, it can be reasonably concluded that the two compounds act on the same signaling pathway, as is also suggested by the absence of any synergic effect on sperm motility (data not shown). However, HCO_3^- stimulation of sAC and AKAP3 phosphorylation appears to be independent of PI3K activity inhibition (Fig. 11A).

Evidence has been reported suggesting that PKA acts upstream of protein tyrosine phosphorylation in mediating sperm capacitation and motility [7–10, 12, 17]. However, conflicting results on H89 ability to affect sperm motility and protein tyrosine phosphorylation in different species are present in the literature [13, 33, 39, 40, 56–58]. In particular, in human sperm, Aitken et al. [39] reported an inhibition of protein tyrosine phosphorylation but not of motility by H89, whereas Bajpai and Doncel [58] and our group [20 and the present paper] demonstrated an inhibition of motility without significant effects on protein tyrosine phosphorylation. Thus, an alternative hypothesis can been proposed according to which, at least in human sperm, PKA

activation is not essential for tyrosine phosphorylation, both processes being essential for motility. Indeed, we report that the time course of cAMP production and protein tyrosine phosphorylation in response to HCO_3^- (present paper) and to LY294002 [20] is very rapid, starting at 1–3 min after addition of the stimulus and thus suggesting that tyrosine phosphorylation and PKA activation may occur simultaneously. The apparent contrast with what has been reported is by other authors [9, 10, 14, 17, 59] can be explained by either species differences or simply because phosphorylation was measured only after longer incubation intervals (15–240 min) with respect to measurement of cAMP production peak (1–3 min, [16, 60]). Moreover, we demonstrate that H89 inhibits sperm motility only and not tyrosine phosphorylation of AKAP3, whereas both processes are blocked by inhibition of tyrosine kinases by erbstatin A, suggesting that tyrosine phosphorylation of AKAP3 is essential for activation of PKA in stimulating sperm motility. However, it cannot be excluded that PKA activation could contribute to potentiating a second wave of tyrosine phosphorylation associated with a general phosphorylation of
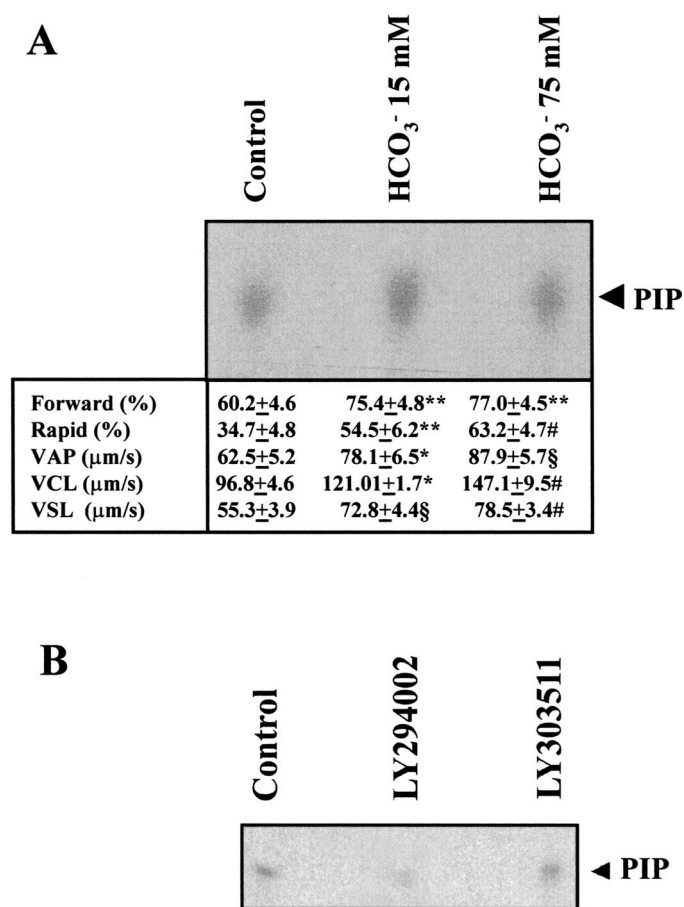
A

	Control	HCO$_3^-$ 15 mM	HCO$_3^-$ 75 mM
Forward (%)	60.2±4.6	75.4±4.8**	77.0±4.5**
Rapid (%)	34.7±4.8	54.5±6.2**	63.2±4.7#
VAP (μm/s)	62.5±5.2	78.1±6.5*	87.9±5.7§
VCL (μm/s)	96.8±4.6	121.01±1.7*	147.1±9.5#
VSL (μm/s)	55.3±3.9	72.8±4.4§	78.5±3.4#

B

FIG. 11. HCO$_3^-$ does not affect PI3K activity. Swim-up-selected spermatozoa were treated for 15 min with HCO$_3^-$ (15 and 75 mM) or with 10 μM LY294002 or LY303511. PI3K activity was evaluated in sperm samples by an in vitro assay after immunoprecipitation with anti-p85 PI3K regulatory antibody. The spots correspond to the PI3K catalytic product [^{32}P] phosphatidyl inositol-phosphate (PIP). **A**) PI3K activity was evaluated in the HCO$_3^-$-treated sperm samples (upper panel) together with sperm motility (rapid and forward) and hyperactivation parameters (VAP, VCL, VSL) as measured by CASA (lower table). Data represent means ± SEM obtained from 12 separate experiments. Dunnet test: *, $P < 0.05$; **, $P < 0.001$; §, $P < 0.005$; #, $P < 0.001$ versus control. **B**) PI3K activity was evaluated in the same spermatozoa treated with the active (LY294002) or the inactive (LY303511) inhibitor of the enzyme for comparison.

PKA substrates, as suggested by Harrison [61]. In conclusion, our study sheds new light on the molecular mechanisms underlying the physiological stimulation of sperm motility by HCO$_3^-$ in human spermatozoa, demonstrating the direct involvement of sAC and tyrosine phosphorylation of AKAP3 acting upstream of PKA activation.

ACKNOWLEDGMENTS

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