Bicarbonate actions on flagellar and Ca\textsuperscript{2+}-channel responses: initial events in sperm activation

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Accepted 16 December 2002

SUMMARY

At mating, mammalian sperm are diluted in the male and female reproductive fluids, which brings contact with HCO\textsubscript{3}– and initiates several cellular responses. We have identified and studied two of the most rapid of these responses. Stop-motion imaging and flagellar waveform analysis show that for mouse epididymal sperm in vitro, the resting flagellar beat frequency is 2-3 Hz at 22-25°C. Local perfusion with HCO\textsubscript{3}– produces a robust, reversible acceleration to 7 Hz or more. At 15 mM the action of HCO\textsubscript{3}– begins within 5 seconds and is near-maximal by 30 seconds. The half-times of response are 8.8±0.2 seconds at 15 mM HCO\textsubscript{3}– and 17.5±0.4 seconds at 1 mM HCO\textsubscript{3}–. Removal of external HCO\textsubscript{3}– allows a slow return to basal beat frequency over ~10 minutes. Increases in beat symmetry accompany the accelerating action of HCO\textsubscript{3}–. As in our past work, HCO\textsubscript{3}– also facilitates opening of voltage-gated Ca\textsuperscript{2+} channels, increasing the depolarization-evoked rate of rise of intracellular Ca\textsuperscript{2+} concentration by more than fivefold. This action also is detectable at 1 mM HCO\textsubscript{3}– and occurs with an apparent halftime of ~60 seconds at 15 mM HCO\textsubscript{3}–. The dual actions of HCO\textsubscript{3}– respond similarly to pharmacological intervention. Thus, the phosphodiesterase inhibitor IBMX promotes the actions of HCO\textsubscript{3}– on flagellar and channel function, and the protein kinase A inhibitor H89 blocks these actions. In addition, a 30 minute incubation with 60 µM cAMP acetoxymethyl ester increases flagellar beat frequency to nearly 7 Hz and increases the evoked rates of rise of intracellular Ca\textsuperscript{2+} concentration from 17±4 to 41±6 nM second\textsuperscript{–1}. However, treatment with several other analogs of cAMP produces only scant evidence of the expected mimicry or blockade of the actions of HCO\textsubscript{3}–, perhaps as a consequence of limited permeation. Our findings indicate a requirement for cAMP-mediated protein phosphorylation in the enhancement of flagellar and channel functions that HCO\textsubscript{3}– produces during sperm activation.

Key words: Capacitation, sAC, cAMP-AM, PKA, Motility, Shear angle, Tan angle, Asymmetry

INTRODUCTION

The bicarbonate anion is a key player in activating sperm when they leave the mammalian epididymis. Early studies (Hamner and Williams, 1964) noted that the bicarbonate anion stimulates respiratory activity of sperm. Later work (Boatman and Robbins, 1991; Garbers et al., 1982; Okamura et al., 1985; Tajima et al., 1987) linked the HCO\textsubscript{3}– content of reproductive fluids to the control of sperm motility and metabolism, and strongly suggested that HCO\textsubscript{3}– increases production of cAMP as a messenger that regulates these functions. Recent cloning of the atypical adenylate cyclase of sperm (Buck et al., 1999) allowed demonstration that HCO\textsubscript{3}– indeed activates the expressed recombinant enzyme (Chen et al., 2000; Jaiswal and Conti, 2001).

An obligatory maturational sequence known as ‘capacitation’ prepares sperm to fertilize the egg (Yanagimachi, 1994). Some events in this multistep process require HCO\textsubscript{3}–, are mediated by cAMP, and follow activation of protein kinase A (PKA). These include the transbilayer movement of plasma membrane phospholipids (Gadella and Harrison, 2000; Harrison and Miller, 2000), lateral redistribution of membrane cholesterol (Flesch et al., 2001) and a delayed tyrosine phosphorylation of several sperm proteins (Visconti et al., 1995a; Visconti et al., 1995b).

Several types of voltage-gated Ca\textsuperscript{2+} channel proteins have been detected in mouse sperm by immunomethods (Quill et al., 2001; Ren et al., 2001; Serrano et al., 1999; Wennemuth et al., 2000; Westenbroek and Babcock, 1999). Past work shows that depolarizing stimuli open some of these channels to allow Ca\textsuperscript{2+} entry, and that this evoked channel activity increases after conditioning of sperm with HCO\textsubscript{3}– (Wennemuth et al., 2000). We characterize this additional action of HCO\textsubscript{3}– more fully and examine its mechanistic basis.

We now show that HCO\textsubscript{3}– also accelerates the flagellar beat, as reported and quantified here for the first time by flagellar waveform analysis. We find that the actions of HCO\textsubscript{3}– on channel and flagellar function are similar in many respects, as
if they are initiated by events that share an involvement of cAMP-mediated protein phosphorylation.

**MATERIALS AND METHODS**

**Materials**

Indo-1 AM and Pluronic 147 were from Molecular Probes (Eugene, OR), H89 from BIOMOL Labs (Plymouth Meeting, PA), and the 8-(4-chlorophenylthio) derivative of cAMP (CPT-cAMP) from Calbiochem (San Diego, CA). The Rp- and Sp-diastereomers of adenosine 3',5'-cyclic monophosphothioate (cAMP-AM) and all other chemicals were from Sigma (St Louis, MO). Rotiblock™ was from Carl Roth (Karlsruhe, Germany), anti-phosphotyrosine antibody clone 4G10 from Upstate Biotechnology (cAMP-AM) and all other chemicals were from Sigma (St Louis, MO), horseradish peroxidase-labeled secondary antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and enhanced-chemiluminescence detection reagents from Amersham (Piscataway, NJ).

**Sperm preparation and incubation conditions**

As in prior work (Wennemuth et al., 2000; Westenbroek and Babcock, 1999), cauda epididymal sperm were prepared from male mice (Swiss Webster, retired breeders) that were euthanized by CO2 asphyxiation. Briefly, the cleaned, excised epididymes were rinsed with medium Na7.4: 135 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid, 1 mM pyruvic acid, adjusted to pH 7.4 with NaOH. The tissue was transferred to 1 ml of a ‘swimout/capacitation medium’ that comprised medium Na7.4 with bovine serum albumin (BSA) (5 mg/ml) and NaHCO3 (15 mM). Semen was allowed to exude (15 minutes at 37°C) adjusted to pH 7.4 with NaOH. The time was transferred to 1 ml of a ‘swimout/capacitation medium’ that comprised medium Na7.4 with bovine serum albumin (BSA) (5 mg/ml) and NaHCO3 (15 mM). Semen was allowed to exude (15 minutes at 37°C) and incubated 90 minutes at 37°C in a 95% air/5% CO2 atmosphere. For subsequent waveform analysis, sperm were washed twice in medium Na7.4 and examined at room temperature.

Potassium-evoked responses were produced with medium K8.6: 135 mM KCl, 5 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 30 mM TAPS, 5 mM glucose, 10 mM lactic acid, 1 mM pyruvic acid, adjusted to pH 8.6 with NaOH, as in past work (Wennemuth et al., 2000).

**Immunoblots**

Total proteins from mouse sperm were extracted by adding an equal volume of a 2× Laemml buffer (Laemmli et al., 1970). After heating for 5 minutes at 100°C, the samples were clarified by centrifugation at 5000 g for 5 minutes and adjusted to 5% mercaptoethanol. Proteins were separated on a 4-12% gradient SDS-PAGE gel and transferred onto nitrocellulose membranes. Prior to the incubation with antibodies, non-specific binding was blocked with PBS containing 10% Rotiblock™ for 1 hour. Blots were incubated with a 1:1000 dilution of the anti-phosphotyrosine primary antibody for 1 hour then washed with 0.1% Tween in PBS (4 × 5 minutes). Under the same conditions, blots were then incubated with horseradish peroxidase-labeled secondary antibody diluted 1:2000 in PBS. Finally, blots were washed with 0.1% Tween in PBS (4 × 5 minutes) and treated with enhanced-chemiluminescence detection reagents for 5 minutes.

**Dye loading and photometry**

Indo-1 AM was dispensed from 2 mM stocks in DMSO, dispersed in 10% Pluronic 147, diluted to 20 μM in 0.25 ml medium Na7.4, then immediately mixed with an equal volume of the sperm suspension. After 15-20 minutes, medium Na7.4 (1 ml) was added and the cells sedimented. After resuspension in 0.25 ml fresh medium, incubation continued for a minimum of 40 minutes and no more than 5 hours before use.

Incubation chambers were constructed from 35 mm tissue culture dishes and 90 glass coverslips. An uncoated, ~5 mm square #00 coverslip was placed in the dish and 10 μl of cell suspension added. After ~5 minutes, the chamber was gently flooded with medium and transferred to the microscope stage. Test solutions were applied by a solenoid-controlled, gravity-fed, multibarreled, local perfusion device with an estimated exchange time of <0.5 second. Photometric measurements were as described (Herrington et al., 1996; Wennemuth et al., 2000). The photometric signals were corrected for background fluorescence and calibrated with the constants Rmin (1.067), Rmax (6.488) and K* (1470 nM) obtained from cells equilibrated in solutions fortified with ionomycin (10 μM) and containing 50 mM EGTA, 15 mM CaCl2, or 20 mM EGTA with 15 mM CaCl2 (calculated free Ca2+ concentration of 251 nM). Automated correction, calibration and kinetic analysis of digital photometric records were performed in Igor (WaveMetrics, Lake Oswego, OR). Statistical analyses were performed in Excel (Microsoft, Redmond, WA). All results are presented as means±s.e.m., except where noted.

**Ester loading of cAMP**

The cAMP-AM was dispensed from a 20 mM stock in DMSO, dispersed in 10% Pluronic 147, diluted to 120 μM in 0.25 ml medium Na7.4, then immediately mixed with an equal volume of a sperm suspension that had or had not received preliminary loading with indo-1 AM. After 30 minutes, an aliquot (5-10 μl) was added to the sample chamber for imaging or photometry. For evoked [Ca2+]i (intracellular Ca2+ concentration) responses, cells were examined with protocols that minimized the duration of perfusion to thereby reduce washout of the membrane-permeant ester (Schultz et al., 1994).

**Waveform analysis**

Images for waveform analysis were collected from cells examined in the sample chambers described above, with a 40×0.65 N.A. objective on an inverted microscope (Nikon Diaphot) in which the stage lamp was replaced by a 10 mm ultrabright (15 candella at 20 mA) red LED (AND90H9P; Newark Electronics, Chicago, IL). Brief flashes of illuminating light were produced by a custom-built stroboscopic power supply that provided 3-5 V pulses of 1-2 ms duration. For visual observation, the flash frequency was 150 Hz. For data collection, the pulse was triggered once-per-frame by a synchronization signal from the controller module of the frame-transfer CCD camera (TCP512; Roper Scientific, Trenton, NJ). Images were collected at 30 Hz from a 128 × 128 pixel region of the camera chip, under the direction of Metamorph (Universal Imaging; West Chester, PA). After initial adjustments of intensity and contrast, images were stored in TIFF format for subsequent analysis with software routines written in Igor (WaveMetrics; Lake Oswego, OR). These routines provide for semiautomated tracing of the flagellum in each image and subsequent fitting of the digitized waveform with a simple sine function. Additional routines fully automated were: (1) regression analysis of the timecourse of the phase lags of the fitted sine functions to provide the flagellar beat frequency; (2) tabulation of the distance along the flagellum (arc length), the angular deviation (tangent angle) evaluated at 0.5 μm intervals along the traced flagellum, and the time-averaged tangent angle data; (3) presentation of the time-averaged tangent angle versus arc length data (shear curves) as a measure of flagellar beat asymmetry (see Brokaw, 1979) [for a general discussion of polymer mechanics and their analysis see Howard (Howard, 2001)]; and (4) tabulation of the maximal excursion of the traced flagellum to define the flagellar beat envelope and the beat amplitude at regular intervals along the beat axis.
RESULTS

Bicarbonate enhances evoked Ca\textsuperscript{2+} entry

Fig. 1 shows typical measurements of Ca\textsuperscript{2+} entry in sperm using indo-1 photometry. The signals monitor spatially averaged [Ca\textsuperscript{2+}]\textsubscript{i} from small cell clusters comprising 3-7 motile sperm loosely tethered to a coverslip. In Fig. 1A, the cells were perfused continuously with the Ringer-like medium Na\textsubscript{7.4}, except during brief 10-second depolarizing stimuli with medium K8.6. [Ca\textsuperscript{2+}]\textsubscript{i} rose abruptly during each stimulus, then returned slowly towards the initial resting level. Recovery from the first stimulus often was incomplete or absent. In a similar experiment (Fig. 1B), the Na\textsubscript{7.4} medium was fortified with 15 mM HCO\textsubscript{3}– for the 2 minute period before the third stimulus. Basal [Ca\textsuperscript{2+}]\textsubscript{i} remained unchanged, but the evoked response was increased dramatically. On an expanded time scale (Fig. 1C), [Ca\textsuperscript{2+}]\textsubscript{i} rose linearly during responses evoked before exposure to HCO\textsubscript{3}– (lower two traces) and more quickly and farther for those evoked afterwards (upper trace). This is the enhancing effect of HCO\textsubscript{3}–. The rate of rise of [Ca\textsuperscript{2+}]\textsubscript{i} is a direct measure of the number of open Ca\textsuperscript{2+} channels. For similar experiments to that shown in Fig. 1A, the rate of rise of the averaged responses to the three control stimuli was 17 nM second\textsuperscript{-1}. For the averaged responses to the two control stimuli of Fig. 1B, the rate was similar (24 nM second\textsuperscript{-1}). However, for the test stimulus applied after treatment with HCO\textsubscript{3}– the rate increased more than fourfold (to 98 nM second\textsuperscript{-1}).

Characterization of bicarbonate action on Ca\textsuperscript{2+} channel activity

In early trials, the rates of evoked Ca\textsuperscript{2+} rise varied considerably between experiments. Fig. 2A identifies the age of the preparation (t=0 marks the beginning of dye loading) as the source of much of this variation. After the 60 minutes of dye-loading, sperm were stored for various periods of at room temperature in medium Na\textsubscript{7.4}, then exposed to 15 mM HCO\textsubscript{3}– for 2 minutes prior to stimulus. In records collected immediately, indo-1 signals were weak (probably indicating that hydrolysis of the indo-1 AM ester was still incomplete) and the rates of rise were ~20 nM second\textsuperscript{-1}, similar to those seen for the control stimuli in Fig. 1. Sperm examined 1-6 hours later had stronger indo-1 signals. During this interval, the mean evoked rates of rise increased more than threefold. Regression analysis shows that the age of the sperm preparation correlates strongly with this increase (r=0.992). Usually, we limit the experimental window to preparations of 2-5 hours in age and compare the rates of rise observed during control and test stimuli applied in protocols like those of Fig. 1.

Our conditions for storage of sperm are intended to prevent capacitation in vitro. As protein tyrosine phosphorylation is considered a marker of progression towards capacitation, we used phosphotyrosine immunoblots to assess the effectiveness of our strategy. Fig. 2B compares extracts of sperm prepared in various ways. Lane 1 is after ‘swimout’ from the epididymal exudate, conditions that include 15 minutes incubation with 15 mM NaHCO\textsubscript{3} at 37°C. Lane 2 is after a further 15 minute incubation in Na\textsubscript{7.4} with 15 mM NaHCO\textsubscript{3} at 22-24°C, a longer exposure to HCO\textsubscript{3}– than we use in actual experiments. Lane 3 is after 90 minutes in the swimout/capacitation medium at 37°C (conditions reported to induce capacitation in vitro) (Visconti et al., 1995a). Lane 4 is after 180 minutes at 22-24°C in Na\textsubscript{7.4} alone, our usual storage conditions. All extracts show a prominent band migrating at ~116 kDa, a less-prominent band migrating at ~200 kDa and minor bands of lower apparent M\textsubscript{r}. The 116 kDa band presumably is the constitutively phosphorylated hexokinase reported by Kalab et al. (Kalab et al., 1994); the identity of the higher and lower M\textsubscript{r} bands is not known. The extracts of sperm incubated under capacitating conditions (lane 3) show large increases in a band migrating at ~90 kDa, and in three bands migrating between 40 and 60 kDa, as reported in prior work (Visconti et al., 1995a). The 90 kDa band also is present but substantially weaker in extracts prepared from cells after 3 hours storage in Na\textsubscript{7.4}. Thus, the gradual increases in evoked Ca\textsuperscript{2+} channel responses that occur during prolonged storage at room temperature (Fig. 2A) are accompanied by some of the increases in protein tyrosine phosphorylation.

Fig. 1. Bicarbonate enhances the rate of depolarization-evoked Ca\textsuperscript{2+} entry. (A,B) Sperm were perfused locally with medium Na\textsubscript{7.4} that contained 0 or 15 mM bicarbonate except during brief (10 second) stimuli with the depolarizing medium K8.6 as shown. [Ca\textsuperscript{2+}]\textsubscript{i} was monitored by indo-1 emission ratio photometry. (C) The initial portions of the averaged responses evoked in these experiments before (■,●) and after (▼) exposure to 15 mM HCO\textsubscript{3}–. The slope of the best-fit regression line with the indicated rate of rise is a measure of the number of Ca\textsuperscript{2+} channels open during the KCl depolarization.
phosphorylation that are produced by incubation under capacitating conditions. However, no increases in phosphorylation were observed for cells incubated with HCO$_3^-$ for 15 minutes, several times longer than the 1-2 minutes exposures that acutely enhance Ca$^{2+}$ channel responses (Fig. 1B).

Fig. 2C examines the timecourse of this enhancing action of HCO$_3^-$ on channel responses. Using a protocol like that of Fig. 1B we varied the duration of exposure to HCO$_3^-$, In this and most subsequent experiments, the rates of Ca$^{2+}$ rise for the test stimulus was normalized to the rate of rise in the preceding control stimulus. The half-time for the action of 15 mM HCO$_3^-$ is ~60 seconds. Fig. 2D shows that HCO$_3^-$ acts even at 1 mM, although this concentration appears to be below the EC$_{50}$ with respect to the rate of rise.

Sperm flagellar waveform analysis

Simultaneously with indo-1 photometry, we observe real-time, red-light video images of cells within the ~25 mm diameter recording window to ascertain that the recorded sperm remain motile. Although these low-quality images show only the sperm head and midpiece, they clearly reveal a rapid increase in movement when cells are perfused with HCO$_3^-$.

Fig. 3A shows the initial six video frames in a time series of images (48 × 48 µm) of a sperm collected at 30 Hz. (B) The flagellar beat envelope was visualized from a composite of all 30 images collected in the complete 1 second series. The flagellar beat amplitude is indicated by the orthogonal arrow placed 25 µm along the midline of the flagellar beat axis. (C) The flagellar waveform in each image in A was fitted with a sine function. The original, superimposed grayscale images were overlaid with the (colored) best-fit curves. (D) Regression analysis of the phase lags of the fitted sine functions in a longer segment of this series yields the flagellar beat frequency. The slope of the regression line (39.8 rad second$^{-1}$) corresponds to a beat frequency of 6.34 Hz.
series. The extremes of flagellar excursion outline the flagellar beat envelope, and the midline of the envelope approximates the flagellar beat axis. Beat amplitude can be estimated from the perpendicular distance between the extremes of the envelope (the double-headed arrow), here measured arbitrarily at 25 µm from the point of attachment.

The image series of Fig. 3A contains much additional information related to the spatial coordinates of the flagellum. Extraction of these coordinates is not a trivial task. The simplest approach begins with manual tracing of projected images to provide a binary bit-map. This is easy to apply, but tedious and time consuming. Image recognition algorithms hold the promise of fully automated determination of flagellar coordinates. However, we examined several and rejected them on the basis of unacceptably high error rates. Instead, we have implemented semi-automated tracing, with a various fully automated calculations.

For the range of conditions explored here, the flagellar waveform is relatively symmetrical and sinusoidal. Analysis therefore includes fitting of a simple sine function to each image. The fit usually is quite good, as shown in Fig. 3C, which contains a grayscale composite of the images of Fig. 3A, each overlaid with a colored, best-fit sine wave. The phase lag of the fitted sine function must change by 2π radians (360°) during each beat cycle. For the cell examined here, regression analysis of the phase lag for 15 successive images indicates a slope of 39.8 rad second–1. Dividing this slope by 2π gives a beat frequency of 6.34 Hz (Fig. 3D).

**Bicarbonate accelerates flagellar beat frequency**

Simple visual observation indicated that HCO₃⁻ stimulates sperm motility. Fig. 4A examines the distribution of flagellar beat frequencies for cells selected at random from samples of the sperm from a single animal that were bathed in media lacking or containing 15 mM NaHCO₃. Without HCO₃⁻, the beat frequencies ranged from 1.5-6.0 with a mean of 3.1±0.2 Hz. With HCO₃⁻, the range was 3.8-10.3 with a mean of 7.9±0.3 Hz. The distributions within both groups were well fitted by single Gaussian functions that showed little overlap. Beat amplitude (Fig. 4B) for the two treatment groups showed larger variability and more overlap. Here, and consistently in subsequent experiments, the trend was toward decreased beat amplitude for sperm exposed to HCO₃⁻. A statistically significant correlation between beat frequency and amplitude was not found here.

In the absence of added HCO₃⁻, the beat frequency of most sperm remained steady for long periods of observation. If the perfusing medium was changed to include 15 mM NaHCO₃, the beat frequency quickly increased by several-fold and remained at this faster rate for many minutes so long as HCO₃⁻ was present (Fig. 4C,D). The effects were reversible. Thus, if HCO₃⁻ was removed after 60 seconds, beat frequency slowly returned to the basal rate in ~10 minutes (Fig. 4E). Then if HCO₃⁻ was reapplied, the sperm responded rapidly and strongly again. Fig. 4C also examines the concentration-dependent kinetics of HCO₃⁻ action on a shorter time scale. At 15 mM, HCO₃⁻ robustly increased beat frequency. Acceleration was detectable within 2 seconds and proceeded linearly, perhaps with a slight overshoot. The half-time for development of response was 8.8±0.2 seconds. At 1 mM HCO₃⁻, the increase in beat frequency was nearly as large but only half as rapid with a half-time of 17.5±0.4 seconds.

**Bicarbonate decreases flagellar beat asymmetry**

Sperm swimming behavior is determined both by the frequency of the flagellar beat and by its symmetry. Flagellar beat symmetry determines linearity of swimming paths (Brokaw, 1979; Cook et al., 1994). Our analysis of flagellar waveform images (like those of Fig. 3A) includes calculation of the angular deviation (tangent angle) along the traced flagellum. Fig. 5A,B show examples of typical data obtained...
increased the averaged tangent angle and the symmetry of the flagellar waveform (Fig. 5C).

Mechanisms of bicarbonate action on Ca\textsuperscript{2+} channel activity and flagellar function

How is HCO\textsubscript{3} coupled to enhancement of evoked Ca\textsuperscript{2+} entry and acceleration of flagellar beat frequency? We used a pharmacological approach to evaluate the hypothesis that stimulation of sperm adenyl cyclase, increased cAMP content, and activation of PKA underlie the actions of HCO\textsubscript{3}.

Fig. 6A shows measurements of Ca\textsuperscript{2+} rise using protocols similar to Fig. 1. Sperm received a pair of control stimuli, then were perfused for 1 minute with medium Na7.4 alone or with 15 mM HCO\textsubscript{3}, or with 15 mM HCO\textsubscript{3} and 0.2 mM of the phosphodiesterase inhibitor IBMX. A third and final test stimulus then was applied. With short 1-minute exposures to HCO\textsubscript{3}, facilitation of Ca\textsuperscript{2+} entry was only increased 2.3±0.2-fold. When cells were perfused with HCO\textsubscript{3} together with IBMX, facilitation increased to 3.3±0.2-fold. Perfusion with IBMX alone produced only a 1.6±0.1-fold enhancement (data not shown). These results are consistent with the interpretation that IBMX enhances HCO\textsubscript{3} action by shifting the balance between competing pathways for synthesis and degradation of the cAMP messenger.

Fig. 6B summarizes experiments testing the ability of the PKA inhibitor H89 to prevent enhancement of Ca channel responses by HCO\textsubscript{3}. In protocols similar to Fig. 1, sperm received first a pair of control stimuli. After brief recovery in Na7.4, alone or with 0-30 \(\mu\)M H89, sperm were perfused for 2 minutes with media that contained HCO\textsubscript{3} (at 15 mM) and the same concentration of H89. The third test stimulus was then applied. As in Fig. 2D, this longer exposure to HCO\textsubscript{3} alone produced a nearly sevenfold increase in the evoked rate of [Ca\textsuperscript{2+}]\textsubscript{i}. H-89 inhibited this response with an IC\textsubscript{50} of ~10 \(\mu\)M, consistent with a required phosphorylation by PKA in the pathway of HCO\textsubscript{3} action.

Similar experiments examined HCO\textsubscript{3} action on flagellar beat frequency. Fig. 6C shows that bath application of 50-200 \(\mu\)M IBMX alone increased beat frequency with an EC\textsubscript{50} of ~50 \(\mu\)M, suggesting that basal phosphodiesterase activity may limit resting beat frequency. Fig. 6D shows that when 30 \(\mu\)M H89 was applied for the 5 minutes before and during exposure to HCO\textsubscript{3}, average beat frequency increased to only 3.3±0.2 Hz. This rate is higher than the 2.3±0.2 Hz of resting cells, but much less than the 7.3±0.2 Hz observed for cells treated directly with HCO\textsubscript{3}. Interestingly, 30 \(\mu\)M H89 alone had little or no effect on beat frequency, suggesting that the basal beat rate is not determined by a dynamic balance between PKA and competing phosphatases. In other experiments to test the reversibility of H89 action (not shown) individual sperm were perfused sequentially with Na7.4 alone, then with 30 \(\mu\)M H-89, then 30 \(\mu\)M H-89 and 15 mM NaHCO\textsubscript{3}, then finally with Na7.4 containing only 15 mM NaHCO\textsubscript{3}. It was difficult to maintain recording during experiments of such long duration. However, in 3 out of 18 trials the results clearly showed that a strong stimulus by HCO\textsubscript{3} returned within 1 minute after removal of H-89, indicating that the inhibitory actions of H89 are readily reversible.

The results with IBMX and those with H-89 are consistent with a simple working hypothesis: bicarbonate acts on flagellar beat frequency and on evoked Ca\textsuperscript{2+} entry by raising cAMP and...
stimulating protein phosphorylation via PKA. This view predicts that membrane-permeant analogs of cAMP should mimic (or block) the actions of HCO₃⁻. Table 1 summarizes several tests of that prediction. Rates of evoked Ca²⁺ rise (first column) were assessed before and after 2 minutes perfusion with various analogs alone or with IBMX. Medium Na7.4 alone or with 15 mM NaHCO₃ served as negative and positive controls. The effects were not those expected. Stimuli applied after perfusion with CPT-cAMP or Sp-cAMPS gave normalized rates of rise ~50% lower than for stimuli applied before exposure to these agents. For Rp-cAMPS the rates were ~50% higher than for the stimuli applied after Na7.4 alone. By comparison, for control experiments in which no treatment was applied before the test stimulus, the normalized rates were elevated by 20%. Thus, the effects, if any, of the analogs are modest compared with the robust nearly 500% increase produced by NaHCO₃.

Table 1. Modest actions of cAMP analogs on evoked Ca²⁺ entry and flagellar activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>Normalized rate of rise</th>
<th>Beat frequency (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.20±0.08 (n=20)</td>
<td>2.72±0.01 (n=147)</td>
</tr>
<tr>
<td>dibutyryl-cAMP</td>
<td>n.d.</td>
<td>2.86±0.03 (n=32)</td>
</tr>
<tr>
<td>8-BrcAMP</td>
<td>n.d.</td>
<td>2.67±0.03 (n=36)</td>
</tr>
<tr>
<td>Sp-cAMPS + IBMX</td>
<td>0.47±0.08 (n=10)</td>
<td>3.04±0.03 (n=36)</td>
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<tr>
<td>Rp-cAMPS</td>
<td>3.36±0.03 (n=11)</td>
<td>n.d.</td>
</tr>
<tr>
<td>CPT-cAMP</td>
<td>1.65±0.22 (n=9)</td>
<td>n.d.</td>
</tr>
<tr>
<td>CPT-cAMP + IBMX</td>
<td>0.41±0.07 (n=10)</td>
<td>2.66±0.03 (n=24)</td>
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<tr>
<td>NaHCO₃</td>
<td>4.92±1.24 (n=11)</td>
<td>6.37±0.01 (n=128)</td>
</tr>
<tr>
<td>Rp-cAMPS + NaHCO₃</td>
<td>n.d.</td>
<td>5.0±0.05 (n=36)</td>
</tr>
</tbody>
</table>

For evoked Ca²⁺ entry, cells perfused with Na7.4 received two 10-second stimuli with K8.6. After 1 minute of recovery in Na7.4, the perfusate was changed to Na7.4 alone or with 15 mM NaHCO₃, or to Na7.4 with the indicated additions of cAMP analogs (1 mM) alone or with 0.2 mM IBMX. A third stimulus was applied after 2 minutes. Rates of rise during the third stimulus were normalized to that of the mean for the first two. Beat frequency was assessed from waveform analysis of cells selected at random from samples bathed for 5-20 minutes in Na7.4 with the indicated additions of analogs (1 mM), alone or with NaHCO₃. To examine the mean of the normalized rates of rise, beat frequencies and the number of cells examined in three independent experiments for each of the two assays. n.d., not determined.

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Fig. 6. Enhancement of the actions of bicarbonate by the phosphodiesterase inhibitor IBMX and blockade by the PKA inhibitor H89. (A) Enhancement by IBMX. Ca²⁺ channel activity was determined as in Fig. 1B, except that the third stimulus followed 1 minute perfusion by medium Na7.4 alone (none), with 15 mM NaHCO₃, or with 0.2 mM IBMX and NaHCO₃, as indicated. The depolarization-evoked rate of rise was normalized to the average of rates for the preceding two control stimuli. Mean values (n=13) were 0.95±0.10, 1.78±0.20 and 2.57±0.45. (B) Inhibition by H-89. In similar experiments, sperm in Na7.4 received a control stimulus, 1 minute of recovery in Na7.4 with 0, 1, 10 or 30 μM H89 then 2 minute exposures to Na7.4 with 15 mM NaHCO₃ and the same concentration of H89. The test stimulus used K8.6 containing the same concentration of H89. Normalized mean values (n=11) were 6.64±0.74, 4.39±0.37, 3.85±0.82 and 2.96±0.39. (C,D) Beat frequencies for cells randomly selected after 5-15 minutes in Na7.4 with: (C) 0, 50, 100 or 200 μM IBMX; (D) no additions, 30 μM H89, 15 mM NaHCO₃ (BC), or 30 μM H89 (5 minutes preincubation) and 15 mM NaHCO₃ as indicated. For each averaged point in C, n=15 cells from three independent experiments. For the data groups in D, n=41-43 cells from three independent experiments. Boxes indicate ±1 s.d. from the mean.
was even stronger than that of NaHCO₃; with cAMP-AM the time-averaged asymmetry parameter became positive over the entire proximal 40 μm of the flagellum.

DISCUSSION

The alterations to mammalian sperm that occur between mating and fertilization (collectively called capacitation) (see Yanagimachi, 1994) are extremely difficult to study. Even the component of capacitation that is most accessible experimentally – the activation of sperm upon release from storage in the male reproductive tract – is not entirely defined. However, several recent insights provide an improved view through this narrow early window of observation. Numerous past reports proposed roles for cAMP as a regulatory messenger in sperm (Kopf and Garbers, 1980), without identifying the primary signal that increases cAMP content. We now know that sperm possess an atypical adenylyl cyclase that is stimulated directly by HCO₃⁻ (Buck et al., 1999; Chen et al., 2000; Jaiswal and Conti, 2001). This advance has sparked renewed interest in the role of the HCO₃⁻ anion as a regulatory signal for other early events in capacitation. These events include activation of sperm motility (Holt and Harrison, 2002; Okamura et al., 1985), the transbilayer movement of aminophospholipids from the inner to the outer leaflet of the plasma membrane (Gadella and Harrison, 2000; Harrison and Miller, 2000) and subsequent lateral redistribution of cholesterol in membranes of the sperm head (Flesch et al., 2001; Gadella and Harrison, 2002). The half-time for externalization of phosphatidylethanolamine is ~5 minutes (Gadella and Harrison, 2002). We now report that HCO₃⁻ facilitates voltage-gated Ca²⁺ channels and increases flagellar beat frequency even more quickly, with half times of ~60 and ~10 seconds.

Pharmacological evidence supports the hypothesis that the activation of a phospholipid scramblase, which underlies HCO₃⁻ action on membrane lipid remodeling, requires protein phosphorylation by the cAMP-dependent protein kinase A (Gadella and Harrison, 2002). We find that, like their actions on HCO₃⁻-mediated lipid remodeling, the phosphodiesterase inhibitor IBMX promotes and the PKA inhibitor H89 blocks the actions of HCO₃⁻ on flagellar beat frequency and the rate of evoked Ca²⁺ entry.

Moreover, we find that incubation with cAMP-AM ester strongly increases rates of evoked Ca²⁺ entry and flagellar beat frequency, presumably indicating an effective elevation of cAMP without involvement of adenylyl cyclase. Unexpectedly, several other analogs of cAMP were less effective in mimicking these actions of HCO₃⁻ than in their actions on sperm reported elsewhere (Gadella and Harrison, 2002; Harrison and Miller, 2000; Visconti et al., 1995b). The most likely explanation for limited efficacy of the cAMP analogs examined in Table 1 is that they are much less permeant than the cAMP-AM ester in sperm. Comparison of the kinetics of responses to cAMP-AM and other analogs in somatic cell preparations support this interpretation (Schultz et al., 1994; Zhang et al., 2001).
If the action of HCO$_3^-$ on flagellar function indeed results from stimulation of adenyl cyclase, we can now also estimate the time scale for this activation. Thus, the region of the signaling pathway between HCO$_3^-$ exposure and elevation of cAMP (perhaps involving only entry of HCO$_3^-$ and direct activation of adenyl cyclase) must operate on a time scale at least as fast as that found for the acceleration of flagellar beat frequency (τ/2 <10 seconds). As the kinetics of Ca$^{2+}$ channel facilitation are slower than those of flagellar responses, the signaling pathway between cAMP and the ultimate target that facilitates Ca$^{2+}$ entry channels may contain additional steps. The ability of concentrations as low as 1 mM HCO$_3^-$ to stimulate these responses is consistent with reported stimulation of the adenyl cyclase of rat spermatogenic cells by 1 mM HCO$_3^-$ (Jaiswal and Conti, 2001).

In concept, targeted disruption of the catalytic (C) subunits of PKA provides an alternate approach to study of the signaling pathways that control sperm function. Skalhegg et al. (Skalhegg et al., 2002) produced mice carrying a null mutation in the widely expressed gene that encodes the C$\alpha$ subunit of PKA. Male C$\alpha$-null mice were runted and few survived to puberty. The spermatogenic cells of the testes of the survivors lacked C$\alpha$ protein. Intact sperm were recovered from the cauda epididymis, but they did not show progressive motility and presumably were unable to fertilize. Subsequent work (Nolan et al., 2002) has produced mice carrying the more selective disruption of the unique C$\alpha$2 transcript that is expressed exclusively in spermatocytes and spermatids (Reinton et al., 2000; San Agustin et al., 2000; San Agustin and Witman, 2001). These animals should provide an improved transgenic model to test the proposed requirement for PKA-mediated phosphorylation in the actions of HCO$_3^-$ during sperm activation.

We thank David Miller for the design and construction of the custom-built LED power supply, Lea Miller and Anke Friedetzky for technical help, and Drs Joe Beavo, Charles Brokaw and Linda Wordeman for critical reading of earlier versions of this manuscript. We especially thank Dr Bertil Hille for providing laboratory space and for his continued encouragement of these studies. This work was supported by the NICHD, National Institutes of Health through a cooperative agreement U54-HD12629 as part of the Specialized Cooperative Centers Program in Reproduction Research, by the W. M. Keck Foundation, and by the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft (We 2344/4-1 DFG, Germany).

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