A Requirement of Bicarbonate for Ca\textsuperscript{2+}-induced Elevations of Cyclic AMP in Guinea Pig Spermatozoa*

David L. Garbers§§, D. Janette Tubb‡, and Ross V. Hyne¶

From the †Howard Hughes Medical Institute and the §Departments of Pharmacology and Physiology, Vanderbilt University Medical Center, Nashville, Tennessee 37232

Ca\textsuperscript{2+} causes less than 2-fold elevations of guinea pig sperm cyclic AMP concentrations when cells are incubated in a minimal culture medium in the absence of bicarbonate (HCO\textsubscript{3}⁻). However, in the presence of HCO\textsubscript{3}⁻, Ca\textsuperscript{2+} increases cyclic AMP by as much as 25-fold within 1 min. The (Ca\textsuperscript{2+}, HCO\textsubscript{3}⁻)-induced elevations occur in either the presence or absence of the permeant anions, pyruvate and lactate. In the absence of extracellular Ca\textsuperscript{2+}, HCO\textsubscript{3}⁻ elevates cyclic AMP only slightly. The effect of HCO\textsubscript{3}⁻ is concentration-dependent, with maximal responses obtained at concentrations of greater than 25 mM. Ca\textsuperscript{2+} (25 mM HCO\textsubscript{3}⁻) at concentrations of less than 100 μM causes one-half-maximal elevations of cyclic AMP. The (Ca\textsuperscript{2+}, HCO\textsubscript{3}⁻)-induced elevations of cyclic AMP are observed at various extracellular pH values (7.5-8.5) and in the absence or absence of extracellular Na\textsuperscript{+} or K\textsuperscript{+}. NH\textsubscript{4}Cl does not elevate sperm cyclic AMP concentrations and does not greatly alter the (Ca\textsuperscript{2+}, HCO\textsubscript{3}⁻)-induced elevations. The putative Ca\textsuperscript{2+} transport antagonist, D-600 (100 μM), completely blocks the (Ca\textsuperscript{2+}, HCO\textsubscript{3}⁻)-induced elevations of cyclic AMP. A23187, in the presence but not in the absence of extracellular Ca\textsuperscript{2+}, increases sperm cyclic AMP but does not further elevate cyclic AMP in HCO\textsubscript{3}⁻-treated cells.


These studies establish that Ca\textsuperscript{2+}-dependent elevations of cyclic AMP in guinea pig spermatozoa are dependent on the presence of HCO\textsubscript{3}⁻ and suggest that HCO\textsubscript{3}⁻ is required for the uptake (exchange) or membrane sequestration of small amounts of physiologically active Ca\textsuperscript{2+}.

Altered Ca\textsuperscript{2+} permeability appears to represent one of the primary signals for induction of the acrosome reaction in spermatozoa (1-5). In mammals, the chemical signal(s) or biochemical changes responsible for the change in Ca\textsuperscript{2+} permeability are not yet resolved (5), whereas in the sea urchin, the component responsible for accelerated uptake of "Ca" appears to be a fucose-sulfate-rich complex associated with the egg jelly coat (6-8). The large, fucose-sulfate-rich polymer not only increases "Ca" uptake, but also markedly increases cyclic AMP concentrations of intact spermatozoa (7). Since other agents (elevated pH, agiectin, and A23187) which increase "Ca" uptake also elevate cyclic AMP concentrations, it has been suggested that increased Ca\textsuperscript{2+} uptake results in the direct or indirect activation of the sperm adenylate cyclase (5, 7, 9).

In 1979, we demonstrated (10) that Ca\textsuperscript{2+} could cause marked elevations of guinea pig sperm cyclic AMP concentrations, and subsequent work demonstrated that Ca\textsuperscript{2+} (micromolar concentrations) could activate the Mg\textsuperscript{2+}-dependent adenylate cyclase of guinea pig spermatozoa (11). Ca\textsuperscript{2+} has also been reported to cause elevations of cyclic AMP in hamster spermatozoa (12). Since sperm cells may not contain a functional GTP regulatory component (5, 13), the regulation of adenylate cyclase by Ca\textsuperscript{2+} could represent a primary mechanism of enzyme regulation in these cells.

In studies designed to determine requirements for the Ca\textsuperscript{2+}-induced elevations of cyclic AMP in guinea pig spermatozoa, we discovered that Ca\textsuperscript{2+} failed to elevate cyclic nucleotide concentrations in the absence of bicarbonate (HCO\textsubscript{3}⁻). We suggest here that bicarbonate ion is a requisite for the specific uptake or membrane binding of low concentrations of Ca\textsuperscript{2+}; this uptake or binding results in large elevations of cyclic AMP and could account, in part, for the reported stimulation of sperm motility by HCO\textsubscript{3}⁻ in some animals (14-16).

**EXPERIMENTAL PROCEDURES**

**Materials**—Common chemicals were of the highest reagent grade available and were purchased from Sigma Chemical Co. or Fisher Scientific Co. D-600 was from Knoll A-G, and 1-methyl-3-isobutylxanthine was from Dr. J. N. Wells (Department of Pharmacology, Vanderbilt University Medical Center).

**Media**—A solution containing 102 mM NaCl, 1.7 mM CaCl\textsubscript{2}, 0.25 mM sodium pyruvate, 20 mM sodium lactate, 1 mM MgCl\textsubscript{2}, and 25 mM triethanolamine buffer at pH 8.0 was used as the basic minimal culture medium. The medium was modified in various experiments to contain various concentrations of NaHCO\textsubscript{3}, 10 mM glucose for pyruvate, lactate, 25 mM NaHCO\textsubscript{3} to replace the triethanolamine buffer, 1 mM 1-methyl-3-isobutylxanthine, or various concentrations of CaCl\textsubscript{2}. When "Ca" was added, approximately 4 × 10\textsuperscript{5} dpm of "Ca" were added to each incubation vessel. For short incubation time periods (1 min), the pH of the various media remained at pH 8.0. When media were gassed with 95.5% (air/CO\textsubscript{2}), the pH was maintained at approximately 7.2 (25 mM NaHCO\textsubscript{3}). In some cases, choline chloride was substituted for NaCl, pyruvic acid for sodium pyruvate, lactic acid for sodium lactate, and KHCO\textsubscript{3} for NaHCO\textsubscript{3}.

**Sperm Preparation and Incubation Procedure**—Spermatozoa were prepared and washed as previously described (10) except in some cases where choline chloride (Na\textsuperscript{+}+free experiments) was used to wash the sperm cells.

Reactions were incubated with either air or air/CO\textsubscript{2} (95:5) as the gas phase. In general, cells collected in 154 mM NaCl were added immediately after collection to an incubation vessel containing the minimal culture medium (total volume = 1 ml). After incubation at 37 °C, the reactions were stopped (cyclic AMP) with 1 ml of 0.5 N perchloric acid. When "Ca" uptake was measured, reactions were stopped by rapid filtration on Whatman GF/C glass fiber filters. In some cases, cells were allowed to preincubate with the buffer for various periods of time at 37 °C prior to the addition of CaCl\textsubscript{2}.

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†Present address, Department of Obstetrics and Gynecology, University of Melbourne, Parkville, 3052 Australia.
Cyclic Nucleotide Determinations—The acidified cell suspensions were frozen and thawed 3-5 times and the cyclic AMP was then purified on acid alumina and Dowex 50-(H') columns as previously described (17). The concentration of cyclic AMP was determined by radioimmunoassay (18) as modified by Harper and Brooker (19).

Ca++ Uptake—At the end of the incubation, cells were diluted with 2 ml of 104 mM NaCl and rapidly added to GF/C glass fiber filters. The cells were washed 3 times with the saline solution by vacuum filtration and the filters were then counted for radioactivity.

HCO3-/CO2 Equilibria—In the experiments where HCO3- was added to the incubation vessels with air as the gas phase, the other species present would be CO2 and H2CO3. However, in most cases, the time of incubation was less than 1 min and, therefore, only major species present was HCO3-. Similarly, in some experiments, cells were gassed with air/CO2 (95:5) in the presence of 25 mM HCO3- or in the presence of triethanolamine buffer (no HCO3- added). When the triethanolamine buffer was used, the incubation times were short such that the pH of the medium did not appreciably change. Thus, under these conditions, only small amounts of HCO3- were present.

RESULTS

Requirement of HCO3-—When spermatozoa were added to a minimal culture medium (pH 8.0) containing Ca++ and HCO3- (25 mM), cyclic AMP concentrations were elevated by as much as 25-fold within 30 s (not shown). The concentrations were maximal between 15 s and 1 min and then declined rapidly toward basal concentrations. If Ca++ was omitted from the culture medium, cyclic AMP concentrations were elevated less than 2-fold. These observations are similar to those reported previously (10). Unexpectedly, however, when HCO3- was omitted from the buffer, cyclic AMP concentrations were elevated less than 2-fold in response to Ca++. Thus, the marked elevations of cyclic AMP in guinea pig spermatozoa appeared to require both extracellular Ca++ and HCO3-. Even when the sperm cell concentration was increased to 2 × 10⁷ cells/ml, both Ca++ and HCO3- were still required for the marked elevations of cyclic AMP (not shown).

The requirement of HCO3- for Ca++-induced elevations of cyclic AMP was concentration dependent (Fig. 1) and did not require the presence of exogenous substrates (Fig. 2). In other experiments, glucose also replaced pyruvate and lactate in the minimal culture medium without alteration of the HCO3- response curve. In these experiments, where the incubation time was 1 min or less, the pH of the medium was maintained constant at pH 8.0 in an air atmosphere. However, in other experiments, cells were incubated for up to 1 h in a 95:5 (air/CO2) atmosphere at pH 7.7 and were shown to remain completely responsive to Ca++ with respect to elevations of cyclic AMP (not shown). Thus, cells remain in a Ca++-responsive state when incubated in a HCO3- based buffer, and HCO3-, itself, cannot duplicate the effect of Ca++ even after long time periods of incubation. When cells were incubated for up to 1 h in a minimal culture medium in the absence of HCO3-, they also did not acquire Ca++ sensitivity (not shown).

Based on these results, it was postulated that the functional role of HCO3- was either to increase intracellular pH which might then be a requirement for a Ca++-dependent response or to serve as a specific activator or counter-ion for Ca++ uptake (or Ca++ binding to the membrane). CO2 rather than HCO3- also remained as a potential mediator of the Ca++ response.

Extracellular pH and HCO3-—The effect of HCO3- on cyclic AMP as a function of extracellular pH is shown in Fig. 3. In these experiments, the gas phase was air. The various
absence of NaHCO₃-, it failed to elevate cyclic AMP. However, when NH₄Cl was added in the absence of CO₂, it elevated intracellular pH, increased intracellular pH being required in order for Ca²⁺ to elevate cyclic AMP. Thus, neither lowered intracellular pH nor COS- in the absence of HCO₃- formed was low and the Con should decrease intracellular pH, intracellular pH. The penneant anions, pyruvate and lactate, would be expected to lower intracellular pH (20), but as already indicated, Ca²⁺ failed to elevate cyclic AMP concentrations in either the presence or absence of these substances when HCO₃- was absent. In other experiments, cells were incubated with 25 mM triethanolamine and gassed with a 95:5 (air/CO₂) gas mixture. Cells were added to the incubation vessels contained 0.25 mM CoCl₂, 10 mM NaCl or KCl, 1.0 mM MgCl₂, and 20 mM lactate, 0.25 mM pyruvate, and 25 mM NaHCO₃. Various concentrations by various concentrations of NaHCO₃ (0-10 mM) and with various concentrations of NaHCO₃ (0-10 mM) for various periods of time. Although Ca²⁺ stimulated the adenylate cyclase 3- to 5-fold as reported in other studies (11), NaHCO₃ did not alter the Ca²⁺ stimulation of the enzyme (not shown).

**Calcium Transport**—Since apparent alterations of intracellular pH did not appear to account for the HCO₃⁻ requirement, the possibility of a HCO₃⁻-activated Ca²⁺ uptake system was investigated.

Low concentrations of extracellular Ca²⁺ were capable of causing the large elevations of cyclic AMP (Fig. 5). Ca²⁺ at concentrations of less than 10 μM caused a positive response, and no Ca²⁺ at 0.1 μM failed to elevate cyclic AMP. In other experiments, the diuretic furosemide at concentrations of 50 μM also failed to block the (Ca²⁺, HCO₃⁻)-induced elevations of cyclic AMP (not shown).

**Adenylate Cyclase**—To determine whether the Mg²⁺-dependent adenylate cyclase was affected by HCO₃⁻, cells were broken and washed as described previously (11). Enzyme was then incubated with various concentrations of Ca²⁺ (0-1.0 mM) and with various concentrations of NaHCO₃ (0-10 mM) for various periods of time. Although Ca²⁺ stimulated the adenylate cyclase 3- to 5-fold as reported in other studies (11), NaHCO₃ did not alter the Ca²⁺ stimulation of the enzyme (not shown).

**Monovalent Cations and HCO₃⁻**—Cells collected and washed in a sodium-free or a potassium-free, choline-substituted medium were much less responsive to Ca²⁺ and HCO₃⁻ than cells prepared in physiological saline. Nevertheless, a Ca²⁺-dependent elevation of cyclic AMP was still observed (Table I). The percent elevation of cyclic AMP caused by Ca²⁺ and HCO₃⁻ was comparable for all treatments. In other experiments, the diuretic furosemide at concentrations of 50 μM also failed to block the (Ca²⁺, HCO₃⁻)-induced elevations of cyclic AMP (not shown).

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**TABLE I**

| Treatment       | TEA-PL medium | Na⁺-free medium | K⁺-free medium |
|-----------------|---------------|-----------------|----------------|
| NaCl or KCl     | 11.9 ± 2.9    | 8.4 ± 1.9       | 8.0 ± 1.9      |
| NH₄Cl           | 7.6 ± 1.1     | 4.7 ± 0.2       | 4.8 ± 0.6      |
| NaHCO₃ or KHCO₃ | 39.5 ± 8.0    | 23.7 ± 7.0      | 22.2 ± 5.6     |
| NH₄HCO₃         | 29.3 ± 8.1    | 13.6 ± 3.6      | 11.9 ± 2.6     |

*Picomoles of cyclic AMP/10⁶ cells.

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**Fig. 4**. The effect of NH₄Cl on the (Ca²⁺, HCO₃⁻)-induced elevations of cyclic AMP. Incubation vessels contained 0.25 mM pyruvate, 25 mM lactate, 25 mM NaHCO₃, 2 μM ethylene glycol bis(di-aminoethyl ether)-N,N′,N″,N‴-tetraacetic acid, and 1.0 mM 1-methyl-3-isobutylxanthine. NaCl (106 mM) was adjusted to 96 mM when NH₄Cl (10 mM) was added. The incubation vessels were continually gassed with a 95:5 (air/CO₂) gas mixture. Cells were added to the buffer (37 °C) and incubated for 10 min prior to the addition of 25 μl of H₂O or 25 μl of CaCl₂ (final concentration = 2.5 mM). At the times after CaCl₂ addition indicated on the abscissa, the reactions were stopped with perchloric acid. The values represent the mean ± S. E. of 4 determinations.

**Fig. 5**. The elevation of guinea pig sperm cyclic AMP concentrations by various concentrations of calcium. The incubation mixtures (1 ml) contained 106 mM NaCl, 1.0 mM MgCl₂, 20 μM ethylene glycol bis(di-aminoethyl ether)-N,N′,N″,N‴-tetraacetic acid, 20 mM lactate, 0.25 mM pyruvate, and 25 mM NaHCO₃. Various concentrations of CaCl₂ were added to the vessels followed by the addition of 100 μl of spermatozoa to give a final concentration of 2 × 10⁶ cells/ml. At 30 s after addition of the sperm cells, the reactions were terminated with perchloric acid.
and concentrations of slightly less than 100 µM caused onehalf-maximal elevations of cyclic AMP.

D-600, a putative Ca²⁺-transport antagonist, completely blocked the (Ca²⁺, HCO₃⁻)-induced elevations of cyclic AMP at 50–100 µM concentrations (Fig. 6).

A23187, the divalent cation ionophore, also was capable of causing elevations of cyclic AMP, although the elevations were generally only 2- to 3-fold (Fig. 7). Nevertheless, its effects on cyclic AMP required extracellular Ca²⁺ and A23187 failed to elevate cyclic AMP further in the presence of HCO₃⁻ (Fig. 7).

In final experiments, the uptake of "Ca⁺⁺" was estimated in the presence or absence of HCO₃⁻. The effect of HCO₃⁻ on "Ca⁺⁺" uptake was variable and statistically not significant (not shown). A23187, in contrast, caused 2- to 4-fold increases in "Ca⁺⁺" uptake (not shown) under similar conditions.

**DISCUSSION**

Ca²⁺ was previously shown to cause marked elevations of cyclic AMP and to activate the Mg²⁺-dependent adenylyl cyclase of guinea pig spermatozoa (10, 11) and to activate motility and elevate cyclic AMP of hamster spermatozoa (12). In the studies reported here, it is shown that one other component, HCO₃⁻, is absolutely required in order for Ca⁺⁺ to elevate cyclic AMP in intact guinea pig spermatozoa. The effect of HCO₃⁻ does not appear to be directly on adenylyl cyclase since it does not stimulate the adenylyl cyclase in broken cell preparations. Instead, the function of HCO₃⁻ appears to be related to the transport of Ca⁺⁺ since D-600 blocks the (Ca⁺⁺, HCO₃⁻)-induced elevations of cyclic AMP, and A23187, at least in part, reproduces the effect of HCO₃⁻. These data also suggest that multiple Ca⁺⁺ uptake systems exist in spermatozoa since Singh et al. (21) have demonstrated that D-600 can facilitate "Ca⁺⁺" uptake by guinea pig spermatozoa. An argument against an effect of HCO₃⁻ on Ca⁺⁺ transport is the failure to detect HCO₃⁻-induced increases in "Ca⁺⁺" uptake. However, certain potential problems with the uptake experiments must be considered. 1) Sperm immediately bind approximately 75 pmol of "Ca⁺⁺"/10⁹ cells when added to the Ca⁺⁺-containing medium; and 2) the uptake of Ca⁺⁺ (assuming it occurs) is probably very rapid and in low amounts. Thus, binding of "Ca⁺⁺" to the sperm membrane itself may represent amounts high enough to prevent detection of a low capacity uptake system. Recently, Hefner and Storey (22) observed a positive effect of Ca⁺⁺ on the motility of mouse spermatozoa and also failed to detect Ca⁺⁺ uptake by three different methods. It is possible, as suggested by Hefner and Storey (22), that a physiologically active Ca⁺⁺ pool exists directly on specific membrane sites. In this case, HCO₃⁻ might facilitate the binding of Ca⁺⁺ to those sites. The failure to observe significant HCO₃⁻-induced increases in "Ca⁺⁺" uptake, however, also could be explained by a concomitant negative effect of HCO₃⁻ on "Ca⁺⁺" uptake by mitochondria (23, 24).

The site of Ca⁺⁺ action to elevate cyclic AMP has not been firmly established, although the sperm adenylyl cyclase appears to represent the most likely target. Methylxanthines synergize with Ca⁺⁺ to elevate cyclic AMP (10) and Ca⁺⁺ activates the Mg²⁺-dependent adenylyl cyclase in guinea pig broken sperm cell preparations (11). Sperm cells contain high quantities of calmodulin (25-29) and adenylyl cyclase is known to be activated by the Ca⁺⁺-calmodulin complex (30). Nevertheless, we have not successfully activated the calmodulin-deficient adenylyl cyclase of guinea pig spermatozoa (11). Recently, Loersztajn et al. (31) have suggested that a Ca⁺⁺-dependent activator of a (Ca⁺⁺, Mg⁺⁺)-ATPase from rat liver may not be calmodulin; this also could be the case for the sperm adenylyl cyclase.

The results reported here emphasize the importance of buffer selection in intact cell studies. In the absence of HCO₃⁻, guinea pig sperm cells survive in vitro, but at least one response, the elevation of cyclic AMP, does not occur. Female oviduct fluids have been reported to contain high concentrations (35-90 mM) of HCO₃⁻ (32, 33), and large elevations of cyclic AMP in response to Ca⁺⁺, therefore, normally would be
motility or metabolic activations. It also has been suggested that small increases in cytosolic Ca²⁺ appear to be absolutely required for motility (36).

Phosphodiesterase inhibitors and cyclic AMP are known to stimulate sperm metabolism and motility in a wide variety of animals including the guinea pig (5). HCO₃⁻, likewise, has been reported to stimulate sperm metabolism and motility in various animals (14-16, 34). Although these effects of HCO₃⁻ could be due in part to fixation of CO₂ into the tricarboxylic acid cycle, at least one report (16) has suggested that HCO₃⁻ stimulates spermatozoa without direct entry into metabolic pathways. If this is true, HCO₃⁻ may stimulate spermatozoa by virtue of its primary effect on Ca²⁺ uptake. Subsequent events such as the elevations of cyclic AMP could explain the motility or metabolic activations. It also has been suggested that small increases in cytosolic Ca²⁺ (35) or Ca²⁺ binding to specific membrane sites (22) may directly stimulate sperm motility. In at least one case, hamster spermatozoa, extracellular Ca²⁺ appears to be absolutely required for motility (36).

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