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Stimulation of partially purified adenylate cyclase from bull sperm by bicarbonate

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Solubilized and partially purified adenylate cyclase from bull sperm was found to be specifically activated (up to 6-fold) by sodium bicarbonate ($NaHCO_3$) and to a lesser extent by $NaNO_3$. Other sodium salts were either ineffective (e.g. NaCOOH) or inhibitory (e.g. $NaHSO_3$, $NaHSO_4$ and $Na_2B_4O_7$). Stimulation by $NaHCO_3$ was dose-dependent in the range of 0–40 mM and was greater when enzyme activity was assayed in the presence of magnesium as compared with manganese ions. Bicarbonate seems to affect maximal enzyme velocity (V_{max}) and has no effect on the K_m of adenylate cyclase for Mn-ATP. Stimulation of adenylate cyclase by $NaHCO_3$ coincided with the elution pattern of the enzyme as recorded following chromatography on DEAE-cellulose or gel filtration on BioGel P-100. These results suggest that in the course of stimulation of sperm adenylate cyclase, bicarbonate is likely to interact directly with the enzyme. Furthermore, this intrinsic and unique property of sperm adenylate cyclase may explain results reported by others on the stimulation of cAMP production by bicarbonate in intact and broken sperm preparations and suggest a biochemical basis for enhanced sperm motility associated with high bicarbonate concentrations.

Adenylate cyclase; Bicarbonate; (Sperm)

1. INTRODUCTION

The motility of ejaculated bull and human sperm was recently shown to be enhanced by the addition of bicarbonate to spermatozoa [1]. Furthermore, low levels of bicarbonate in human semen have been shown to be associated with poor motility [2], and 3′,5′-cyclic AMP (cAMP) levels in guinea pig sperm were shown to increase upon addition of bicarbonate. It was argued that the effect of bicarbonate is related to calcium transport or to the facilitation of Ca^{2+} binding to physiologically relevant sites [3]. Thus, elevation of endogenous cAMP could be associated with enhanced sperm motility. Subsequent studies showed that membrane-bound adenylate cyclase (AC) in sperm membrane preparations of various species could also be stimulated by the addition of bicarbonate [1,4]. It was suggested that AC activity may be affected directly, by bicarbonate, but indirect effects by Ca^{2+} and/or other membrane components have not been ruled out.

We have recently solubilized and partially purified bull sperm AC [5,6] and studied the effect of bicarbonate on its activation. In this study, we show that such enzyme preparations can be stimulated by bicarbonate even when resolved from the membrane, thus suggesting a direct regulatory effect of bicarbonate on the enzyme itself.

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2. MATERIALS AND METHODS

Frozen ejaculated bull sperm pellets were obtained from the Israel Artificial Insemination Center at Hafetz Haim. Adenylate cyclase assay reagents were described [7]. All other reagents used were of analytical grade.

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2.1. *Adenylate cyclase assay*

Adenylate cyclase activity was determined in a final volume of 50 μl which contained 25 mM Tris-acetate, pH 7.6, 10 mM $MgCl_2$ (MgAC) or 10 mM $MnCl_2$ (MnAC), 0.5 mM [α-^{32}P]ATP (2.5–6.5 × 10^6 cpm/assay), 0.1 mM isobutylmethylxanthine (IBMX), 0.5 mg/ml bovine serum albumin (BSA), test substance and enzyme. The reaction was initiated by the addition of enzyme (50–100 units MgAC and 1000–2000 units MnAC) and incubation at 37 or 30°C was for 40 or 10 min assaying MgAC or MnAC activity, respectively. The reaction was terminated and [^{32}P]cAMP quantitated as described [7]. Assays were performed in duplicate or triplicate.

One unit of AC is defined as the formation of 1 pmol cAMP/min under standard incubation conditions.

2.2. *Solubilization and partial purification*

Adenylate cyclase was solubilized from bull sperm membranes using 400 mM NaCl and 0.2% Triton X-100. A partially purified AC preparation was obtained by sequential chromatography on DE-52 DEAE cellulose, lens cullinaris hemagglutinin and phenyl-Sepharose as described [5,6]. Using this procedure enzyme activity was purified 2000–4000-fold with 3% yield. Purification is expressed relative to calmodulin content.

Calmodulin was determined by its ability to stimulate *B. pertussis* AC [8].

3. RESULTS

3.1. *Stimulation of adenylate cyclase activity by $NaHCO_3$*

When the activity of partially purified adenylate cyclase from bull sperm membranes was determined at increasing concentrations of $NaHCO_3$, a significant and dose-dependent stimulation of AC activity was observed. Fig.1 depicts the effects of increasing $NaHCO_3$ concentrations on AC activity as compared to an identical range of increasing NaCl concentrations. In part A, MnAC was measured and the degree of stimulation reached 2-fold at 10 mM $NaHCO_3$ whereas at concentrations which exceeded 30 mM, a decrease of AC activity was observed. In the presence of NaCl, which served as control, AC activity remained constant throughout the concentration range tested.

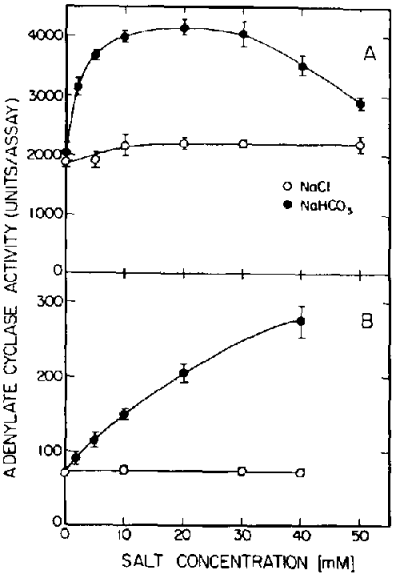

Fig.1. Effect of increasing $NaHCO_3$ concentration on activity of partially purified sperm AC. 950 units MnAC activity (A) and 75 units MgAC activity (B) were assayed in the presence of the indicated concentrations of $NaHCO_3$ (●—●) or NaCl (○—○) as control. Data are expressed as mean ± SE of triplicate determinations. All other details are as described in section 2.

When MgAC activity (part B) was assayed in the presence of the same concentrations of bicarbonate and NaCl, a 4-fold increase in enzyme activity was observed at 40 mM $NaHCO_3$ (the highest concentration tested), while no change in activity occurred when NaCl was included in the assay mixture.

The kinetic basis of this phenomenon was further explored by determination of MnAC activity at increasing concentrations (0.2–10 mM) of MnATP, keeping free $MnCl_2$ concentration at 10 mM (fig.2). It was found that the maximal velocity (V_{max}) of MnAC increased 2.4-fold from 2300 units in the absence of bicarbonate (20 mM NaCl) to 5500 units in the presence of 20 mM $NaHCO_3$. In contrast, the K_m values for MnATP remained unchanged 0.55 and 0.57 mM, respectively. In similar experiments we have tested the effect of 40 mM $NaHCO_3$ on MgAC activity in the range 0.2–10 mM MgATP with 10 mM free $MgCl_2$. Although we obtained significant stimulation of enzyme activity (6-fold), MgAC activity increased linearly with substrate concentration but

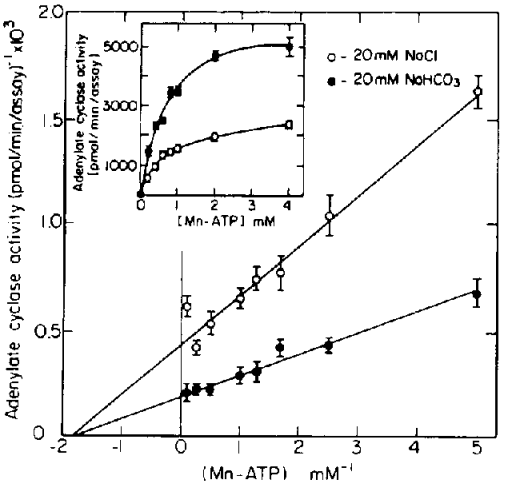

Fig.2. Effect of $NaHCO_3$ on the activity of partially purified adenylate cyclase from bull sperm at varying Mn-ATP concentrations. MnAC activity was determined at varying Mn-ATP concentrations as indicated (10 mM free Mn^{2+}) in the presence of 20 mM $NaHCO_3$ (●—●) or 20 mM NaCl (○—○) as control. MnAC activity as a function of Mn-ATP concentration (inset) or its double reciprocal are presented. Data are expressed as mean ± SE of triplicate determinations. All other details are as described in section 2.

Table 1

The effects of various sodium salts on MgAC activity

Addition	MgAC activity (%) ± SE (n = 3)
NaCl	100
$NaHCO_3$	347 ± 19
$NaNO_3$	156 ± 8
NaCOOH	107 ± 5
$NaHSO_4$	63 ± 2
$Na_2B_4O_7$	42 ± 3
$NaHSO_3$	19 ± 2

86 units of MgAC were assayed in the presence of 20 mM of the indicated salts. Tris-acetate buffer concentration was kept at 45 mM, pH 7.6. 100% activity was defined in the presence of 20 mM NaCl. All other details were as described in section 2

saturation was not reached. Consequently, K_m and V_{max} could not be determined (not shown).

We next compared the stimulatory effects of $NaHCO_3$ on MgAC activity with that of other anions (table 1). MgAC activity in the presence of 20 mM NaCl was defined as 100% (control). All anions (as sodium salts) were used at final concen-

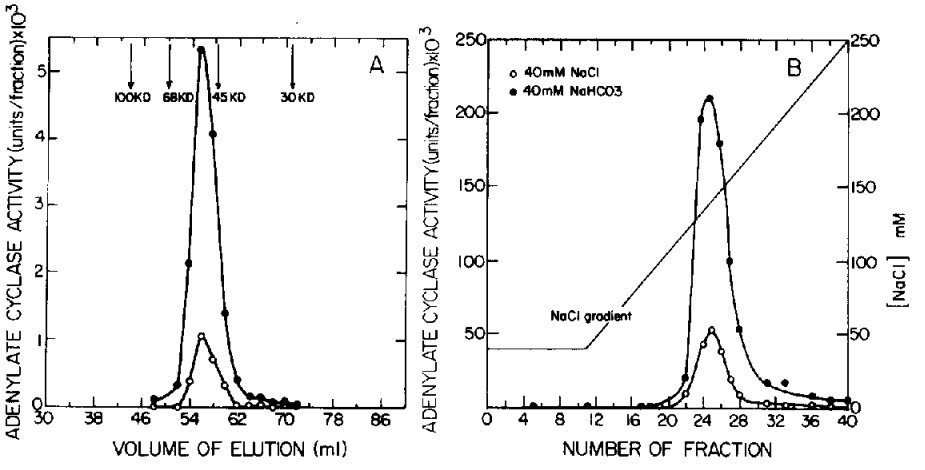

Fig.3. (A) Gel filtration of bull sperm AC. 4095 units of MgAC in 0.7 ml of no.25 from the DEAE column (B) were loaded onto a Bio-Gel P-100 column (1.8 × 80 cm). Elution at 4°C (1 ml/6 min) was in 50 mM Tris-acetate, pH 7.6, 100 mM NaCl, 1 mM DTT, 1 mM $NaHCO_3$ and 0.02% NaN_3 collecting 2 ml fractions. MgAC activity was assayed in the presence of 40 mM $NaHCO_3$ (●—●) or in the presence of added 40 mM NaCl (○—○) as control. All other details were described in section 2. (B) Fractionation of sperm AC on DEAE-cellulose. 232000 units of MgAC were loaded onto a DEAE cellulose (DE-52) column (1 × 18 cm) in 40 mM NaCl buffer and further eluted (3.5 ml/fraction) with linear NaCl gradient (40–250 mM). MgAC activity was assayed (30 μl samples/assay) in the presence of 40 mM $NaHCO_3$ (●—●) or control (○—○) in which assays were supplemented with 40 mM NaCl instead.

trations of 20 mM and the Tris buffer concentration in the assay was increased to 45 mM to keep the pH constant at 7.6. As can be seen, out of the sodium salts tested, only $NaNO_3$, apart from $NaHCO_3$ stimulated AC (1.5-fold) while sodium formate was essentially without effect. In contrast, the other anions tested were found to inhibit MgAC activity increasingly in the order $NaHSO_4 < Na_2B_4O_7 < NaHSO_3$.

3.2. *Distribution of AC coincides with its stimulation by bicarbonate in two chromatographic procedures*

In order to examine the possibility that bicarbonate acts by direct association with AC we tested for the distribution of enzyme activity and its ability to be stimulated by bicarbonate following ion-exchange chromatography on DEAE-cellulose (DE-52) and gel filtration on Bio-Gel P-100.

Solubilized AC was diluted 10-fold to reduce NaCl concentrations to 40 mM. Following absorption of enzyme to DEAE-cellulose, activity was selectively eluted using an NaCl gradient (40–250 mM). MgAC eluted as a single peak at 150 mM NaCl (fig.3B). As can be seen, the elution profiles of enzyme activity assayed in the absence or presence of 40 mM bicarbonate coincided precisely. The average degree of stimulation throughout the peak was 4-fold. We next subjected the DEAE peak fraction (no.25) to gel filtration (fig.3A). In full agreement with the results of fig.3B the elution profiles of MgAC activity as assayed in the absence or presence of 40 mM bicarbonate, eluted coincidentally as a single protein with an apparent M_r of 49000. Enzyme activity was stimulated by bicarbonate by an average of 6-fold throughout this peak.

4. DISCUSSION

Presently, the mode of regulation of adenylate cyclase in sperm is obscure. It was, therefore, of interest to examine whether the basis for the elevation of cAMP levels in intact sperm [1], or stimulation of AC in sperm membrane preparations [3,4] by $NaHCO_3$, is intrinsic to sperm AC. In order to eliminate any indirect effects of $NaHCO_3$ on sperm AC which may be mediated by a host of membrane associated phenomena [3], we tested the effects of bicarbonate on soluble preparations of this enzyme. We were surprised to find that throughout advanced stages of purification this enzyme retains the ability to be significantly (figs 1–3) and selectively (table 1) stimulated by bicarbonate. Although MgAC activity comprises only 2–4% of MnAC activity the effect of bicarbonate is seen under both assay conditions, yet the degree of stimulation of MgAC (3–6-fold) is greater than that observed for MnAC (2–3-fold stimulation). In view of these results, the lack of stimulation of MnAC in sperm membrane preparations derived from guinea pig, as reported by Okamura and Sugita [4], may result from an intrinsic insensitivity of the membrane-bound form of MnAC to $NaHCO_3$. Alternatively, free Mn^{2+} may also precipitate the form of $MnCO_3$ (solubility product constant = 2.5×10^{-7} M) at $NaHCO_3$ concentrations >30 mM, as we have noticed. This situation might lead to limiting Mn^{2+} concentrations and hence to a reduction in MnAC activity (fig.1A). Thus, stimulation of MnAC by $NaHCO_3$ may only be seen under conditions where free Mn^{2+} concentrations are not rate limiting. The kinetic basis for the effect of $NaHCO_3$ on sperm AC seems to involve an increase in V_{max} without any effect on the K_m for MnATP as demonstrated with MnAC (fig.2). In a parallel experiment performed with MgAC a similar analysis could not be performed since enzyme activity did not saturate even at concentrations of up to 10 mM MgATP. Yet, enzyme velocity was stimulated 6-fold (not shown). It is proposed that bicarbonate ions interact directly with the catalyst since responsiveness of AC to stimulation by these ions coeluted precisely and homogenously throughout both ion-exchange and gel filtration chromatographic steps (fig.3). However, the possibility that a putative $NaHCO_3$ metal-ATP complex may increase catalytic efficiency cannot be ruled out at this point. The molecular mass of this enzyme preparation from bull sperm, 49 kDa (fig.3A), is slightly higher than that reported for ram sperm, 38 kDa (gel filtration) or 46 kDa (sucrose gradient) [9,10]. Species differences and the fact that the ram enzyme was proteolytically solubilized may account for these deviations. The effect of bicarbonate appears to be specific and is not seen with several other sodium salts, as has also been reported by others, using membrane-bound sperm AC [1]. Bicarbonate in

solution has been described to form infinite chains of composition $(HCO_3)_n$, which are laterally linked by Na^+. Furthermore, the planar equilateral structures of bicarbonate and NO_3^- have been suggested to be geometrically similar and different from the pyramidal PO_3^{3-}, SO_3^{2-} and ClO_3^- of the elements of the second short period [11]. It is thus possible that stimulation of solubilized sperm AC by $NaHCO_3$ and $NaNO_3$ (table 1) relates to their common ability to form semi-stable structures in solution. These may specifically interact with the enzyme and lead to increased catalytic rate. The stimulatory effect of the $NaHCO_3$ seems to be unique to sperm AC since under identical assay conditions, the addition of $NaHCO_3$ did not stimulate AC in turkey erythrocyte membranes assayed in the absence or presence of isoproterenol, or $GTP\gamma S$ (not shown). Erythrocytes are actively handling bicarbonate as part of their metabolic function [12] but similar negative results concerning AC stimulation by $NaHCO_3$ have also been reported for other tissues [1]. The significance of these findings and the possible role of bicarbonate in sperm physiology is also compatible with reports that $NaHCO_3$ is present in high concentrations in cauda epididymal fluids (4 mM) and in semen (20 mM). In the absence of $NaHCO_3$, sperm becomes immotile. Furthermore, the addition of $NaHCO_3$ to bicarbonate-depleted semen leads to increased sperm motility [2]. Interestingly, these findings are also consistent with high concentrations of $NaHCO_3$ (30 mM) in the vagina [13,14].

Finally, numerous studies have demonstrated a positive correlation between cAMP levels and sperm motility. Treatment of semen with methylxanthine derivatives which is often used clinically, leads to increased levels of cAMP in sperm presumably due to inhibition of cyclic nucleotide phosphodiesterase [15–17]. In contrast to this mechanism (cyclic nucleotide phosphodiesterase inhibition) the results of this study strongly support the view that by penetrating sperm [3] bicarbonate ions elevate intraspermial cAMP levels and enhance sperm motility by directly stimulating endogenous AC activity. Thus bicarbonate is suggested to be a physiological allosteric regulator of AC. The exact molecular mechanism by which such regulation is achieved remains yet to be explored.

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