# SPECIFIC EFFECTS OF SULFATE ION ON VASOPRESSIN-SENSITIVE ADENYLATE CYCLASE : A REEVALUATION OF THE MAGNESIUM REGULATORY ROLE[≠]

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Summary : Substitution of Tris-$H_2SO_4$ buffer for Tris-HCl buffer in the incubation medium of pig kidney plasma membranes led to a decrease in basal adenylate cyclase activity and induced a positive cooperativity towards ATP. These two effects of sulfate ions could not be overcome by the addition of GTP(0.03 mM) or NaCl(500 mM).

The adenylate cyclase activity as a function of magnesium concentration was highly dependent on the anion which accompanied magnesium. Taking into account the relative effects of $Cl^-$ and $SO_4^{2-}$ ions on the velocities of basal and vasopressin-sensitive enzyme activities, the regulatory role of magnesium was reevaluated.

Addition of various salts to the incubation medium of adenylate cyclases from various sources was found to modify their enzyme activity (1-3). Dual effects of salts were usually observed : stimulation of enzyme activity at low concentrations and inhibition at high concentrations. The stimulatory effects of salts was attributed to the anionic species. Among the anions tested, $Cl^-$ was one of the more efficient. Usually, large amounts of $Cl^-$ ions are introduced in the adenylate cyclase incubation medium together with the buffer system used (Tris-HCl). The existence of a stimulatory effect of $Cl^-$ ions might interfere with the action of adenylate cyclase effectors such as hormone (1,3), nucleotides (3) and magnesium. Thus it might be of interest to substitute less potent ions for $Cl^-$. The present paper describes the effects of sulfate ions on pig kidney adenylate cyclase. The data obtained may provide explanations for the conflicting data of the literature on the cooperative behavior of adenylate cyclase towards ATP. They also led to a reevaluation of the regulatory role of $Mg^{2+}$ ions on the vasopressin-sensitive adenylate cyclase.

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Materials and Methods : Sources of all materials were specified in an earlier report(4). Pig kidney medulla plasma membranes were prepared as previously described (4). (8-lysine) vasopressin (vasopressin) was obtained from Sandoz (Basle). Adenylate cyclase activity was assayed by measuring the conversion of[$\alpha$-$^{32}$P] ATP into labeled cyclic AMP. Incubations were performed at 30°. The incubation medium (final volume 50$\mu$l) contained :Tris buffer,pH 8.00, 100 mM; cAMP, 1 mM; creatine kinase, 1mg/ml; creatine phosphate, 10 mM. $Mg^{2+}$ and ATP (0.5-1$\mu$Ci) concentrations are indicated in the legend to Figs. When present vasopressin was added at a concentration eliciting a maximum response.Adenylate cyclase was initiated by the addition of membranes(50-100$\mu$g).Cyclic AMP production was found to be linear over the incubation periods tested (5-20 min).The reaction was stopped as already described (5). Cyclic [$^{32}$P] AMP was separated from labeld ATP according to Salomon et al (6). Protein content was estimated as described by Lowry et al (7). All results are expressed in pmoles cAMP formed/5 min/mg protein. The concentration of ATP present at the end of the incubation was determined after separation of labeled ATP from other labeled nucleotides by TLC on PEI-cellulose plates using the solvent : $CH_3COOH$,M; LiCl,4 M (8:2, v/v).

Results : As shown in Fig 1 (left part) substitution of Tris-$H_2SO_4$ buffer for Tris-HCl buffer in the incubation medium resulted in a change in the kinetic behavior of adenylate cyclase as a function of ATP concentration. When using Tris-HCl, the enzyme behavior towards ATP obeyed classical Michaelis-Menten kinetics. However, when using Tris-$H_2SO_4$ , or when increasing the $Na_2SO_4$ concentration in the presence of Tris-HCl (Table 1) the Hill coefficient for ATP of the adenylate cyclase system was greater than unity. The sulfate effect could be detected with sulfate concentrations above 5 mM and was not further increased with higher concentrations. Sulfate-induced cooperative behavior of adenylate cyclase was not altered under maximal stimulation by vasopressin (Table 1). Furthermore the presence of GTP in the incubation medium did not modify the kinetic behavior of adenylate cyclase as a function of ATP concentration when assayed in either Tris-HCl or Tris-$H_2SO_4$ buffer (Fig 1, right part). The observed effects of substituting Tris-$H_2SO_4$ buffer for Tris-HCl buffer cannot be accounted for by a reduction of $Cl^-$ concentration in the incubation medium.These effects are not suppressed by the addition of 300 mM NaCl.

It was checked that the cooperative behavior of adenylate cyclase assayed in the presence of sulfate was not not due to a sulfate-induced decrease in the efficiency of the ATP regenerating system. This might have led to a reduction in the effective substrate concentration, especi-

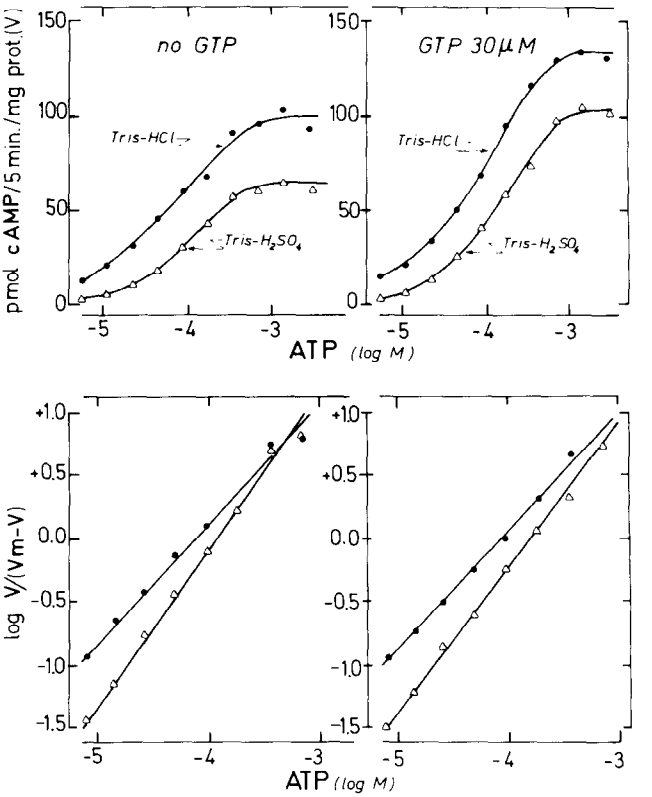


Figure 1 : Adenylate cyclase activity as a function of ATP concentration. Basal adenylate cyclase activity was measured in the presence of 100 mM Tris buffer pH 8.0 either Tris-HCl (●) or Tris-$H_2SO_4$ (▽). The $Mg^{2+}$ concentration added as $MgCl_2$ was 4 mM. The Hill representations of the velocity (v) -concentration relationship are set out in the lower panels. The value ± SD of the Hill coefficients were the following : 1.00 ± 0.02 (Tris-HCl); 1.30 ± 0.05 (Tris-$H_2SO_4$); 0.97 ± 0.03 (Tris-HCl + GTP) and 1.32 ± 0.05 (Tris-$H_2SO_4$ + GTP). The values measured in the presence of Tris-$H_2SO_4$ were statistically different from control values ($p < 0.001$). Note that the concentration of ATP leading to 50% of the maximum velocity is higher in the presence of Tris-$H_2SO_4$.

ally when small amounts of ATP were used. In fact for the entire range of ATP concentrations tested, the concentration of labeled ATP at the end of the incubation period was not dependent on the nature of the buffer used. In the presence of Tris-HCl buffer the amounts of ATP remaining at the end of the incubation period were 59% , 72% and 86% of the initial values for 0.024 mM, 0.13 mM and 1 mM ATP respectively. The corresponding values measured in the presence of Tris-$H_2SO_4$ buffer were 51%, 74% and 86%.

Table I : Sulfate-induced cooperativity of renal adenylate cyclase towards ATP.

Adenylate cyclase activity was measured as a function of ATP concentration both under the basal condition and in the presence of vasopressin (2 $\mu$M). (For experimental details, see Methods and legend to Fig 1). Values in the table are Hill coefficient of the velocity-substrate concentration curves. Each value is accompanied by the SD value of the calculated linear regression line. They were compared to unity using the Student t test. Numbers between brackets refer to the same experiment. ** significantly different from unity ($p<0.001$).

| Buffer (100 mM) | salt added | Hill coefficient for ATP | |
|---|---|---|---|
| | | basal | vasopressin |
| Tris-HCl | — | 1.00 ± 0.02 (1) | 0.99 ± 0.02 (2) |
| " | $Na_2SO_4$ , 1 mM | 1.00 ± 0.04 (1) | 1.02 ± 0.06 (2) |
| " | $Na_2SO_4$, 5 mM | 1.32 ± 0.06** (1) | 1.02 ± 0.06 (2) |
| " | $Na_2SO_4$ , 25 mM | 1.33 ± 0.03** (1) | 1.64 ± 0.02** (2) |
| " | $Na_2SO_4$ , 50 mM | 1.36 ± 0.03** (1) | 1.53 ± 0.03** (2) |
| Tris-$H_2SO_4$ | — | 1.77 ± 0.08** (3) | 1.57 ± 0.03** (3) |
| " | NaCl , 300 mM | 1.55 ± 0.05** (3) | 1.69 ± 0.08** (3) |

As already apparent from the data presented in Fig 1 , the maximal velocity of the adenylate cyclase reaction measured under basal condition or in the presence of GTP was lowered when using Tris-$H_2SO_4$ buffer. The buffer substitution was without significant effect on the maximal velocity of vasopressin-stimulated enzyme. As a consequence the stimulation ratio (vasopressin-stimulated/basal activities) was higher in the presence of $SO_4^{2-}$ As shown by Fig 2 the inhibition of basal adenylate cyclase activity by sulfate ions was not overcome by increasing amounts of $Cl^-$ ions added as NaCl. However the relative stimulatory effect of NaCl was unaffected by the presence of sulfate. The effect of NaCl on vasopressin-stimulated adenylate cyclase activity was not dependent of the buffer used (Fig 2).

From the above described results it can be concluded that $SO_4^{2-}$ ions exert two specific effects on pig kidney adenylate cyclase: 1/ sulfate induces a cooperative behavior of the enzyme as a function of ATP concentration and 2/ it decreases the enzyme activity measured under basal conditions or in the presence of GTP but did not affect the vasopressin-stimulated

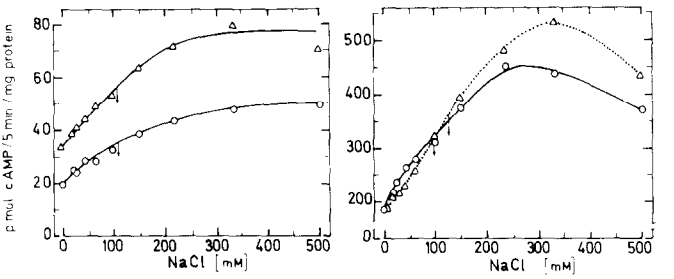


Figure 2 : Basal and vasopressin-stimulated adenylate cyclase activities as a function of NaCl concentration.
Incubation medium was buffered with either Tris-HCl (∇) or with Tris-$H_2SO_4$ (0). $MgCl_2$ concentration was 1 mM. ATP concentration was 0.25 mM. Arrows on curves indicate the concentration of NaCl leading to a 50% increase in enzyme activity. Left panel : basal activity ; right panel : vasopressin (1 μM) stimulated activity.

activity.

Such specific anionic effects can complicate the interpretation of cation effects in particular those of $Mg^{2+}$. The data presented in Fig 3 clearly show that the apparent effect of $Mg^{2+}$ on basal activity was different depending on wether $Mg^{2+}$ was added as a chloride or as a sulfate salt while the apparent effect on vasopressin-stimulated activity was independent of the accompanying anion. The hormonal effect expressed in terms of activation ratio remained almost constant in presence of $Mg^{2+}$ added as $MgSO_4$; it fell from 10 to 3 when increasing the $MgCl_2$ concentration from 1 to 50 mM.

## DISCUSSION

The above described results deserve special comments about the regulatory role of $Mg^{2+}$ ions on the adenylate cyclase system and on the kinetic behavior of the enzyme as a function of ATP concentration. $Mg^{2+}$ is a necessary cofactor for the adenylate cyclase reaction (for review, see 8). It is generally accepted that the Mg-ATP complex is the substate of the enzyme (9-11). On several adenylate cyclase system, it was observed that increasing the $Mg^{2+}$ concentration above that needed to complex ATP present led to a further increase in enzyme activity (9, 12, 13). The existence of a $Mg^{2+}$ regulatory site was therefore suggested. Furthermore, it was assumed that the affinity of $Mg^{2+}$ might be modulated by the addition of

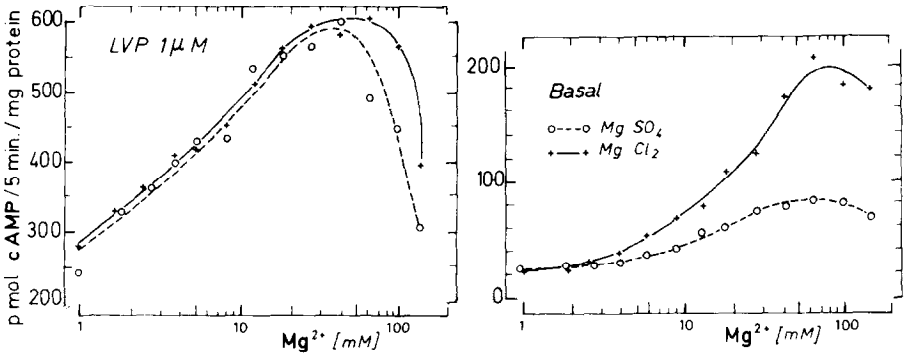


Figure 3 : Basal and vasopressin-stimulated adenylate cyclase activities as a function of $MgCl_2$ or $MgSO_4$ concentrations.
Assay medium was buffered with 100 mM Tris-HCl. ATP concentration was 0.25 mM. Magnesium was added as sulfate salt (0- - -0) or as chloride salt (+—— +).

specific regulatory hormones (12, 14, 15). The latter conclusion was mainly derived from the observation that basal and hormone-stimulated adenylate cyclase activities followed different evolution pattern as a function of $Mg^{2+}$ ions in the incubation medium. At least in the case of the vasopressin sensitive adenylate cyclase from the pig kidney, it is clear that these different evolution patterns for basal and vasopressin-sensitive activities were highly dependent on the $Mg^{2+}$ accompanying anion (Fig 3). The existence of interactions between anionic effects and $Mg^{2+}$ effect(s) makes it difficult to unequivocally define the precise role of $Mg^{2+}$ in the adenylate cyclase reaction.
change as a function of ATP concentration.

At present it is not possible to define the site of action of anions. Basal adenylate cyclase activity and NaF- and vasopressin-sensitive activities are equally sensitive to sulfate when its effect was judged by a modification of cooperativity as a function of ATP concentration. Thus it is conceivable that the anion site of action is on the adenylate cyclase catalytic unit. This interpretation is further confirmed by the observation that detergent solubilized adenylate cyclase is also sensitive to sulfate (G. Guillon; unpublished observation). However the possibility that sulfate might interact with some other component(s) present in the membrane preparation cannot be excluded.

Depending on the adenylate cyclase system studied, the evolution of the enzyme activity as a function of ATP concentration exhibits either Michaelis-Menten kinetics (9, 16-18) or negative cooperativity (11, 14, 19). It is important to note that depending on the ATP regenerating system used the same adenylate cyclase system may exhibit as a function of ATP concentration these two types of behavior (13). Most probably, albeit rarely mentioned by authors, when pyruvate kinase was used as regenerating system , the enzyme was not desalted before use. The enzyme being stored in ammonium sulfate at least 2 M, the amount of sulfate brought to the incubation medium may vary from 5 to 35 mM depending on the enzyme concentration used. Such sulfate concentrations are sufficient to induce a cooperativity

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