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SPECIFIC Mg^{2+} AND ADENOSINE SITES INVOLVED IN A BIREACTANT MECHANISM FOR ADENYLATE CYCLASE INHIBITION AND THEIR PROBABLE LOCALIZATION ON THIS ENZYME'S CATALYTIC COMPONENT*

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Summary : The specificity of adenosine sites involved in adenylate cyclase inhibition (P sites) is identical on membrane-bound and on solubilized enzyme. Kinetic analysis indicates that in addition to a low affinity Mg^{2+} site involved in adenylate cyclase stimulation (Km = 10 mM), there is a high affinity Mg^{2+} site (Km = 2.10^{-4}M) involved together with P sites in a bireactant mechanism for triggering adenylate cyclase inhibition. Guanyl nucleotide-binding protein does not seem to be implicated in this inhibition. We were not able to separate the catalytic component of adenylate cyclase from P sites, either on a sucrose density gradient or in gel filtration experiments. It is suggested that P sites are located on the catalytic component of the enzyme.

Londos et al (1) showed that adenylate cyclase was regulated by two adenosine receptor sites with different stereospecificities : R sites, which interact with adenylate cyclase like other hormonal receptors, i.e. they are localized on extracellular membrane and their coupling with the catalytic component (C) of the enzyme requires GTP and is suppressed by solubilization (2). R sites are localized in a limited number of tissues and trigger either stimulation or inhibition of the enzyme (1). The second type of receptor sites are P sites which have been shown to be present in all mammalian tissue so far tested, and always inhibit adenylate cyclase(1). Several authors have noted that the inhibition triggered by P sites is increased by Mg^{2+} and is present in soluble membrane extracts (1,3). The aim of this work was 1) to compare the specificity of P sites located on membrane and on soluble extract ; 2) to analyze in detail the characteristics of Mg^{2+} and adenosine interaction, and 3) to attempt physical separation of P sites from the catalytic component of adenylate cyclase.

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Enzyme : adenylate cyclase or ATP pyrophosphate-lyase (cyclizing) (EC 4.6.1.1.)

Abbreviation used : Gpp(NH)p = guanylylimidodiphosphate

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MATERIALS AND METHODS - Enzyme sources : a) rat brain. Homogenates from rat striatum and cerebral cortex were prepared as previously described (4). When specified, the adenylate cyclase in the homogenate was solubilized with either triton X 100 (0.3 %, v/v) or cholate (0.9 % w/v) in 2 mM Tris maleate (pH 7.2) and 2 mM EGTA ; 100 mg fresh homogenized tissue was solubilized in 2 ml of the above solution. The solubilized homogenate was centrifuged for 30 min at 100 000 X G. The supernatant was a soluble extract. b) Enzyme from pig kidney medulla . Pig kidney membranes were prepared as already described (5) and solubilized (Triton X 100 0.7 % v/v) either directly, or after 30 min preincubation at 30°C in the presence of 10^{-5}M Gpp(NH)p (6). Adenylate cyclase assays : The composition of the incubation mediums for enzymes prepared from rat brain and pig kidney has been described (2,5). Sucrose density gradient for ultracentrifugation of soluble extracts. A linear gradient (6.2 ml) was prepared from 3 and 15 % sucrose solutions buffered with 100 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 % (v/v) triton X 100, and 0.1 % (v/v) β -mercapto ethanol. 300 µl aliquots of solubilized extract were layered on the top of the gradient together with protein markers. Centrifugation was allowed to proceed for 7 H at 41,000 rpm at 4°C (rotor SW 41 Ti). 200 µl fractions were then collected from the gradient. Gel filtration of solubilized material. 1 ml of solubilized extract was layered together with protein markers on the top of an ultrogel ACA_{34} column (1.6 X 40 cm) equilibrated with an elution buffer identical to the one used for gradient, except for the sucrose concentration (75 mM). Flow rate was 10 ml per hour and fraction volume, equal to 1 ml. Calibration proteins. The protein markers used for the gradient and column calibration were : cytochrome C, alcohol deshydrogenase, catalase and β-galactosidase. For assay conditions and hydrodynamic parameters see reference 6.

RESULTS AND DISCUSSION : As in other systems, adenosine and several of its analogs inhibited membrane-bound and soluble adenylate cyclase from striatum or cerebral cortex by reducing maximal reaction velocity without changing affinity for the substrate ATP - Mg^{2+}. We checked that under steady state conditions, adenosine inhibition was maximal and after addition of 0.4 IU/ml adenosine-deaminase, completely reversed in less than 1 min.

Specificity of the adenosine P site involved in the inhibition of membrane-bound and soluble adenylate cyclase from rat striatum. Adenylate cyclase inhibition kinetics were hyperbolic when measured as the function of adenosine concentrations. The double reciprocal plots of these kinetics were linear (fig. 1, D) thus allowing calculation of an apparent affinity and a maximal inhibition. Table I shows that when measured at 10 mM Mg^{2+}, such inhibition varied from 55 to 70 % both on membranes and soluble enzyme. The affinities and specificity of P sites for different purine derivatives were also comparable for the two enzyme preparations (Table 1). In particular, N^6 -derivatives which act as agonists on R sites (2) were ineffective on P sites. Previous report indicated that theophylline, a good competitive inhibitor of R sites (K_I = 20 µM,(4) did not interact with P sites (1). We found that high doses of theophylline competitively reduced the inhibition produced by 5'-Deoxyadenosine with a low K_I (4.10^{-3}M) (data not shown). These results confirm that after membrane solubiliza-

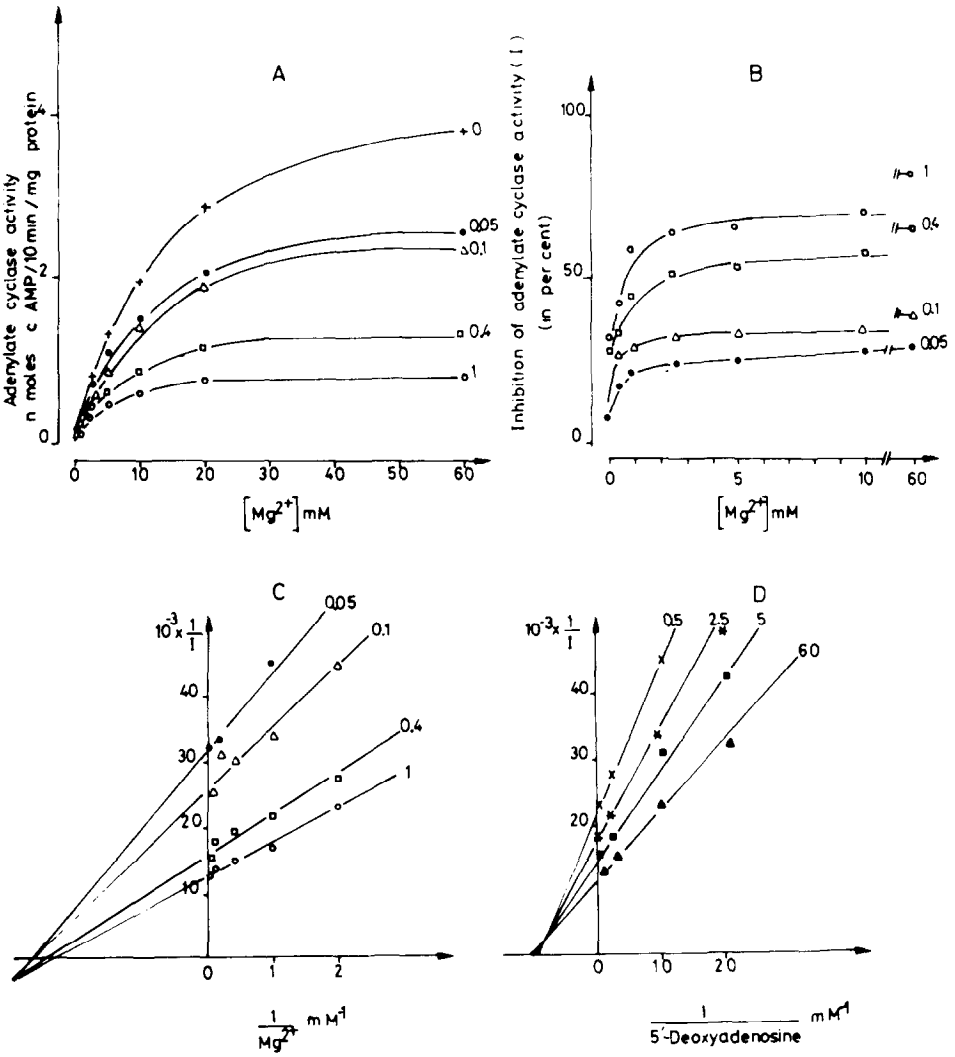

Figure 1 - Kinetics of 5'-Deoxyadenosine inhibition of soluble adenylate cyclase from rat striatum. ATP concentration was 5.10^{-5}M.
A) Adenylate cyclase activity as function of free Mg^{2+}, measured in the absence or presence of different 5'-Deoxyadenosine concentrations (indicated in mM on each curve.
B) Data are the same as in fig. 1,A. The ordinate gives the inhibition produced by 5'-Deoxyadenosine (in % of adenylate cyclase activity measured in the absence of inhibitor).
C) Double reciprocal plot of B.
D) Double reciprocal plot with respect to inhibitions and 5'-Deoxyadenosine concentrations determined at different Mg^{2+} concentrations, indicated in mM on each curve.

tion by Triton X 100, the inhibition by purine compounds ismaintained. They further indicated that stereospecificity of P sites did not alter during solubilization.

Effects of Mg^{2+} on adenylate cyclase inhibition by 5'-Deoxyadenosine. To analyze in detail the effect of Mg^{2+} on the inhibition triggered by P sites, we used rat

Table I - Specificity of P sites on membrane and soluble adenylate cyclase from rat striatum. Mg^{2+} and ATP concentrations were 10^{-2} and 5.10^{-4}M respectively. Apparent K_I ($K_I$app) and maximal inhibition were calculated as described in fig. 1 D. Non-inhibitory adenosine derivatives were tested up to 1 mM. (+) These compounds were tested in the absence of adenosine deaminase. Values are means ± SEM of three separate experiments.

	MEMBRANE BOUND		SOLUBLE	
	K_I app	maximal inhibition (%)	K_I app	maximal inhibition (%)
Adenosine (+)	70 ± 10	64 ± 5	220 ± 50	55 ± 7
2-Chloroadenosine	120 ± 35	61 ± 6	260 ± 58	56 ± 6
2'-Deoxyadenosine (+)	50 ± 11	65 ± 5	80 ± 10	60 ± 7
5'-Deoxyadenosine	70 ± 12	65 ± 4	90 ± 20	70 ± 8
Adenine	NI		NI	
Inosine	NI		NI	
N^6-Methyladenosine	NI		NI	
N^6-Dimethyladenosine	NI		NI	
N^6-Phenylisopropyladenosine	NI		NI	
S - Adenosylmethionine	NI		NI	

striatum soluble adenylate cyclase, a preparation in which stimulating R sites are uncoupled, we then confirmed the results on membrane-bound adenylate cyclase from cerebral cortex and pig kidney membranes, from which R sites are absent (2). Soluble adenylate cyclase from rat striatum was activated by free Mg^{2+} following a Michaelis-Menten reaction (Fig. 1, A). This confirms the existence of an activating Mg^{2+} site already described in many adenylate cyclase systems (7). 5'-Deoxyadenosine produced uncompetitive inhibition of the Mg^{2+} stimulation, i. e. a decrease in the Vmax and an increase in the apparent affinity for Mg^{2+} (Fig. 1, A). The apparent Km for Mg^{2+} was 10 mM in the absence of 5'-Deoxyadenosine and 9, 8, 6 and 4 mM in the presence of 0.05, 0.1, 0.4 and 1 mM 5'-Deoxyadenosine respectively (Fig. 1, A). Plotting of the inhibitions produced at different 5'-Deoxyadenosine concentrations against concentrations of free Mg^{2+} clearly showed that they were Mg^{2+} dependent (Fig. 1, B). However, the Mg^{2+} concentrations needed for half-maximal inhibition were much lower than those required for half-maximal stimulation of the adenylate cyclase (compare figs 1,A and B). This suggests that activation and inhibition of the enzyme were triggered by two different Mg^{2+}sites. The double reciprocal plots for the inhibition curves in figure 1, B, were linear and intersected at one point located on the left of the ordinate, under the abscissa (3 separate experiments) (Fig. 1 C). Thus, 5'-Deoxyadenosine slightly reduced the affinity of free Mg^{2+} for the Mg^{2+} sites

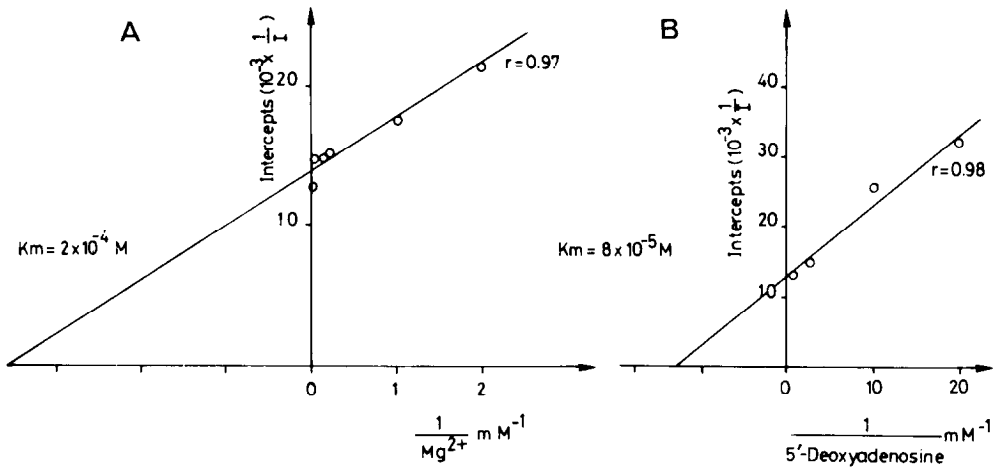

Figure 2 - Secondary plots of Figs 1, C and D.
Each line of the reciprocal plots in Figs. 1, C and D, the secondary plot shown here and the intercepts are computer-derived from least square regression analysis.

involved in the inhibition. Similarly, the double reciprocal plots with respect to the inhibitions and 5'-Deoxyadenosine concentrations obtained at different Mg^{2+} concentrations were also linear and intersected at one point to the left of the ordinate, above the abscissa (3 separate experiments) (Fig. 1, B). Consequently Mg^{2+} slightly modified the affinity of 5'-Deoxyadenosine for P sites. Replots of the intercepts with the ordinate of the plots in figures 1 C and D were also linear allowing calculation of the Mg^{2+} and 5'-Deoxyadenosine affinities for their sites (2.10^{-4} and 8.10^{-5}M respectively) (Fig. 2 A and B). This kinetic analysis confirms that in addition to the Mg^{2+} sites implicated in adenylate cyclase activation (Km = 10 mM), there is another Mg^{2+} site whose affinity is fifty times higher (Km = 2.10^{-4}M). This high-affinity Mg^{2+} site is involved together with 5'-Deoxyadenosine in a bireactant mechanism for adenylate cyclase inhibition. The mechanism seems to be general since we found it in membrane-bound adenylate cyclase from cerebral cortex and pig kidney (data not shown). While the present work was in progress, a report by Johnson et al (3) indicated that in platelet membranes, adenosine inhibits adenylate cyclase and raises the apparent affinity of the Mg^{2+} stimulatory site. However, these authors did not analyse this finding kinetically.

Attempted separation of the catalytic component C from P sites : 5'-Deoxyadenosine also inhibited soluble adenylate cyclase from pig kidney membranes (50-70 % in the presence of 5.10^{-4}M 5'-Deoxyadenosine and 10 mM Mg^{2+}). Adenylate cyclase is a multicomponent system which comprise at least three molecular entities, a catalytic component (C) a GTP binding protein (G) and in some case, an hormonal

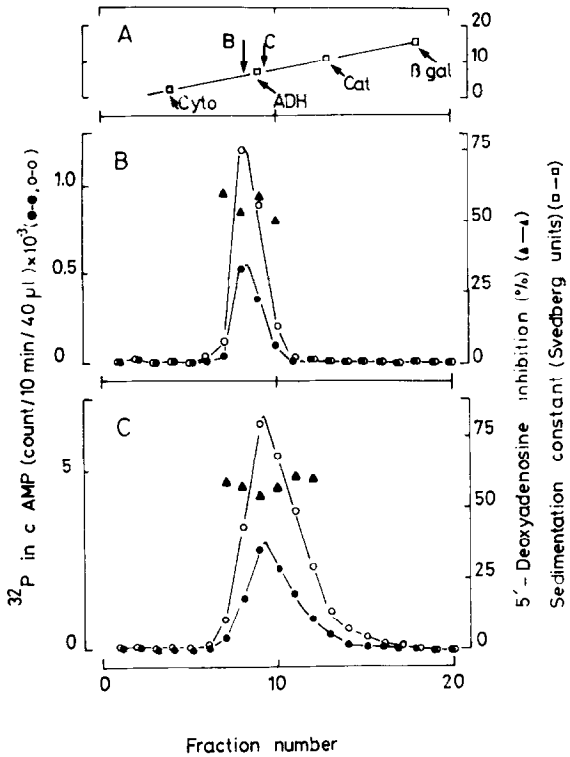

Figure 3 - Absence of separation between C and P sites on a sucrose density gradient.
Pig kidney membranes were incubated with or without Gpp(NH)p (C and B respectively) and solubilized (see methods). Soluble extracts and protein markers were layered together on the top of a sucrose density gradient. Centrifugation and gradient fractionation were carried out as described in methods. In each fraction, adenylate cyclase activity was measured in the absence (o-o) and in the presence (•-•) of 5.10^{-4}M 5'-Deoxyadenosine and enzyme inhibition was calculated (▲).
Sedimentation constant calibration curve was based on the elution pattern of cytochrome C, alcohol deshydrogenase, catalase and β-galactosidase (panel A). Apparent standard sedimentation constants for soluble adenylate cyclase prepared from Gpp(NH)p-treated or control membrane are indicated by arrows C and B.

receptor (8). The GTP binding protein allows the coupling between the receptor and C. GTP or Gpp(NH)p activate the catalytic activity of C by interacting with the GTP binding protein (8). When soluble adenylate cyclase from pig kidney membranes was resolved on a sucrose density gradient, C is no more stimulated by Gpp(NH)p and has a sedimentation constant of 6 S. Moreover, when membranes were pretreated with Gpp(NH)p before solubilization, the apparent sedimentation constant increase from 6 to 7.6 S suggesting that G remained bound to C (6). In these conditions, C remained activated. Figure 3 shows that either separated from G (panel B ; sedimentation constant 6 S) or associated with G (panel C; sedimentation constant 7.6 S) the catalytic component was always inhibited by 5'-Deoxyadenosine.

Table II- Non-requirement of GTP for 5'-Deoxyadenosine inhibition. Mg^{2+} and ATP concentrations were 10^{-2} and 5.10^{-4}M respectively. Membranes from rat striatum were centrifuged twice at 10,000 G for 30 min in hypotonic medium (2 mM EGTA, 2mM Tris-maleate pH 7.2).

	ADENYLATE CYCLASE	
	Stimulation by dopamine (10^{-4}M) (% control)	Inhibition by 5'-Deoxyadenosine (% control)
Homogenate	117	67
Washed membranes	7	64
Washed membranes + GTP 3.10^{-6}M	71	64

We eliminated GTP from rat striatum membranes by washing. The adenylate cyclase from these membranes was no longer stimulated by dopamine unless GTP was added, but was nevertheless inhibited by 5'-Deoxyadenosine (Table II). The above results indicate that GTP as well as G are not involved in adenosine inhibition. Gel filtration of soluble enzyme, prepared from pig kidney medulla or rat striatum, showed that 5'-Deoxyadenosine inhibited the included peak of enzyme activity (data not shown). Similar results were obtained when cholate (a ionic detergent) was used as the solubilizing agent for rat striatum. Thus no separation was obtained between P sites and C either on sucrose density gradient or with gel filtration, suggesting that these inhibitory sites are on the catalytic component of adenylate cyclase.

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