AN APPROACH TO THE IDENTIFICATION OF ADENOSINE'S INHIBITORY SITE ON ADENYLATE CYCLASE

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Received 25 January 1982

1. Introduction

Adenosine inhibits stimulated forms of adenylate cyclase non-competitively with respect to metal–ATP substrate [1,2] and increases Mg^{2+} ion affinity at the divalent cation binding site [2]. This inhibitory effect of adenosine is mediated by the 'P'-site* and is evident in the absence of a functional guanine nucleotide regulatory component [3]. Thus, the suggestion was made that this inhibitory site for adenosine is located on the catalytic subunit of the cyclase [3]. However, since all adenylate cyclases are not inhibited by adenosine [4,5], the participation of additional subunits remains possible. To help distinguish between these alternatives and to approach the problem of isolating the component(s) mediating adenosine inhibition, we have utilized the 'P'-site affinity of the cyclase for the partial purification of the enzyme by affinity chromatography. The results of these studies are presented in this report.

2. Materials and methods

2.1. *Enzyme preparation*

Partially purified plasma membranes were prepared from rat liver as in [6], with 5 mM EDTA in homogenizing media and sucrose solutions. The EDTA was subsequently removed by repetitive centrifugation–resuspensions prior to storage of membranes at −70°C.

Washed particulate and Lubrol-PX-dispersed adenylate cyclases were prepared from cerebellum [7]. The dispersed enzyme was concentrated about 12-fold by ultrafiltration (Amicon PM-10) and was chromatographed on DEAE-Sephadex as in [8].

2.2. *Adenylate cyclase assay*

Adenylate cyclase activity was determined [2] in a reaction mixture containing 50 mM glycylglycine (pH 7.5), 1 mM 3-isobutyl-1-methylxanthine (IBMX), 100 μM MnATP, 400–800 μM excess $MnCl_2$, [α-^{32}P]-ATP [9] (0.5–1.5 × 10^6 cpm), 2 mM purified creatine phosphate [6], creatine kinase (100 μg/ml), and myokinase (100 μg/ml) in a volume of 200 μl. Reactions were for 2 min at 37°C and were initiated by the addition of enzyme. Reactions were stopped by the $ZnCO_3$ precipitation method [10] and the labelled cAMP was purified as in [11].

3. Results

To optimize inhibition of adenylate cyclase by adenosine prior to solubilization, we thought we could take advantage of the striking sensitizing effect of hormone stimulation. Although stimulation of the platelet enzyme by prostaglandin E_1 (PGE_1) decreased the K_i from >1 mM for basal activity to ~10 μM [2], Londos and Preston [1] did not observe such a sensitization by glucagon of the liver enzyme. However, in contrast with [1], we found marked and varied effects of modulators on the enzyme's sensitivity to inhibition by the nucleoside (fig.1). As expected, basal activity

* Effects of various analogs of adenosine on cellular cAMP levels and adenylate cyclase activity have suggested the presence of distinct binding sites for adenosine. High affinity sites requiring an intact ribose moiety have been found to activate or inhibit various adenylate cyclases. These opposing effects on adenylate cyclase have led to the designation of 'R'-site subclasses, R_a and R_i [18], or A_2 and A_1 [19], for activation and inhibition, respectively. $R_i(A_1)$ sites are high affinity inhibitory receptors that are distinct from low affinity 'P'-sites. In this paper we have adopted the designation (R_a and P) of [18]

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

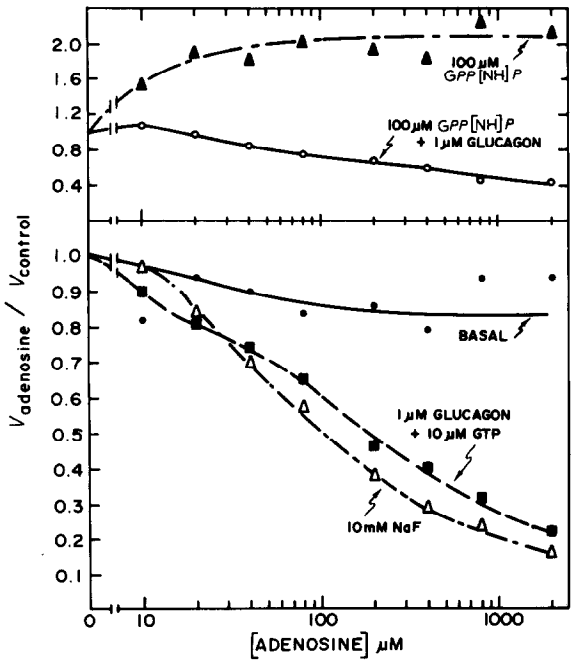

Fig.1. Effects of modulators of liver adenylate cyclase on sensitivity to inhibition by adenosine. Activity was determined with 100 μM MnATP and 400 μM $MnCl_2$. Values are ratios of cAMP accumulation with adenosine to that without adenosine.

was marginally suppressed by adenosine, with <20% inhibition observed with 2 mM adenosine. However, activity stimulated by F^- or glucagon + GTP was significantly more sensitive to adenosine inhibition, with 50% inhibition observed with 75–200 μM. The enzyme stimulated by 100 μM guanosine 5′-[β,γ-imido]triphosphate (*GPP*[NH]*P*) + glucagon was considerably less sensitive to inhibition, whereas the enzyme stimulated by *GPP*[NH]*P* alone was actually enhanced about 2-fold by adenosine (fig.1, upper panel).

In other experiments we found that GTP alone did not enhance sensitivity to inhibition by adenosine and that PGE_1, which stimulated the liver cyclase about 3-fold (not shown), caused an increase in sensitivity to inhibition equivalent to that shown in fig.1 for glucagon or F^-. At concentrations from 0.1–100 μM, cAMP did not increase sensitivity to inhibition by adenosine. Thus, the extents of stimulation by the various agents were quite different and there was no evidence for a correlation between the extent of stimulation of adenylate cyclase and its sensitivity to inhibition by adenosine.

The contrasting effect of *GPP*[NH]*P* to elicit stimulation by adenosine was curious since the conditions (1 mM IBMX) precluded stimulation through adenosine 'R_a'-sites. Not only did adenosine appear to shorten the lag phase of *GPP*[NH]*P* activation, but also a distinct transition from stimulation by adenosine to inhibition was observed as activation by *GPP*[NH]*P* proceeded (fig.2, lower panel). The general character of this developing inhibition was less a function of the adenosine concentration than of time

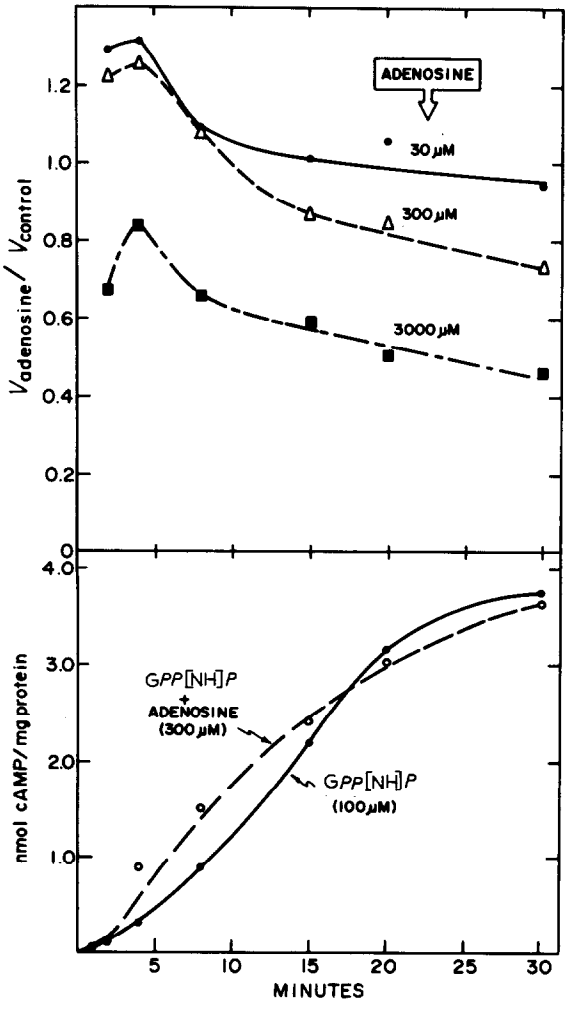

Fig.2. Time-dependent effects of *GPP*[NH]*P* on adenosine modulation of liver adenylate cyclase. Upper panel: cAMP accumulation was measured with 100 μM MgATP, 4 mM $MgCl_2$, 100 μM *GPP*[NH]*P*, and the indicated concentrations of adenosine. Values are ratios of cAMP accumulation with adenosine to that without adenosine. Lower panel: cAMP accumulation was measured with 100 μM MnATP, 400 μM $MnCl_2$, 100 μM *GPP*[NH]*P*, and 300 μM adenosine as indicated.

Table 1
Retention of adenosine inhibition following preactivation and solubilization of hepatic adenylate cyclase

Enzyme	Adenosine[b] (300 μM)	Pretreatment conditions[a]			
		NaF	*GPP*[NH]*P*	*GPP*[NH]*P* + Glucagon	*GPP*[NH]*P* + Glucagon + NaF
		pmol cAMP formed (2 min . mg protein)$^{-1}$			
After treatment (30°C for 30 min)	–	1630	1400	2250	2510
	+	432	535	754	781
	v_+/v_-	0.27	0.38	0.34	0.31
Lubrol-PX Supernate[c]	–	2680	2010	2410	3460
	+	1140	842	1140	1450
	v_+/v_-	0.43	0.42	0.47	0.42

[a] Liver plasma membranes (1.5 mg protein/ml) were pretreated for 30 min at 30°C in 700 μl containing 50 mM glycylglycine (pH 7.5), 10 mM $MgCl_2$, and 10 mM NaF, 100 μM *GPP*[NH]*P*, or 1 μM glucagon as indicated. Aliquots (20 μl) were taken for assay of adenylate cyclase as in section 2

[b] Activities were determined without or with 300 μM adenosine in the reaction mixture. v_+/v_- indicates the ratio of activity determined with adenosine (+) to that determined without adenosine (–)

[c] To 500 μl aliquots of membranes pretreated as under (a) was added 1 ml of 1% (w/v) Lubrol-PX. The mixtures were homogenized with 10 passes of a Teflon-plastic homogenizer and were then centrifuged at 29 000 × *g*, r_{av}, for 30 min. Supernatant fractions were then assayed for adenylate cyclase

and therefore was a characteristic of *GPP*[NH]*P* action (fig.2, upper panel).

Since stimulation of adenylate cyclase by F^- or *GPP*[NH]*P* results in increased sensitivity to inhibition by adenosine, and since pretreatment with these agents also leads to stable activation of the enzyme, it was necessary to ascertain that inhibition occurred after preactivation of the cyclase and also after its dispersion by detergent. Adenosine inhibited liver adenylate cyclase that had been preactivated with Mg^{2+} and F^-, or with *GPP*[NH]*P*, without or with glucagon (table 1). When the preactivated enzyme was dispersed into a 29 000 × *g* supernatant fraction, it retained sensitivity to inhibition by adenosine. Preincubation with the combination of agents (Mg^{2+}, F^-, *GPP*[NH]*P*, glucagon) always led to the greatest activity, which was also reflected in the activity seen after solubilization.

We observed a similar inhibition by adenosine of the detergent-dispersed enzyme from rat brain (table 2). The brain cyclase is principally a calmodulin–Ca^{2+}-regulated enzyme which can be made deficient in calmodulin and functionally deficient in the guanine nucleotide regulatory component by chromatography of the dispersed enzyme on DEAE-Sephadex in the presence of chelator [8,12]. While an enzyme treated in this manner is an essentially Mn^{2+}-dependent enzyme, unresponsive to F^- or *GPP*[NH]*P* [12,13], we found that it retained sensitivity to inhibition by adenosine (table 2). Thus, preactivated and/or dispersed adenylate cyclases from either brain or liver are inhibited by adenosine.

These and earlier [3] studies suggest the possibility that adenosine 'P'-site inhibition may be a direct effect on the catalytic subunit of the cyclase, whereas the

Table 2
Effect of adenosine on soluble enzyme from rat brain

Additions	Control	300 μM Adenosine	Average v_{ado}/v_{cont}
	nmol cAMP (10 min . mg protein)$^{-1}$		
None	31.2, 44.4[a]	13.8, 18.6	0.43
NaF (10 mM)	32.7, 44.1	13.5, 20.5	0.44
GPP[NH]*P* (100 μM)	25.7, 37.9	13.2, 18.4	0.50

[a] Activity was determined with 100 μM MnATP, 800 μM $MnCl_2$, 0.5 mg bovine serum albumin per ml, 3 mM dithiothreitol, and otherwise as in section 2. Values are from two different enzyme preparations, in different experiments, each assayed in triplicate

Table 3
Partial purification of hepatic adenylate cyclase on adenosine agarose

Enzyme	Protein	Adenylate cyclase activity[a]	
		Total	Specific
	mg	nmol/min	nmol (min . mg)$^{-1}$
After preactivation and solubilization	1.00	0.76	0.76[b]
First supernate[c]	0.40	0.14	0.34
Adenosine-wash supernate	0.08	0.31	3.73

[a] Liver plasma membranes were preactivated and solubilized as described for table 1 with $MgCl_2$, G*PP*[NH]*P*, glucagon, and NaF. Lubrol-PX was added to a final concentration of 1.2%, giving a detergent/protein ratio of 6

[b] The specific activity of 0.76 was obtained with 190 μM adenosine, the concentration carried into the assay from the adenosine-wash supernatant fraction. Without adenosine initial activity was 1.76

[c] One ml aliquots of solubilized adenylate cyclase was mixed with 1 ml adenosine(C-8)-agarose and was rotated for 15 min at 4°C. The agarose suspension was centrifuged and the supernatant fraction (1 ml) was removed and used as 'first supernate'. The agarose was then washed with 3 ml of 25 mM glycylglycine (pH 7.5), and was again centrifuged. The second supernatant fraction was discarded. Then 1 ml of 1 mM adenosine was added and the suspension was again centrifuged. The resulting supernatant fraction (Adenosine-wash) was assayed for protein and cyclase

lack of effect of adenosine in other systems [4,5] suggests the possible participation of additional components. To approach this question detergent-dispersed adenylate cyclase was chromatographed on N^6-and C-8-linked adenosine-hexane–agaroses. When liver adenylate cyclase was preactivated and dispersed as per table 1, or detergent-dispersed cerebellar cyclase was either treated batch-wise or applied to a column of adenosine–agarose, significant adenylate cyclase activity bound. In experiments with adenosine(C-8)-agarose 86 ± 7% (n = 5) of applied activity, but only 33 ± 10% of the protein bound. With a 5′-AMP-agarose as a control, only 37 ± 9% (n = 4) of applied activity and 22 ± 14% of applied protein bound. An example of such an experiment is shown in table 3. In the experiment shown 92% of applied activity and 60% of applied protein bound to the adenosine–agarose. When the agarose was washed with buffer containing 1 mM adenosine, 41% of applied activity could be eluted and resulted in a 5-fold increase in adenylate cyclase specific activity. In no instance was the adenylate cyclase, which either bound or did not bind to the adenosine–agaroses, rendered insensitive to inhibition by adenosine by this treatment.

4. Discussion

The data presented in this report suggest several conclusions regarding the locus and mechanisms mediating inhibition of adenylate cyclase by adenosine. First, the enhancing effect that stimulatory agents have on adenosine inhibition may well be due to their combined effects to increase metal ion affinity of adenylate cyclase. Adenosine inhibition of adenylate cyclase is metal dependent and is accompanied by a 20-fold increase in the enzyme's affinity for free metal at its divalent cation site [2]. Seemingly paradoxically, activation of the liver cyclase by F^-, glucagon + GTP, or, with time, G*PP*[NH]*P*, is also coincident with a decrease in the apparent K_m of the enzyme for free metal (e.g., [14–17]). Thus, it may not be the degree of stimulation of adenylate cyclase that would correlate with increased sensitivity to inhibition by adenosine, but rather the degree to which metal ion affinity may be increased.

Second, sensitivity to inhibition by adenosine was retained following preactivation of the liver cyclase by Mg^{2+} + F^- and/or G*PP*[NH]*P* + glucagon and was retained following its subsequent dispersion with

Lubrol-PX. Thus, the molecular configuration of adenylate cyclase elicited by these agents, allowing the stable activation of the enzyme, also allows for inhibition by adenosine.

Third, adenosine affinity chromatography may be a useful addition to other methods for the purification of adenylate cyclase. Detergent-dispersed enzyme from both brain and liver bound to and could be partially purified on adenosine-agaroses. It may be that by the use of adenosine analogs as insolubilized ligands which exhibit higher affinity and specificity for the 'P'-site than adenosine, e.g. 2',5'-dideoxyadenosine, a considerably greater degree of purification than that shown here could be attained.

And fourth, since adenosine was also found to inhibit a brain cyclase which had been made functionally deficient in the guanine nucleotide regulatory component [8,12,13], this component appears unnecessary for 'P'-site inhibition by adenosine, consistent with [3]. Thus, the data suggest that either adenosine inhibits the catalytic subunit directly, or that the component mediating inhibition of adenylate cyclase is tightly bound to it.

Acknowledgements

This work was supported by grant AM 21170 from the United States National Institutes of Health. The author has greatly appreciated the excellent technical help of Ms Janette Welden and Ms Rebecca Williams.

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