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Mn^{2+} DOES NOT UNCOUPLE ADENOSINE "R_a" RECEPTORS FROM THE LIVER ADENYLATE CYCLASE*

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SUMMARY: Stimulation of hepatic adenylate cyclase by "R"-site selective analogs of adenosine was blocked by methylxanthines and required GTP, consistent with "Ra"-receptor initiated coupling through the guanine nucleotide regulatory component. In contrast to the effect of high concentrations of Mn^{2+} to block activation of the liver cyclase by glucagon, activation of the enzyme by N^6(phenylisopropyl)adenosine was not diminished by concentrations of Mn^{2+} up to 40 mM. The data suggest that Mn^{2+}-induced uncoupling of receptor-cyclase interactions, common to stimulation of the cyclase by other hormones, does not apply to the adenosine "R_a"-receptor activation of the enzyme.

INTRODUCTION

High concentrations of Mn^{2+} are known to inhibit various hormone stimulated adenylate cyclases (e.g. 1-5). This effect of Mn^{2+} has been attributed to a functional uncoupling of interactions between the catalytic subunit of adenylate cyclase and the guanine nucleotide regulatory component, with Mn^{2+} not affecting interactions of this component with hormone receptors (5). Although these latter studies (5) were done only with the catecholamine-sensitive cyclase of erythrocytes, the widespread observation of Mn^{2+} inhibition of hormone activation of the enzyme has suggested that the phenomenon may be a general feature of receptor-cyclase coupled systems.

Adenosine is a putative hormone or neurotransmitter (see 6 for reviews) and receptor-mediated effects of adenosine on adenylate cyclase have been

1) Effects on cellular cAMP levels and adenylate cyclase activity of various analogs of adenosine 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 (21), 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 Londos et al. (18).

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observed in numerous in vitro enzyme preparations. The character of the effect, inhibition or stimulation, and differences in the respective structure activity relationships have led to the designation of sub-classes of adenosine receptors.[1] Although stimulation of the liver adenylate cyclase by adenosine via "R_a" receptors has been reported (7), and this is known to be a function of parenchymal cells (8), the mechanisms by which this enzyme is stimulated by adenosine have not been clarified. Sensitivity of the cyclase to adenosine is absolutely dependent on guanine nucleotide, and therefore presumably requires the guanine nucleotide regulatory component. However, as we report here, rather than being blocked, as is activation of the enzyme by glucagon, activation of the liver enzyme by the "R"-site selective analog of adenosine, N^6(phenylisopropyl)adenosine, is actually improved by high concentrations of Mn^{2+}. These and other observations suggest first, that the general phenomenon of Mn^{2+}-induced uncoupling does not obtain for adenosine "R_a"-receptors, and second, that these receptors are coupled to cyclase catalytic units by mechanism(s) distinct from those of other hormone receptors.

MATERIALS AND METHODS

Enzyme preparation. Partially purified plasma membranes were prepared from rat liver essentially as previously described (9), except that 5 mM EDTA was included in all homogenizing media and sucrose solutions (10). The EDTA was then removed by repetitive centrifugation-resuspension steps prior to storage of the membranes at -70°.

Adenylate cyclase assay. Adenylate cyclase activity was determined as previously described (10) in a reaction mixture containing 50 mM glycylglycine, pH 7.5, 1 mM 3-isobutyl,1-methylxanthine (IBMX)[2] or 0.5 mM Ro 20-1724, 100 µM MnATP or MgATP, 0.01 to 40 mM excess $MnCl_2$ or $MgCl_2$, [α-^{32}P]ATP (0.5 to 1.5 x 10^6 cpm), 2 mM purified creatine phosphate (10), creatine kinase (100 µg/ml), myokinase (100 µg/ml), and adenosine deaminase (5 U/ml) in a reaction volume of 200 µl. Reactions were 2 min at 37° and were initiated by the addition of enzyme to reaction mixtures that had been thermally equilibrated at 37° for 2 min. Reactions were stopped by the $ZnCO_3$ precipitation method (11) and the labeled cAMP was purified as described by Salomon et al. (12).

Other methods. [α-^{32}P]ATP was prepared enzymatically by the method of Walseth and Johnson (13). Protein was determined by a modification of the procedure of Lowry et al. (14).

Materials. Carrier-free (^{32}P)phosphoric acid was obtained from either ICN Pharmaceuticals or New England Nuclear at the highest concentation avail-

2) Abbreviations used: IBMX, 3-isobutyl,1-methylxanthine; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PIA, N^6(phenylisopropyl)adenosine; GPP(NH)P, guanylyl (β-γ imino)diphosphate.

TABLE I. Comparison of activators of liver adenylate cyclase.

Additions	ADENYLATE CYCLASE ACTIVITY[a]		
	Basal	GTP (10 μM)	GPP(NH)P (100 μM)
	pmol cAMP (2 min·mg protein)$^{-1}$		
None	19 ± 6	18 ± 7	74 ± 9
NaF (10 mM)	429 ± 45	b	b
Glucagon (1 μM)	41 ± 10	249 ± 34	329 ± 33
Prostaglandin E_1 (10 μM)	17 ± 6	45 ± 7	144 ± 6
2-Cl-Adenosine (30 μM)	20 ± 9	52 ± 9	165 ± 24

a) Activities were determined in a reaction mixture containing 50 mM glycylglycine, pH 7.5, 0.5 mM Ro 20-1724, 100 μM MgATP (GTP-free), and 4 mM excess $MgCl_2$. Values represent the mean ± S.E.M. of seven experiments, each done in triplicate, from six different membrane preparations.
b) Activity was not determined with F^- and either GTP or GPP(NH)P.

able. Glucagon was a generous gift from Dr. W.W. Bromer, Eli Lilly Co., Indianapolis, Indiana. Ro 20-1724 was a generous gift from Dr. W.E. Scott, Hoffmann-LaRoche, Nutley, N.J. Other reagents were from commercial sources and were of the highest purity available.

RESULTS

Adenosine sensitivity. Stimulation of the liver adenylate cyclase by optimal concentrations of 2-Cl-adenosine was dependent on guanine nucleotide (Table I). The nearly 3-fold stimulation seen with 30 μM 2-Cl-adenosine + GTP was appreciably less than seen with fluoride (22-fold) or glucagon + GTP (14-fold), but was comparable to that seen with prostaglandin E_1 + GTP and was routinely about twice that seen with epinephrine + GTP (not shown). These observations were true with each of the adenosine analogs used (2-Cl-adenosine, N^6(phenylisopropyl)adenosine, and adenosine N^1-oxide). The data suggest that the relative intrinsic activities of the respective receptors to activate the cyclase catalytic unit were glucagon > adenosine > catecholamine in this tissue.

To help establish that the stimulation observed with these adenosine analogs was mediated by adenosine "R"-sites, the effects of methxlxanthines were determined. From a basal activity (with 10 μM GTP) of 19 pmol(2 min·mg protein)$^{-1}$, 30 μM PIA caused a roughly 5-fold stimulation (95 pmol(2 min·

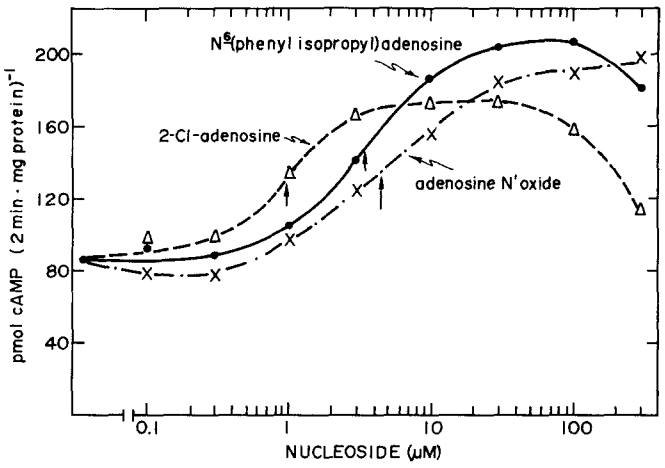

Figure 1. Concentration-dependent activation of liver adenylate cyclase by adenosine analogs. Activity was determined with 0.5 mM Ro 20-1724, 100 μM MnATP, 800 μM excess $MnCl_2$, and 10 μM GTP.

mg protein)$^{-1}$), which was completely blocked by 1 mM IBMX (17 pmol(2 min·mg protein)$^{-1}$) or 10 mM theophylline (8 pmol(2 min·mg protein)$^{-1}$). IBMX was itself without effect on activity, whereas theophylline was somewhat inhibitory (9 pmol(2 min·mg protein)$^{-1}$). Nearly identical results were also seen with 2-Cl-adenosine.

2-Cl-Adenosine consistently stimulated the liver adenylate cyclase at concentrations lower than either PIA or adenosine N^1-oxide (Figure 1). But at higher concentrations 2-Cl-adenosine consistently showed a reduction in activity, suggestive of a "P"-site inhibitory effect not usually seen with the other ligands at concentrations up to 300 μM. There was little or no difference in the maximal extent of stimulation by any of these analogs.

Effects of metals. Adenosine stimulation of platelet adenylate cyclase was reported to be greatest with low concentrations of free metal; increasing free metal concentration reduced the extent of stimulation by adenosine (15). This inhibitory aspect of adenosine's action was considerably more pronounced with Mn^{2+} than with Mg^{2+} and is presumably due to the metal-dependent inhibition of adenylate cyclase via the adenosine "P"-site (15-18). However, the extent of stimulation of the liver adenylate cyclase by PIA, an "R"-site specific analog, was if anything enhanced by increasing concentrations of free metal (Figure 2). This discrepancy was particularly evident with Mn^{2+},

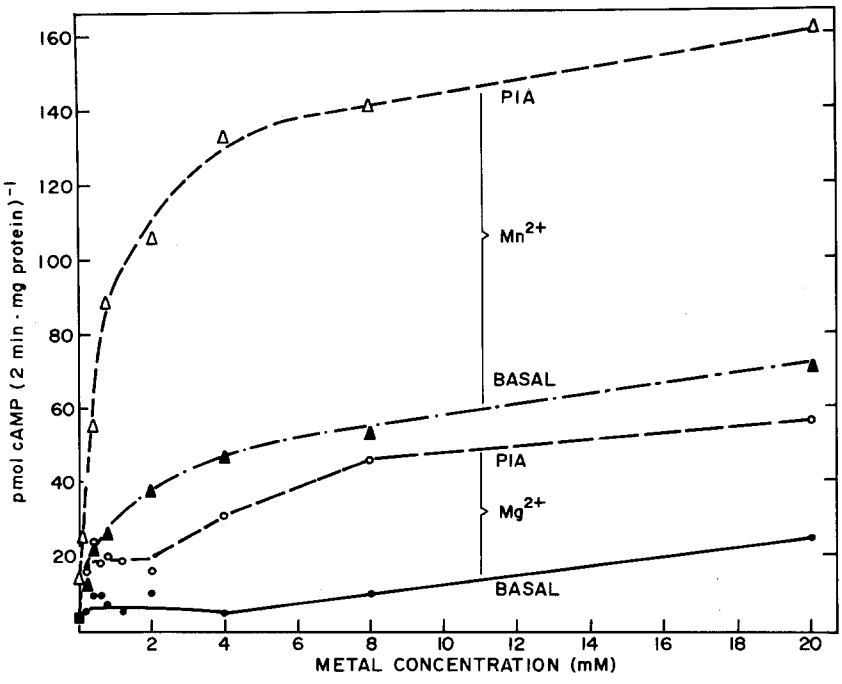

Figure 2. Metal-ion dependency of PIA stimulation of liver adenylate cyclase. Activity was determined with 0.5 mM Ro 20-1724, 10 μM GTP, either 100 μM MnATP or 100 μM MgATP, and the indicated concentrations of metal chloride. PIA was 30 μM.

even at concentrations up to 40 mM. This lack of inhibition of PIA-stimulated activity by high concentrations of Mn^{2+} is in striking contrast with the behavior of other hormone-sensitive cyclases. For example, under conditions where stimulation of the liver adenylate cyclase by PIA + GTP was unaffected by high concentrations of Mn^{2+}, stimulation by fluoride was also unaffected, but stimulation by glucagon + GTP was blocked (Figure 3). While these effects of high concentrations of Mn^{2+} on fluoride- and glucagon-stimulated cyclase activities are not new (cf. 2), they emphasize that the distinct behavior of the adenosine-sensitive cyclase was not due to differences in tissue source, membrane preparations, or assay methods used in our studies. The data indicate that Mn^{2+} does not disrupt coupling of the PIA-sensitive liver adenylate cyclase system.

DISCUSSION

Stimulation of hepatic adenylate cyclase by adenosine analogs behaves as a receptor-initiated process coupled through the guanine nucleotide regulatory

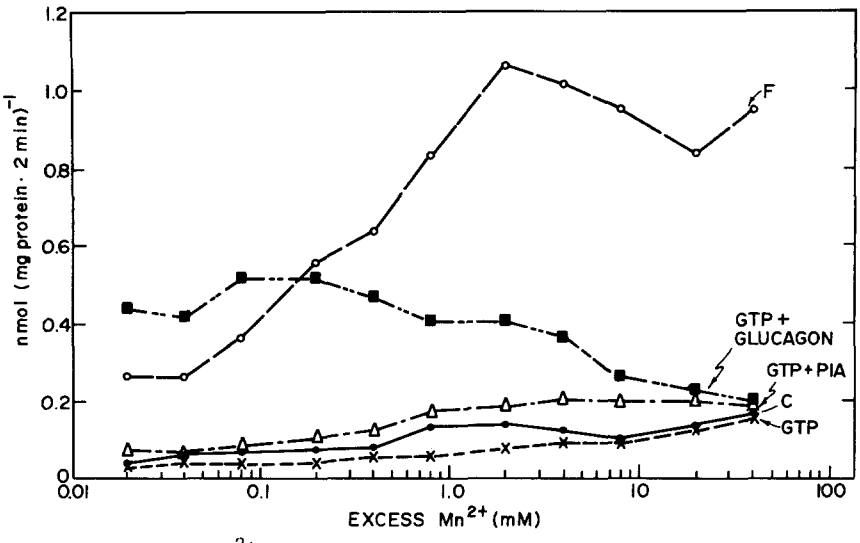

Figure 3. Effect of Mn^{2+} on stimulation of the liver adenylate cyclase by various agents. Activity was determined with 0.5 mM Ro 20-1724, 100 µM GTP-free MnATP, and the indicated concentrations of $MnCl_2$. Additions were 10 µM GTP, 10 mM NaF, 1 µM glucagon, and 30 µM PIA.

component. Stimulation is blocked by methylxanthines, is seen with analogs of adenosine selective for the"R"-site, and it requires guanine nucleotide. In general, activation of the enzyme by adenosine analogs shared properties with activation of numerous adenylate cyclases by other hormones. However, the susceptibility to inhibition by high concentrations of Mn^{2+} seen with other systems was not observed with this adenosine-sensitive cyclase. This observation suggests that Mn^{2+}-induced "functional uncoupling", suggested by Limbird et al.(5) for the catecholamine-sensitive cyclase, does not occur for adenosine "R_a"-receptor-cyclase interactions. That is, adenosine "R_a"-receptor coupling to the cyclase is a clear exception to a generally observed phenomenon.

The findings reported here give support to the idea that activation of the cyclase by adenosine may involve mechanisms distinct from those involved with glucagon or catecholamine activation. This conclusion is supported by several other lines of evidence. First, the pH optimum of the liver cyclase was not shifted to lower values by PIA as was seen upon stimulation with glucagon, epinephrine, fluoride, and vanadate (19). Second, the PIA-sensitive cyclase from rat brain striatum was found to be considerably less sensitive to phospholipases A_2 and C and to Lubrol-PX than was the dopamine-sensitive enzyme (20). And third,

a cyclase fully responsive to PIA, but completely unresponsive to dopamine, could be extracted by low concentrations of non-ionic detergent from striatal membranes. Thus, the adenosine receptor mediating activation may be tightly coupled to the cyclase catalytic unit and therefore coupling is less susceptible to interruption (by Mn^{2+}, detergents, phospholipases) than is catecholamine receptor-cyclase coupling. Alternatively, it is possible that the process of receptor cyclase coupling may be a cascade of enzyme catalyzed reactions and the adenosine receptor affects the system at a site distinct from that through which the catecholamine receptor acts.

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