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METAL ION AND GUANINE NUCLEOTIDE REGULATION OF THE INHIBITION OF LUNG ADENYLATE CYCLASE BY ADENOSINE ANALOGS

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Summary

Adenosine inhibits guinea-pig lung adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) through a 'P' type regulatory site. The inhibition is of a non-competitive type. Divalent cations which activate the enzyme (Mg^{2+} and Mn^{2+}) and also those which inhibit (Ca^{2+}) increase the inhibitory potency of 'P' site analogs at this site. Guanine nucleotides also increase the inhibitory potency at this regulatory site but this does not appear to be directly related to the ability of the guanine nucleotides to activate the enzyme. Other regulators of lung adenylate cyclase, epinephrine and isoproterenol, do not affect the adenosine inhibitory process when examined at physiological concentrations. These studies demonstrate that two types of ligand which regulate the catalytic activity of the lung adenylate cyclase (metal ions and guanine nucleotides) also have a role in regulating the inhibition of the enzyme by adenosine.

Introduction

We have demonstrated that adenosine potentiates antigen-induced histamine release from chopped guinea-pig lung tissue [1]. During the course of these studies, it was of interest to relate the effects observed with this nucleoside on the release process with the inhibitory effects of adenosine, described by Weinryb and Michel [2], on guinea-pig lung adenylate cyclase. It was thought that the two effects might be related because decreases in cyclic AMP levels are thought to be associated with histamine release from mast cells [3—6]. To

Abbreviations: GppNHp, 5'-guanylylimidiphosphate; GpCpp, 5-guanylyl-α,β-methylenediphosphonate; GppCp, 5-guanyl-β,γ-methylenediphosphonate; TSH, thyroid-stimulating hormone.

determine whether such a relationship existed, we characterized the sites involved in each process as being either 'R' or 'P' sites, according to the classifications established by Londos and Wolff [7]. 'R'-type adenosine sites can only tolerate adenosine analogs with unmodified ribose moieties while 'P' sites only tolerate analogs with intact purine rings. Our investigation demonstrated that the sites involved in mediator release ('R' site) and inhibition of adenylate cyclase ('P' site) were not directly related [1]. This paper describes a further outgrowth of these studies, a new examination of those factors which influence the ability of adenosine to inhibit guinea-pig lung adenylate cyclase at the 'P' regulatory site. We have observed that the sensitivity of this adenylate cyclase system to adenosine is modulated by the concentration and type of metal ion in the assay as well as by guanine nucleotides. Other types of enzyme activator, i.e., epinephrine and isoproterenol, at physiological concentrations have no effect on the adenosine inhibition process.

Materials and Methods

Materials. The following chemicals were purchased from the Sigma Chemical Co: adenosine; adenosine 5′-triphosphate; guanosine 5′-triphosphate; creatine phosphate; creatine phosphokinase; dithiothreitol; adenosine 3′,5′-cyclic monophosphate; 2′-deoxyadenosine; 2-chloroadenosine; adenine and adenosine deaminase. ICN provided 9-β-D-arabino-furanosyl adenine, GppNHp and GpCpp. GppCp was obtained from Miles Laboratories. PL Biochemicals supplied the 5′-deoxyadenosine and 2′,5′-dideoxyadenosine. Erythro-9-(2-hydroxy-3-nonyl) adenine was obtained from Burroughs Wellcome Co. [α-^{32}P]ATP and cyclic[^{3}H]AMP were obtained from New England Nuclear.

Adenylate cyclase assay. For assay of adenylate cyclase activity, a membrane fraction was prepared from the lungs of actively sensitized male guinea-pigs *. All procedures were carried out at 0—4°C. The lungs were dissected away from the major airways and blood vessels, cut into small pieces using scissors, and homogenized in 9 vols. of 50 mM Tris-HCl (pH 7.5)/1 mM dithiothreitol/0.25 M sucrose. After filtering the homogenate through cheesecloth, it was centrifuged at 40 000 × *g* for 20 min in a Sorvall RC 2B centrifuge. The pellet obtained from this centrifugation was resuspended in the original volume of homogenizing buffer and stored under N_2 gas at −80°C. The enzyme was stable in this form for at least 1 month. Protein determinations on this membrane preparation were performed using the Bio-Rad method [8]. Bovine serum albumin was the protein standard used.

Adenylate cyclase activity was assayed according to the method of Solomon et al. [9]. The assay reaction contained in a volume of 100 μl, 30 mM Tris-HCl (pH 7.5), 7 mM creatine phosphate, 2.8 units of creatine phosphokinase, 0.1 mM EGTA, 1 mM dithiothreitol, 0.1 mM [α-^{32}P]ATP (20—200 cpm/pmol), 0.04 mM cyclic AMP, approx. 50 μg of membrane protein, and varying con-

* The animals were injected intraperitoneally with 10 mg of egg albumin in 1 ml of 0.9% NaCl and killed 28—45 days later. Sensitized guinea-pigs were used in the studies reported in this paper because these experiments were being conducted in parallel with studies reported elsewhere [1] describing adenosine effects on antigen-induced mediator release from lung. We noticed no difference between the lungs from sensitized and non-sensitized animals with regard to the properties described in this paper.

centrations of metal ions and adenosine analogs as described in the figure legends. The reactions were initiated by the addition of membrane protein and carried out at 37°C for 10 min (under these conditions the assay was linear with time and membrane protein). Reactions were terminated by the addition of 100 μl of 'stopping solution' containing 2% sodium dodecyl sulfate (SDS)/ 40 mM ATP/1.4 mM cyclic AMP at pH 7.5. cyclic[^{3}H]AMP was added to the reaction mixture to monitor recovery during sequential column chromatography through Dowex AG50-WX4 and Alumina WN-3.

Each experiment reported in this paper was repeated a minimum of two times with similar results being obtained each time. The data reported for a given assay condition is the average of triplicate determinations. The standard deviation observed within a given set of determinations was never more than 10%.

Results and Discussion

As can be seen in Table I, in the guinea-pig lung adenylate cyclase system, the relative order of inhibitory potency of a wide variety of 'P' site adenosine analogs is as follows: 2′,5′-dideoxyadenosine > 5′-deoxyadenosine > 2′-deoxyadenosine ≈ 9-β-D-arabinofuranosyl adenine > adenosine. This order of potency is similar to that observed for the 'P' site associated with other adenylate cyclase systems [10,11]. Inclusion of the adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine, did not affect the relative order of sensitivity to the analogs. Thus, metabolism of adenosine or the metabolizable analogs (9-β-D-arabinofuranosyl adenine) by endogenous adenosine deaminase does not account for their apparent lower inhibitory potency in the system.

To characterize further the inhibitory site as a 'P' site, we have observed that theophylline does not block the ability of adenosine to inhibit adenylate cyclase when used at concentrations less than 1 mM (concentrations at which theophylline is an 'R' site antagonist [12]). As previously reported, higher concentrations of theophylline (1—10 mM) inhibit the enzyme [13]. We have observed that this inhibition does not appear to be related to an effect at an adenosine site on the enzyme since this effect of theophylline is not antagonized by adenosine or either 'R' or 'P' site adenosine analogs. Also, while theophylline's ability to inhibit the enzyme is influenced by metal ions (as will be

TABLE I

INHIBITION OF GUINEA-PIG LUNG ADENYLATE CYCLASE BE ADENOSINE 'P' SITE ANALOGS

The adenylate cyclase assay was performed as described in the 'Materials and Methods'. The concentration of Mg^{2+} used in the assay was 5 mM.

Analog	IC_{50} (M)
Adenosine	$6 \cdot 10^{-4}$
9-β-D-Arabinofuranosyladenine	$1.8 \cdot 10^{-4}$
2′-Deoxyadenosine	$1.6 \cdot 10^{-4}$
5′-Deoxyadenosine	$4.8 \cdot 10^{-4}$
2′,5′Dideoxyadenosine	$2.8 \cdot 10^{-5}$

shown below to be also true of adenosine 'P' site analogs), these effects do not correlate with an effect of theophylline at an adenosine site. For example, we have observed theophylline inhibits best at low metal ion concentrations while adenosine inhibits best at high metal ion concentrations.

Kinetic studies were performed to examine the type of inhibition being exhibited by the 'P' site analogs. Fig. 1 is a double reciprocal plot of the activity of guinea-pig lung adenylate cyclase activity at various substrate concentrations when the enzyme is assayed in the presence and absence of $5 \cdot 10^{-5}$ M and $1 \cdot 10^{-5}$ M 5'-deoxyadenosine. GTP was also included in the assay medium in order to eliminate the interaction of ATP (or contaminating GTP) at the guanine nucleotide regulatory site. It has previously been demonstrated in both the adrenal [14] and hepatic [15] systems that if the assay medium is not supplemented with GTP for the above reason, non-linear Lineweaver-Burk plots are obtained. On the basis of this analysis, the 'P' site analogs appear to be non-competitive inhibitors, as has also been found to be true in other cyclase systems [10,11]. Similar kinetic analysis of the inhibition exhibited by 9-β-D-arabinofuranosyl adenine and 2',5'-dideoxyadenosine yielded identical results. Weinryb and Michel [2] have previously reported that 9-(tetrahydro-2-furyl) adenine was a non-competitive inhibitor of guinea-pig lung adenylate cyclase. Presumably, this analog, too, acts through the 'P' regulatory site. Their kinetic analysis with this analog yielded non-linear Lineweaver-Burk plots possibly because the assay was not performed in the presence of GTP.

Metal ions increase the inhibitory potency of 'P' site analogs [2,10,11]. Fig. 2A demonstrates this effect on the 'P' site inhibition observed with 5'-deoxyadenosine. Similar results were also obtained with other 'P' site inhibitors. The concentration range over which Mg^{2+} increased the inhibitory potency of 5'-deoxyadenosine is also the concentration range over which Mg^{2+} activates

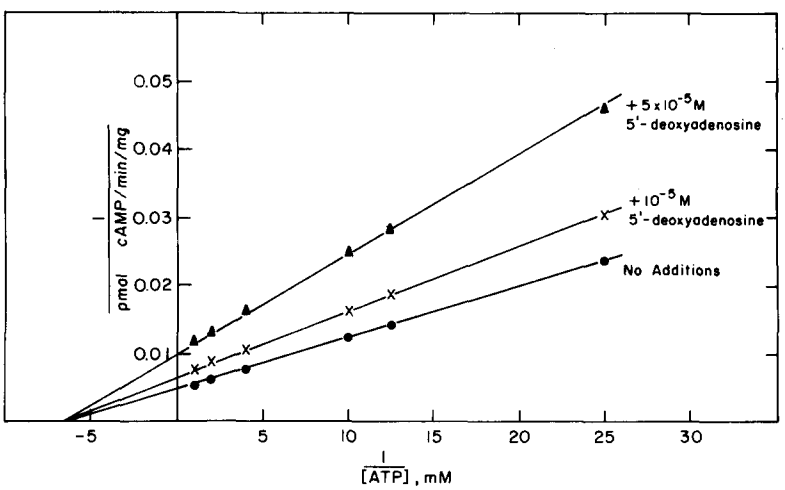


Fig. 1. Lineweaver-Burk analysis of the inhibitory properties of 5'-deoxyadenosine. Studies were performed at $1 \cdot 10^{-5}$ M (X———X) and $5 \cdot 10^{-5}$ M (▲———▲) 5'-deoxyadenosine. Guinea-pig lung adenylate cyclase was assayed as described in Materials and Methods except that ATP concentrations varied between 0.04 mM and 1.0 mM and all assays were carried out in the presence of 50 μM GTP and 5 mM $MgCl_2$. cAMP, cyclic AMP.

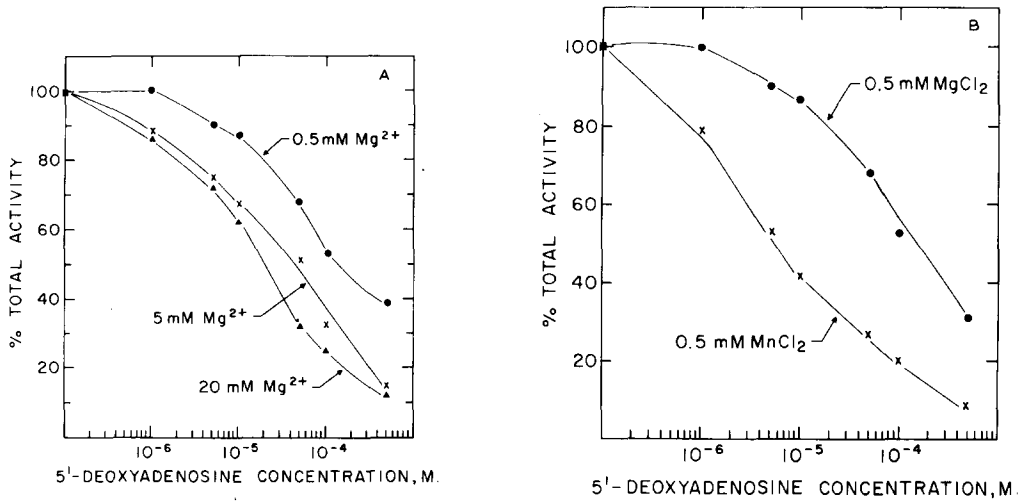


Fig. 2. Effect of increasing metal ion concentration on the sensitivity of guinea-pig lung adenylate cyclase to inhibition by 5'-deoxyadenosine. Adenylate cyclase activity was assayed in the presence of: A, 0.5 mM Mg^{2+} (●———●); 5 mM Mg^{2+} (X———X); 20 mM Mg (▲———▲); B, 0.5 mM Mg (●———●); 0.5 mM Mn (X———X). Enzymatic activities in the absence of 5'-deoxyadenosine at the various metal ion concentrations were A, 0.5 mM Mg^{2+}, 21.6 pmol/min per mg; 5 mM Mg^{2+}, 65.5 pmol/min per mg; 20 mM Mg, 114.0 pmol/min per mg; or B, 0.5 mM Mg^{2+}, 21.6 pmol/min per mg; and 0.5 mM Mn^{2+}, 24.1 pmol/min per mg.

the enzyme (Fig. 3). This has been seen in other cyclase systems and it has been suggested that an increase in inhibitory potency may be associated with activation of the enzyme by metal ion [10,11,22]. It was first observed in the liver adenylate cyclase system that the inhibition observed for 'P' site analogs is greater when the enzyme is assayed in the presence of Mn^{2+} rather than Mg^{2+} [10]. Fig. 2B demonstrates this same phenomenon in the guinea pig lung system. In the liver cyclase system, Mn^{2+} was also observed to be a 50—100-fold more potent activator of adenylate cyclase than Mg^{2+} [16]. This observation led to the suggestion that the metal ion site regulating enzyme activity and inhibition were one and the same. In the lung system, we did not observe such a difference in the activating potency of Mn^{2+} and Mg^{2+} (Fig. 3); that is, while we did observe that the V obtained with Mn^{2+} was greater than that obtained with Mg^{2+}, the K_m values for metal ion were nearly the same. Therefore, our data suggest that in the lung the metal ion sites regulating enzyme activity and those regulating adenosine inhibition may be distinct. Interestingly enough, we have also observed that Ca^{2+}, an inhibitory metal ion in the guinea-pig lung adenylate cyclase system (Fig. 4), also increases the inhibitory potency at the regulatory 'P' site (Fig. 5). Thus, from this type of analysis, it appears that in the lung cyclase system, both activating and inhibitory metal ions can effect 'P' site inhibition.

A common feature of adenylate cyclase systems is their ability to be activated by guanine nucleotides. The guinea-pig lung cyclase system also exhibits this characteristic (Fig. 6). The highest fold-stimulation by these nucleotides occurred with GppNHp, but GpCpp and GppCp also gave higher activity states than GTP. This pattern is typical of that seen with other cyclase

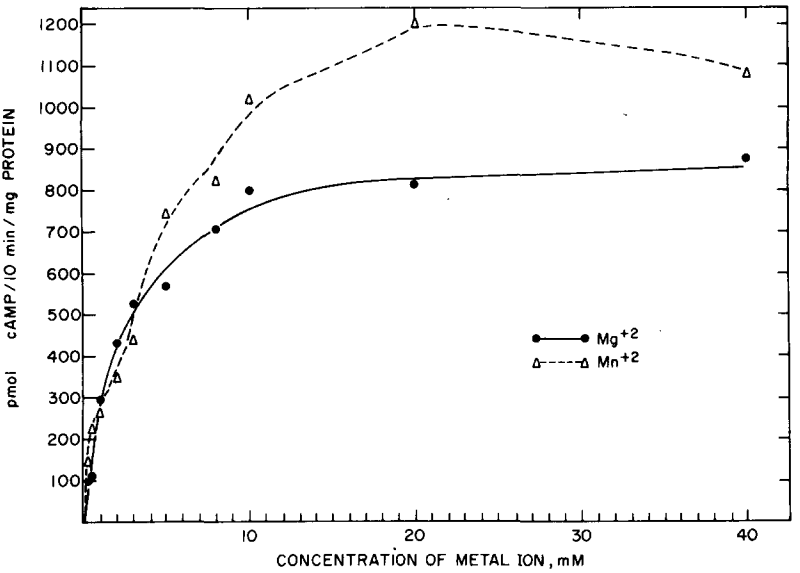


Fig. 3. Effect of metal ion concentration on the activity of lung adenylate cyclase. Enzymatic activity was assayed in the presence of varying concentrations of Mg^{2+} (●———●) and Mn^{2+} (△———△). Adenosine deaminase (0.8 units/ml) was included in the assay mixture to remove endogenous adenosine from the assay mixture. Because of the metal ion dependence of the adenosine inhibition process, endogenous adenosine might affect the pattern of the metal ion activation curve.

systems and has been attributed to the presence of a GTPase activity at the guanine nucleotide activating site [17,18,19]. In the lung cyclase system, guanine nucleotides also increase the potency of adenosine 'P' site analogs (Fig. 7). This phenomenon has been seen in other adenylate cyclase systems [10,11,20]. A comparison of the abilities of guanine nucleotides to activate lung adenylate cyclase and influence 'P' site inhibition (compare Figs. 6 and 8) demonstrates, however, that there appears to be no direct relationship in the ability of guanine nucleotides to influence the two phenomena. It has been suggested that multiple guanine nucleotide regulatory sites are associated with

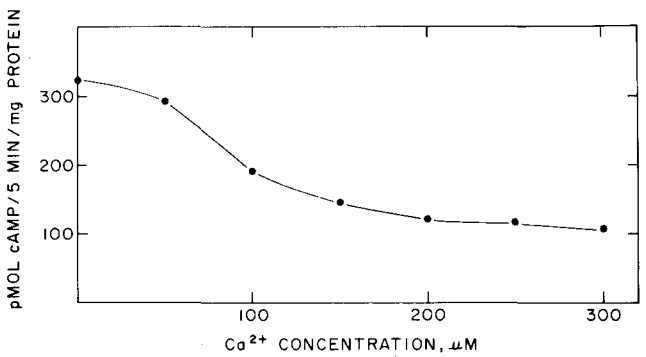
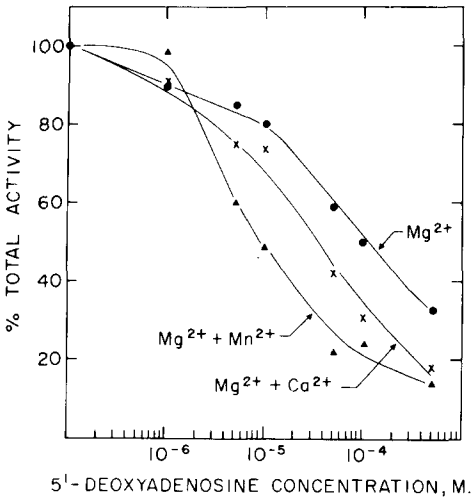


Fig. 4. Effect of Ca^{2+} on activity of guinea-pig lung adenylate cyclase. Enzymatic activity was assayed in the presence of 5 mM Mg^{2+} and increasing concentrations of Ca^{2+}.



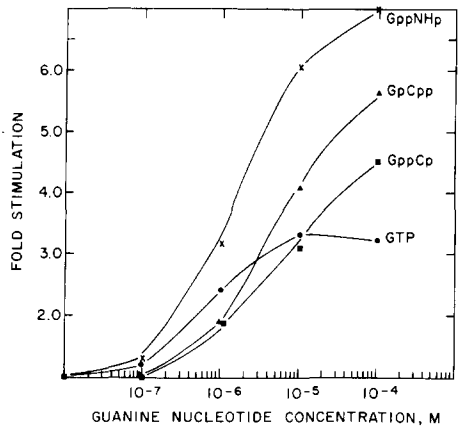


Fig. 5. Effect of Mn^{2+} or Ca^{2+} on the sensitivity of lung adenylate cyclase to 5'-deoxyadenosine. Assays were carried out in the presence of 0.5 mM Mg^{2+} (●———●) or 0.5 mM Mg^{2+} in the presence of 0.2 mM Ca^{2+} (X———X) or Mn^{2+} (▲———▲). Enzymatic activity with 0.5 mM Mg^{2+} alone was 25.2 pmol/min per mg.

Fig. 6. Effect of increasing concentrations of guanine nucleotides on lung adenylate cyclase activity. Enzyme assays were carried out in the presence of 0.5 mM Mg^{2+} and varying concentrations of GTP (●———●), GppCp (■———■), GpCpp (▲———▲) or GppNHp (X———X). The basal enzymatic activity was 43.7 pmol/min per mg.

other adenylate cyclase systems [21]. If this is true also in the lung system, one could explain this discrepancy by postulating that the site which regulates enzymatic activity is independent of that regulating 'P' site inhibition.

We have also observed that guanine nucleotides affect the inhibitory characteristics of different adenosine analogs in different ways; that is, plots of the increase in the present inhibition by $1 \cdot 10^{-4}$ M 5'-deoxyadenosine, $1 \cdot 10^{-4}$ M

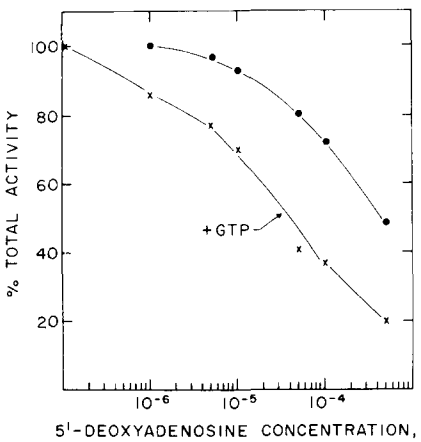


Fig. 7. Effect of $1 \cdot 10^{-4}$ M GTP on the sensitivity of lung adenylate cyclase to 5'-deoxyadenosine. All assays were carried out in the presence of 0.5 mM Mg^{2+} alone (●———●) or in the presence of GTP (X———X). Basal enzymatic activity was 10.9 pmol/min per mg; GTP-stimulated activity was 67.8 pmol/min per mg.

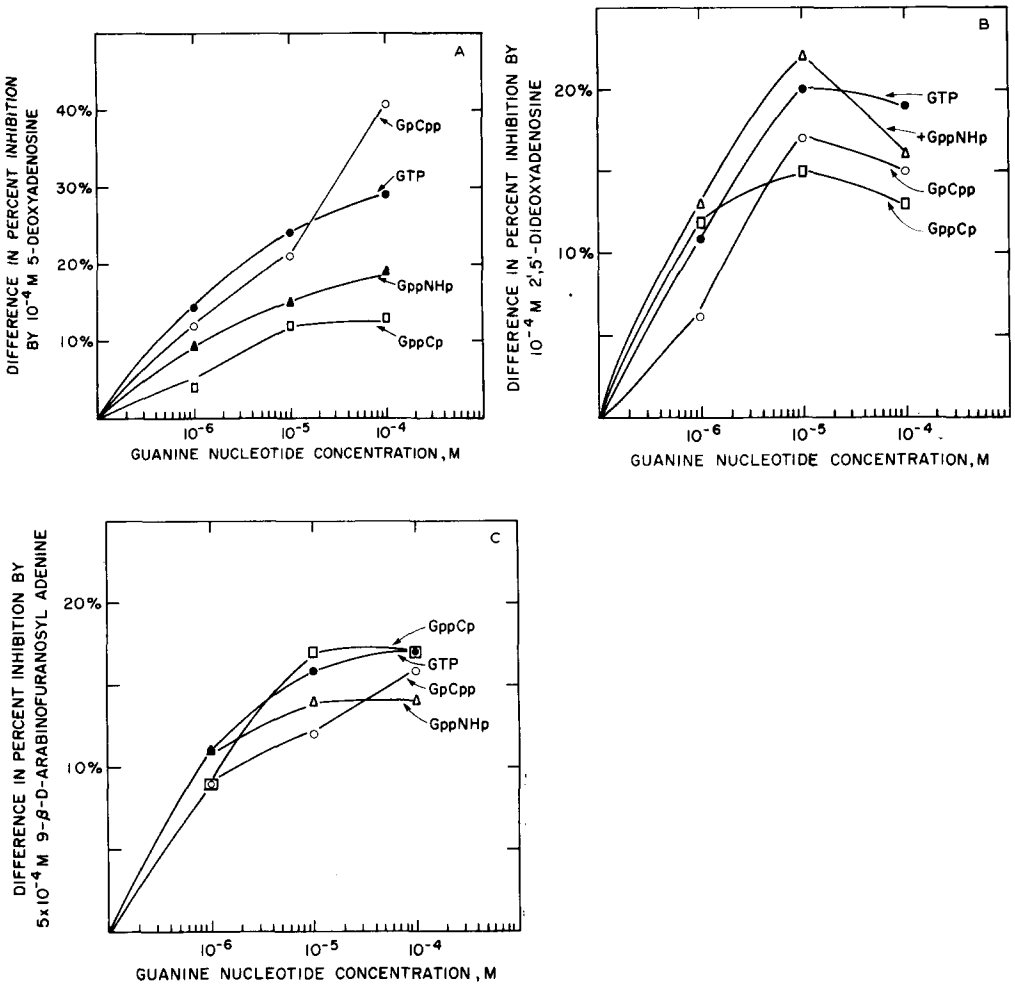


Fig. 8. The difference in the percent inhibition of lung adenylate cyclase observed with (A) $1 \cdot 10^{-4}$ M 5'-deoxyadenosine, (B) $1 \cdot 10^{-4}$ M 2',5'-dideoxyadenosine, and (C) $5 \cdot 10^{-4}$ M adenine arabinoside in the presence of increasing concentrations of GTP (●———●); GppNHp (▲———▲); GppCp (□———□) and GpCpp (○———○). All assays were carried out in the presence of 0.5 mM Mg^{2+}. The concentrations of adenosine analogs used in these experiments were the IC_{50} values at 0.5 mM Mg^{2+}. The basal enzymatic activities in each case were (A) 43.7 pmol/min per mg, (B) 43.7 pmol/min per mg, and (C) 23.4 pmol/min per mg.

2',5'-dideoxyadenosine and $5 \cdot 10^{-4}$ M 9-β-D-arabinofuranosyl adenine (the IC_{50} values of the compounds at 0.5 mM Mg^{2+}) in the presence of varying concentrations of guanine nucleotides have strikingly different patterns (Fig. 8). Thus, the various guanine nucleotides also have the ability to alter the relative affinity of the enzyme for the different adenosine analogs.

The hormones glucagon and TSH have been shown to increase the sensitivity of adenylate cyclase to 'P' site inhibitors in liver [10] and thyroid [11] plasma membranes, respectively. Catecholamines, such as epinephrine and isoproterenol, are analogous stimulators of lung adenylate cyclase. These catecholamines stimulate the enzyme over the concentration range of $1 \cdot 10^{-8}$—

$1 \cdot 10^{-5}$ M. Within this concentration range, they have no effect on inhibition by adenosine at the 'P' site. Jakobs et al. [20] have recently reported a similar observation for β-adrenergics in the adenylate cyclase system of human platelets. We have observed in the lung system that high concentrations of the catecholamines (greater than $1 \cdot 10^{-4}$ M) actually decrease the sensitivity of the system to 'P' site analogs. Since this effect did not occur over the concentration range within which epinephrine or isoproterenol were activating the enzyme and since this process was not blocked by propranolol or phentolamine, it is difficult to speculate on the physiological relevance of this observation.

Our studies have demonstrated that adenosine inhibits guinea-pig lung adenylate cyclase through 'P' type regulatory sites, as defined by the work of Londos and Wolff [7]. The potency of this inhibition appears to be modulated by metal ions and guanine nucleotides, as been described for the 'P' sites associated with other adenylate cyclase systems [10,11,20,22]. It has been suggested from work with other cyclase systems that there is a relationship between the ability of a ligand to increase the inhibitory potency of 'P' site analogs and its ability to stimulate cyclase activity [10,11,22]. In the lung system, this is not strictly true. For example, in the case of metal ions, both activating metal ions (Mg^{2+} and Mn^{2+}) and inhibiting metal ions (Ca^{2+}) increase 'P' site inhibition. Also, we have seen that although guanine nucleotides both activate the enzyme and increase inhibitory potency, there is no direct correlation between the fold-stimulation of enzymatic activity attained and the degree of 'P' site inhibition observed. It has been shown that receptor, catalytic and guanine nucleotide components of adenylate cyclase systems have a complex interrelationship. Our studies in lung tissue and also the studies of others in tissues such as liver [10], thyroid [11], adrenal [22] and blood platelets [7,20] seem to demonstrate that the same is true with regard to the interrrelationship of these components to adenosine 'P' sites.

In other tissues, it has been postulated that 'P' sites are located intracellularly and possibly function in a type of negative feedback inhibition of adenylate cyclase to maintain cellular cyclic AMP levels within certain limits. This may be true in lung tissue also, We had undertaken the studies described here and in a previous paper [1] with another thought in mind that in lung tissue the 'P' regulatory site might be associated with adenosine's ability to potentiate allergic mediator release. This did not prove to be true, however, since only 'R' site analogs participate in the mediator release process. Thus, the physiological role of the 'P' site in lung and other cellular types, at present, remains an enigma.

References

1 Welton, A.F. and Simko, B.A. (1980) Biochem. Pharmacol. 29, 1085—1092
2 Weinryb, I. and Michel, I.M. (1974) Biochim. Biophys. Acta 334, 218—225
3 Marquardt, D.L., Parker, C.W. and Sullivan, T.J. (1978) J. Immunol. 120, 871—878
4 Sullivan, T.J. and Parker, C.W. (1976) Am. J. Path. 85, 437—463
5 Sullivan, T.J., Parker, K.L., Eisen, S.A. and Parker, C.W. (1975) J. Immunol. 114, 1480—1485
6 Kaliner, M. and Austen, K.F. (1974) J. Immunol. 112, 664—674
7 Londos, C. and Wolff, J. (1977) Proc. Natl. Acad. Sci. U.S.A. (1977) 74, 5482—5486
8 Bio-Rad Inc., (1977) Technical Bulletin No. 1051, April 1977
9 Solomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541—548

10 Londos, C. and Preston, M.S. (1977) J. Biol. Chem. 252, 5951—5956
11 Wolff, J., Londos, C. and Cook, G.H. (1978) Arch. Biochem. Biophys. 191, 161—168
12 Londos, C., Wolff, J. and Cooper, D.M.F. (1979) in Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides (Baer, H.P. and Drummond, G.I., eds.), pp. 271—281, Raven Press, New York
13 Weinryb, I. and Michel, I.M. (1971) Experientia 27, 1386
14 Londos, C. and Rodbell, M. (1975) J. Biol. Chem. 250, 3459—3465
15 Lin, M.C., Salomon, Y., Rendell, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 4246—4252
16 Londos, C. and Preston, M.S. (1977) J. Biol. Chem. 252, 5957—5961
17 Salomon, Y., Lin, M.C., Londos, C., Rendell, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 4239—4245
18 Londos, C., Lin, M.C., Welton, A.F., Lad, P.M. and Rodbell, M. (1977) J. Biol. Chem. 252, 5180—5182
19 Welton, A.F., Lad, P.M., Newby, A.C., Yamamura, H., Nicosia, S. and Rodbell, M. (1978) Biochim. Biophys. Acta 522, 625—639
20 Jakobs, K.H., Saur, W. and Johnson, R.A. (1979) Biochim. Biophys. Acta 583, 409—421
21 Lad, P.M., Welton, A.F. and Rodbell, M. (1977) J. Biol. Chem. 252, 5942—5946
22 Glynn, P. and Cooper, D.M.F. (1978) Biochim. Biophys. Acta 526, 605—612