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Metal and Metal-ATP Interactions with Human Platelet Adenylate Cyclase: Effects of *Alpha* Adrenergic Inhibition

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SUMMARY

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In human platelet lysates, metal and metal-ATP interactions with adenylate cyclase have been studied, using Mg^{++} as divalent cation. The kinetic pattern followed Michaelis-Menten kinetics and conformed to a bireactant mechanism in which free metal served as a requisite activator. The *alpha* adrenergic component of epinephrine (20 μM) reduced V_{max} of the enzyme by about 60% without changing the apparent affinities for the substrate, Mg-ATP, and the activator, free Mg^{++}. The inhibition was partial and noncompetitive, with an apparent K_I value of 2 μM for epinephrine.

INTRODUCTION

Formation of cyclic AMP catalyzed by the adenylate cyclase (ATP:pyrophosphate lyase, cyclizing, E.C. 4.6.1.1) requires the presence of metal to form the active substrate, metal-ATP. Further, free metal is required at a distinct activator site (1-3). The mechanisms by which the catalytic activity of adenylate cyclase is regulated under physiological conditions by hormones that stimulate the enzyme are still unclear by may involve altered affinities for free metal or for the substrate, metal-ATP (for review see Ref. 4).

We have recently demonstrated an inhibition of adenylate cyclase in lysates (5) and particles (6) of human platelets by epinephrine and norepinephrine. This inhibition was reversed by *alpha* adrenergic blocking agents such as dihydroergotamine and phentolamine and was enhanced by *beta* adrenergic blocking agents such as propranolol and pindolol; therefore, this inhibitory effect was assumed to involve *alpha* adrenergic receptors.

We have studied the effects of the *alpha* adrenergic component of epinephrine on the kinetic characteristics of adenylate cyclase in human platelet lysates. This paper shows 1) that in platelet lysates adenylate cyclase conformed to a bireactant sequential mechanism with no apparent cooperative behavior and 2) that epinephrine reduced the maximal velocity of the enzyme with no changes of the kinetic constants for free Mg^{++} or Mg-ATP, indicating partial, noncompetitive inhibition.

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MATERIALS AND METHODS

Reagents. ATP, creatine phosphate and creatine kinase (E.C. 2.7.3.2) were from Boehringer Mannheim. 3-Isobutyl-1-methylxanthine was from Aldrich Chemical Corp. Carrier-free [^{32}P]H_3PO_4 was obtained from New England Nuclear Corp. [α-^{32}P]-ATP was prepared by a chemical method according to the procedure of Symons (7), as modified by Nakai and Brooker (8), with

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a specific activity of 50 to 100 Ci/mmol at the time of preparation. (−)-Epinephrine bitartrate was purchased from Sigma Chemical Company. (±)-Pindolol hydrochloride was provided by Sandoz AG, Nürnberg and Basel. Solutions of epinephrine and pindolol were prepared shortly before experiments. Neutral aluminum oxide (type 90, activity grade I) was obtained from E. Merck, Darmstadt. All other reagents were obtained from commercial sources and were of the highest purity available.

Enzyme preparation. Lysates of human platelets were prepared essentially as described previously (5). Platelets were prepared by differential centrifugation of blood from healthy volunteers who had not taken any medication for at least one week prior to collection. The isolated platelets were suspended and washed in 150 mM NaCl solution containing 10 mM triethanolamine-HCl buffer, pH 7.4. Lysates of platelets were obtained by freezing small aliquots in liquid nitrogen and thawing shortly before assay of adenylate cyclase.

Adenylate cyclase assay. Platelet adenylate cyclase activity was determined in a reaction mixture containing 6 to 200 μM ATP, [α-^{32}P]ATP (0.6 to 0.8 μCi per tube), 0.4 to 10 mM Mg-acetate, an ATP-regenerating system consisting of 5 mM creatine phosphate and 400 μg/ml of creatine kinase, 1 mM isobutylmethylxanthine, and 50 mM triethanolamine-HCl, pH 7.4, in a final volume of 100 μl. Incubations were initiated by the addition of platelet lysate (120 to 170 μg of protein) to reaction mixtures that had been incubated for 5 min at 37°. Reactions were conducted in triplicate for 10 min at 37°. Under these conditions, product accumulation was linear for at least 20 min at all ATP and metal concentrations tested. The *beta* adrenergic blocking agent, pindolol (10 μM), was present under each condition. Epinephrine was added to some reactions at the indicated concentrations.

Reactions were terminated by the addition of 500 μl of 120 mM Zn-acetate. The labeled cyclic AMP formed was purified by $ZnCO_3$ coprecipitation of the 5′-nucleotides and subsequent chromatography on neutral alumina, essentially as described previously (5). More than 90% of ATP remained at the end of the incubation periods, as determined by isolation on polyethyleneimine cellulose columns (9) after dissolving the $ZnCO_3$-precipitate in acetic acid.

Other methods. Protein was determined essentially as described by Lowry et al. (10), with bovine serum albumin as standard. All kinetic data were analyzed by linear regression analysis with a Diehl Alphatronic calculator. Kinetic constants were determined from the slopes and intercepts of secondary plots, as suggested by Cleland (11). To maintain essentially constant concentrations of free Mg^{++}, excess Mg^{++} was added to the reaction at the chosen concentrations above the Mg-ATP concentration, as had been done in studies of Garbers and Johnson (2). Free Mg^{++} introduced into the incubation medium together with the enzyme preparation was estimated from data of various mammalian tissues (12) to be 10 μM or less and was therefore neglected.

RESULTS

It was possible to determine meaningful initial velocities of adenylate cyclase in human platelet lysates, and thereby, to evaluate the reaction kinetics. Even at very low substrate concentrations, adenylate cyclase activity could be determined under conditions in which product accumulation was linear with respect to the time and protein concentrations and in which ATP levels were maintained essentially unchanged during the incubation period. There were no measurable effects of adenosine deaminase or of one of its inhibitors on the enzyme's apparent activity.[1] These findings indicate that there was neglible hydrolysis of ATP to adenosine, which could conceivably have interfered with these kinetic evaluations[2] (13).

The kinetic behavior of adenylate cyclase in platelets is shown in Figs. 1 and 2. The figures present double reciprocal plots of adenylate cyclase activity as function of Mg-ATP and free (excess) Mg^{++} concentrations, in the presence and absence of 20

[1] Jakobs, K. H., Saur, W. & Johnson, R. A. Submitted for publication.

[2] Johnson, R. A., Saur, W. & Jakobs, K. H. Submitted for publication.

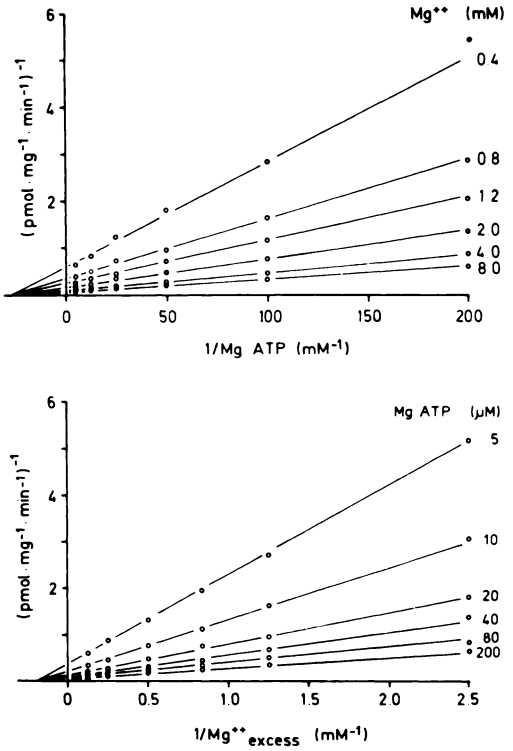

FIG. 1. *Mg-ATP and free Mg^{++} kinetics of basal adenylate cyclase in human platelet lysate*

Upper panel: Double reciprocal plot of adenylate cyclase activity as a function of Mg-ATP at the indicated concentrations of excess Mg^{++}. Lower panel: Double reciprocal plot of adenylate cyclase activity as a function of excess Mg^{++} at the indicated concentrations of Mg-ATP.

μM epinephrine. The data obtained, whether plotted as function of Mg-ATP or of free Mg^{++}, resulted in linear plots; the intersection was to the left of the ordinate on the abscissa. The lines showed no obvious tendency to be concave upward or downward, indicating no evidence of cooperativity. The resulting secondary plots (Fig. 3) were linear, whether plotted as a function of free Mg^{++} or of Mg-ATP. The findings that the primary plots were linear and intersected to the left of the ordinate and that the secondary plots were also linear suggest a bireactant mechanism in which free cation is a requisite activator of the enzyme (2, 11). This behavior of enzyme activity was unaffected by the presence of 20 μM epinephrine (compare Figs. 1 and 2).

The kinetic constants for platelet lysate adenylate cyclase were determined from the secondary plots of the slopes and intercepts of the double reciprocal plots (Table 1). Epinephrine (20 μM) reduced the maximal velocity (V_{max}) of the enzyme by about 60%. The apparent K_m value (Michaelis-Menten constant) and the K_i value (dissociation constant) for Mg-ATP (both about 40 μM) and the apparent K_m value and the K_i value for free Mg^{++} (both about 4 mM) were not significantly changed by treatment with 20 μM epinephrine. This indicates that epinephrine lowers the enzyme activity without changing the affinities for metal-ATP or for free metal.

Figure 4 shows the kinetics of inhibition of adenylate cyclase by epinephrine. At each concentration tested, primary double

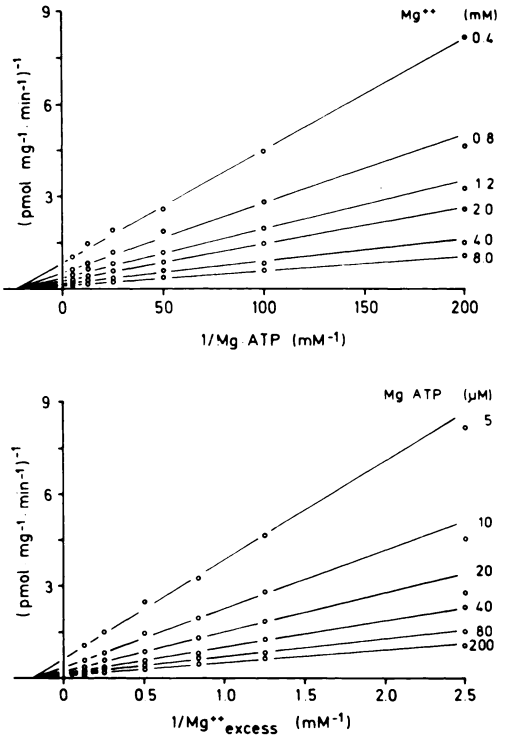

FIG. 2. *Mg-ATP and free Mg^{++} kinetics of epinephrine-inhibited adenylate cyclase in human platelet lysate*

Epinephrine concentration was 20 μM. Upper panel: Double reciprocal plot of adenylate cyclase activity as a function of Mg-ATP at the indicated concentrations of excess Mg^{++}. Lower panel: Double reciprocal plot of adenylate cyclase activity as a function of excess Mg^{++} at the indicated concentrations of Mg-ATP.

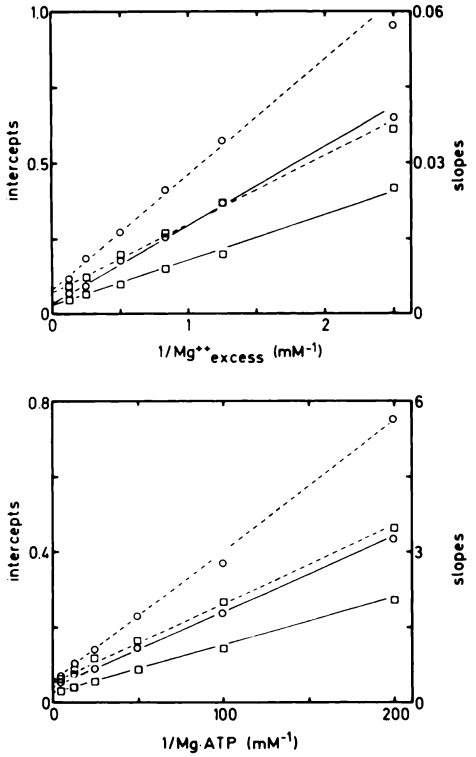

FIG. 3. *Replots of slopes and intercepts derived from the double reciprocal plots in Figs. 1 and 2*

Slopes (□) and intercepts (○) taken from the upper and lower panels of these figures are plotted as functions of 1/excess Mg^{++} and 1/Mg-ATP, respectively. Solid lines, control conditions; interrupted lines, in the presence of 20 μM epinephrine.

TABLE 1

Kinetic Constants for Adenylate Cyclase in Human Platelet Lysate

Data are derived from experiments shown in Figs. 1-3. Epinephrine concentration was 20 μM. K_m, Michaelis-Menten constant; K_i, dissociation constant.

	Control	Epinephrine
	pmol·mg⁻¹·min⁻¹	
V_{max}	22.5	9.2
	μM	
K_m:Mg-ATP	37.1	35.1
K_i:Mg-ATP	39.2	35.1
	mM	
K_m:excess Mg^{++}	4.2	3.5
K_i:excess Mg^{++}	4.4	4.5

reciprocal plots were linear and intersected to the left of the ordinate on the abscissa at different Mg-ATP and free Mg^{++} concentrations. The data indicate that inhibition by epinephrine was noncompetitive with respect to either Mg-ATP or Mg^{++}.

The secondary plots of the slopes and intercepts of the data shown in Fig. 4 produced concave downward lines, reaching a plateau at high concentrations of epinephrine (not shown). Similarly, concave downward curves that reached a plateau at high concentrations of epinephrine were also obtained when reciprocal adenylate cyclase activities were plotted versus the concentration of epinephrine, both at different Mg-ATP (Fig. 5, upper panel) and at different free Mg^{++} (Fig. 5, lower panel) concentrations. These findings indicate partial noncompetitive enzyme inhibition. The in-

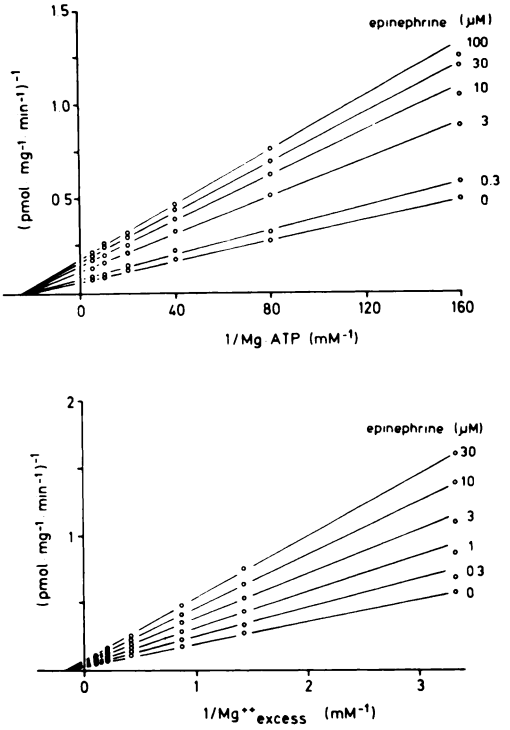

FIG. 4. *Effects of epinephrine on the Mg-ATP and free Mg^{++} kinetics of adenylate cyclase in human platelet lysate*

Upper panel: Double reciprocal plot of adenylate cyclase activity as a function of Mg-ATP at the indicated concentrations of epinephrine at a constant concentration (5 mM) of excess Mg^{++}. Lower panel: Double reciprocal plot of adenylate cyclase activity as a function of excess Mg^{++} at the indicated concentrations of epinephrine at a constant concentration (0.1 mM) of Mg-ATP.

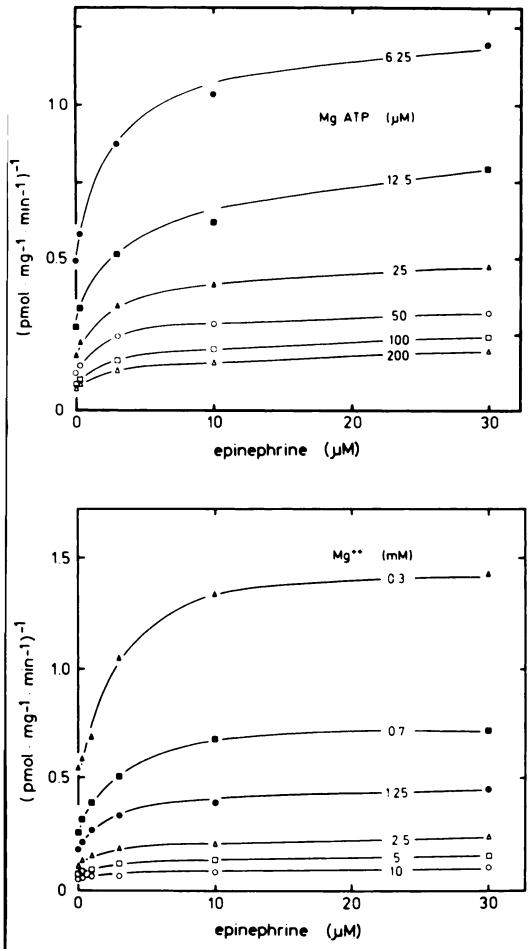

FIG. 5. *Dixon plot for inhibition of platelet adenylate cyclase by epinephrine*

The data are the same as shown in Fig. 4. Upper and lower panels give reciprocal plots of activity versus epinephrine concentration at the indicated concentrations of Mg-ATP and excess Mg^{++}, respectively.

hibitor constant (K_I) for epinephrine, calculated from these data (14), was about 2 μM.

DISCUSSION

Because of various interfering substances and enzymatic activities, kinetic studies of the membrane-bound adenylate cyclase have been difficult. In some systems studied, the enzyme kinetics appeared to follow Michaelis-Menten behavior and conform to a bireactant sequential mechanism (11) in which free metal serves as a requisite activator. This has been shown for the dispersed adenylate cyclase from rat cerebellum (2), for the sea urchin sperm adenylate cyclase (4), for the pig platelet enzyme,[2] and for the human platelet adenylate cyclase. Given the multifactorial nature of the enzyme system, stimulatory hormone effects have been studied, applying similar kinetic approaches. The kinetic basis of the stimulatory effects of hormones and neurotransmitters on adenylate cyclase, however, is still unclear. Until now, no clear hormone effects on the affinities to the substrate, metal-ATP, and to free metal have been demonstrated. Data from several laboratories indicate that stimulation of adenylate cyclase may be coincident with increased affinity of the enzyme for free cation, allosteric or other modifications, resulting in altered cooperative behavior, or a combination of these effects (for review see Ref. 4).

Epinephrine reduced the V_{max} of human platelet adenylate cyclase but did not change the affinities of the enzyme for either the substrate, Mg-ATP, or the activator, free Mg^{++}. The noncompetitive, partial inhibition caused by epinephrine can be interpreted in several ways: a) there are two or more populations of the platelet enzyme, one of which is inhibited by epinephrine while the other is not affected; b) *alpha* adrenergic receptors are coupled to the entire population of catalytic subunits of the adenylate cyclase systems and *alpha* adrenergic agonists convert all adenylate cyclase molecules into a less active form. This question will probably be answered by understanding the coupling mechanism between the *alpha* adrenergic receptor and the catalytic subunit of the adenylate cyclase. Although it has recently been shown (6) that GTP is required for epinephrine-induced inhibition of platelet adenylate cyclase, the coupling process is not understood.

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