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CONTROL OF ADENYLATE CYCLASE BY DIVALENT CATIONS AND AGONISTS

ANALYSIS OF INTERACTIONS BY THE HILL EQUATION

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Summary

The analysis by the Hill equation of the results of adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) activation in human granulocytes resulted in the following findings: the adenylate cyclase agonists have no effect on guanyl nucleotide and divalent cation activation or inhibition of the adenylate cyclase activity since the Hill coefficients for Gpp(NH)p, Mg^{2+} and Ca^{2+} were not affected by (±)-isoproterenol, histamine or prostaglandin E_1. The fact that the Hill coefficient for five adenylate cyclase agonists (prostaglandin E_1, (±)-isoproterenol, histamine, (—)-adrenalin and (±)-noradrenalin was approximately 0.5 or less rules out the possibility that there is a cooperation among the catalytic subunits of the adenylate cyclase.

The inhibitory action of Ca^{2+} on the adenylate cyclase activity can be attributed to a competition between Ca^{2+} and Mg^{2+} that results in a replacement of Mg^{2+} by Ca^{2+} at the intracellular Mg^{2+} binding site. The Hill coefficient for Mg^{2+} was 1.8, 2.1 and 1.7 at 0, 0.1 and 0.5 mM Ca^{2+} but decreased significantly to 1.1 at 1 mM Ca^{2+}. The exposure of whole cells to Mg^{2+}, Ca^{2+}, prostaglandin E_1 and ionophore A23187 has indicated a diverse action of divalent cations on the cyclic AMP formation. Our data suggest that Ca^{2+} and Mg^{2+} potentiate the prostaglandin E_1 stimulatory effect on cyclic AMP production, Ca^{2+} at the extracellular and Mg^{2+} at the intracellular site of the adenylate cyclase complex. In contrast, prostaglandin E_1-stimulated cyclic AMP formation was inhibited when Ca^{2+} and Mg^{2+} acted at the reverse sites.

Abbreviation: Gpp(NH)p, guanyl-5′-yl-imidodiphosphate.

Introduction

Relatively little is known about the mechanism by which the activity of adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) is affected by the positive or negative effectors. There are several current hypotheses that include dephosphorylation of an active state [1], conformational changes of the enzyme following the effector binding [2], involvement of the membrane lipids [3] and the interaction of the mobile hormone receptor with the catalytic subunit of the adenylate cyclase [4]. It has been shown that the active enzyme complex contains separate binding units for agonist [5,6], positive regulators (guanine nucleotide and Mg^{2+}) [7—10], substrate ($MgATP^{2-}$) [5,11], negative regulator (Ca^{2+}) [12,13], associated proteins [14] and adenosine [9,15].

We have shown [12] by the analysis of the Hill equation [16] that there is no direct competition in the adenylate cyclase complex of polymorphonuclear leukocytes for the binding sites occupied by $MgATP^{2-}$ or guanine nucleotide, $MgATP^{2-}$ or Ca^{2+}, and Ca^{2+} or guanine nucleotide. Our results suggest that there are at least three separate binding sites on the catalytic subunit of the adenylate cyclase, one of each for substrate ($MgATP^{2-}$, Ca^{2+} and guanine nucleotide.

Recent studies indicate that there is an interdependence among the ligands that occupy the distinct binding sites of the adenylate cyclase complex [10, 17—20]. We would like to demonstrate that it is possible to gain valuable insight about the basic features of the adenylate cyclase by the Hill equation analysis of the ligand interactions.

Materials and Methods

Materials. [α-^{32}P]ATP (10 Ci/mmol) was purchased from ICN. Pyruvate kinase and Gpp(NH)p[1] were obtained from Boehringer. ^{3}H-labeled cyclic AMP (37.7 Ci/mmol) was purchased from New England Nuclear. Alumina (WN3), aminophylline, (—)-adrenalin, (±)-isoproterenol, (±)-noradrenalin, dithiothreitol, phospho*enol*pyruvate, ATP and cyclic AMP were obtained from Sigma. Bovine serum albumin was purchased from Calbiochem. Prostaglandin E_1 was a kind gift of Dr. J. Pike, The Upjohn Co. Ionophore A23187 was kindly donated by Dr. R.J. Hosley, The Eli Lily Co. The buffy coats for the isolation of polymorphonuclear leukocytes were purchased from Central Blood Bank of Pittsburgh, PA.

Preparation of cell homogenate. The protocol for preparation of pure populations of human polymorphonuclear leukocytes (granulocytes or neutrophils) has been described previously [12]. Briefly, the granulocytes were isolated from peripheral blood of blood donors by centrifugation. The erythrocytes were separated from white blood cells by dextran sedimentation and lysis in hypotonic NaCl. The while blood cells were then fractionated by Ficoll-Hypaque gradient, and the pellet containing 95—99% granulocytes, collected. The cells were washed in buffer containing: NaCl, 123 mM; $CaCl_2$, 0.45 mM; KH_2PO_4, 0.8 mM; KCl, 4.9 mM; $MgSO_4$, 1.23 mM; Tris-HCl (pH 7.4 was adjusted at room temperature), 21 mM; 0.1 μg of NaI/ml; and 1 mg of

glucose/ml. The granulocytes were incubated in buffer at room temperature for 1 h followed by 37° C for 15 min. After centrifugation, the cell pellet was homogenized by sonication (sonic dismembrator, Quigley-Rochester, Inc. setting 80 for 30 s) in 0.5 to 1.0 ml buffer without Ca^{2+} to give a concentration of approximately $3.2 \cdot 10^8$ granulocytes or 20 mg of protein/ml. Aliquots were used immediately for the adenylate cyclase assay.

Adenylate cyclase assay. The assay medium contained the following ingredients: dithiothreitol, 1 mM; [^{32}P]ATP (ATP level was determined at pH 7.0 on the Gilford spectrophotometer at 259 nm using the molar absorbance coefficient of 15 400), 1 mM; $MgCl_2$, 5 mM; bovine serum albumin, 400 μg/ml; aminophylline, 10 mM; Tris-HCl (pH 7.6 was determined at ambient room temperature), 21 mM; phospho*enol*pyruvate, 16 mM; pyruvate kinase, 50 μg/ml and granulocyte proteins, approximately 8 mg/ml. The agonists and divalent cations were present at concentrations indicated in the legends to figures. The reaction was started by addition of cell homogenate. The incubation time at 37° C was 10 min and the total incubation volume was 0.05 ml. The adenylate cyclase activity was terminated by the addition of 0.05 ml of a solution containing: ATP, 5 mM; cyclic AMP, 5 mM; and 20 000 cpm of cyclic [^{3}H]AMP and the immersion of the tubes with the incubation mixture in a boiling water bath for 3 min. Cyclic [^{32}P]AMP was measured after the passage of the clear supernate through an Alumina column [21]. The recovery was corrected according to the recovery of cyclic [^{3}H]AMP (50—80%). Samples were counted in 15 ml of Bray's solution [22] in a Packard Tri-Carb scintillation counter. The results are reported as pmol cyclic AMP $\cdot$ min^{-1} $\cdot$ mg^{-1} protein. The Hill plots were drawn according to the equation:

$$\log Y/(1 - Y) = \log S_{50} - N \log (L)$$

In this equation, S_{50} is the half-maximal concentration of ligand (L) when $Y/(1 - Y)$ is equal to 1.0. $(1 - Y)$ represents the maximal activity (1) minus the fraction activity (Y) of the adenylate cyclase. N is the Hill coefficient.

All experiments were performed in triplicate and repeated at least three times using different granulocyte preparations. Protein was determined in the granulocyte homogenate according to Lowry et al. [23] using bovine serum albumin as standard.

Assay of adenosine 3',5'-monophosphate. The exact protocol has been presented previously [12]. Briefly, approximately $2 \cdot 10^7$ granulocytes were incubated in triplicate in buffer with or without divalent cations, agonist and ionophore A23187. 1 mM aminophylline was always included in each experiment. After incubation, the cells were separated by centrifugation (1600 × *g* for 2 min). The granulocytes were sonicated in trichloroacetic acid and the centrifugation pellet discarded. The cyclic AMP level was determined in the ether-extracted supernate by Gilman's method [24].

Results

Effect of divalent cations and agonists on adenylate cyclase activity in cell homogenate

Ca^{2+}/Mg^{2+} interaction. The effect of Ca^{2+} was studied at 0, 0.1, 0.5 and

1 mM level. The $Mg^{2}1$ concentration ranged between 0—25 mM. As shown in Fig. 1, the graded Ca^{2+} concentration decreased the adenylate cyclase activity in relation to Mg^{2+} levels. The calculated Hill coefficient for Mg^{2+} was 1.8, 2.1 and 1.7 at 0, 0.1 and 0.5 mM Ca^{2+} but decreased significantly to 1.1 at 1 mM Ca^{2+}. Also, the half-maximal concentration (S_{50}) of Mg^{2+} for the adenylate cyclase activition was about 2 mM at 0, 0.1 and 0.5 mM Ca^{2+} but increased to 5.5 mM Mg^{2+} at 1 mM Ca^{2+}.

Effect of agonists on Mg^{2+} activation of adenylate cyclase. The addition of agonists to the cell homogenate caused a dose-related increase of the adenylate cyclase activity. As shown in Figs. 2 and 3, prostaglandin E_1 and (±)-isoproterenol at 10 μM level have no effect on the Hill coefficient for Mg^{2+} as the N values were 1.9 and 2.0. On the other hand, the S_{50} for Mg^{2+} was decreased by both agonists from a control value of 2 mM to 1.1 mM.

Effect of agonists on Ca^{2+} inhibition of adenylate cyclase. The effect of 10 μM prostaglandin E_1 and 10 μM (±)-isoproterenol on the activity of adenylate cyclase in relationship to Ca^{2+} levels is shown in Fig. 4. The adenylate cyclase activity decreased approximately 10 times with 0.1—1.0 mM concentrations of Ca^{2+} when agonists were present in the cell homogenate. On the other hand, the Hill coefficient and S_{50} for Ca^{2+} were not significantly changed by 10 μM concentrations of prostaglandin E_1 or (±)-isoproternol.

The control, prostaglandin E_1 and (±)-isoproterenol-treated granulocyte homogenates had Hill coefficients and S_{50} values of 1.5, 1.8, and 1.8 and 0.26, 0.26, and 0.28 mM Ca^{2+}, respectively.

Effect of agonists on guanine nucleotide activation of adenylate cyclase. The effect of (±)-isoproterenol (10 μM), prostaglandin E_1 (2 μM) and histamine (50 μM) on Gpp(NH)p adenylate cyclase activation in the cell homogenate is shown in Fig. 5. The adenylate cyclase activity increased with increasing concentra-

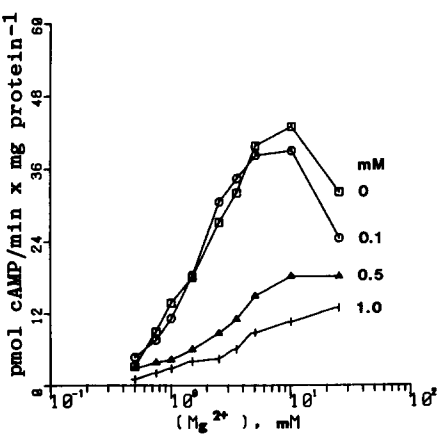

Fig. 1. The effect of increasing Ca^{2+} concentrations on Mg^{2+} activation of adenylate cyclase activity. The Ca^{2+} levels were 0, 0.1, 0.5 and 1.0 mM. The experimental conditions are given in the Experimental Procedure section. The granulocyte homogenate was incubated with divalent cations and substrate at 37°C for 10 min to study the adenylate cyclase activity. This time interval was used in all experiments. Each point represents a mean of triplicate determinations from four experiments.

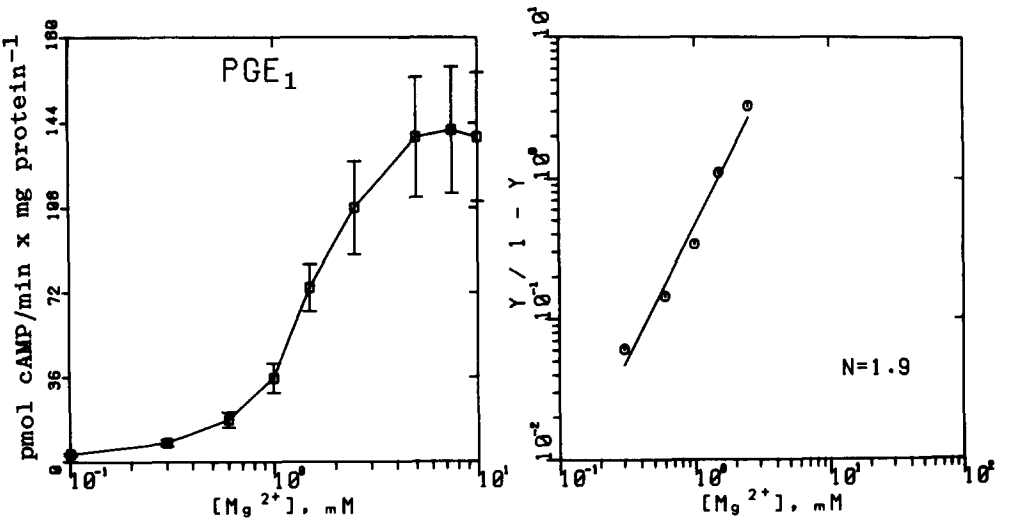

Fig. 2. The effect of 10 μM prostaglandin E_1 on Mg^{2+} activation of adenylate cyclase activity in granulocyte homogenate. Data points represent the mean ± S.D. of triplicate determinations from three experiments. The right panel shows the results expressed by the Hill equation. Y = the fractional activity of adenylate cyclase when the maximal activity is equal 1.0. N = the Hill coefficient for Mg^{2+}.

tions of Gpp(NH)p. The Hill coefficient and S_{50}, however, for Gpp(NH)p were similar in the presence of (±)-isoproterenol, prostaglandin E_1 or histamine. The values of Hill coefficients and S_{50} values for Gpp(NH)p were: 0.9, 1.0 and 1.1, and 10, 40 and 10 μM Gpp(NH)p. The Hill coefficient and S_{50} for Gpp(NH)p in the control incubations without agonists were 0.8 and 10 μM Gpp(NH)p.

Negative cooperativity of agonists. The effect of five positive effectors on

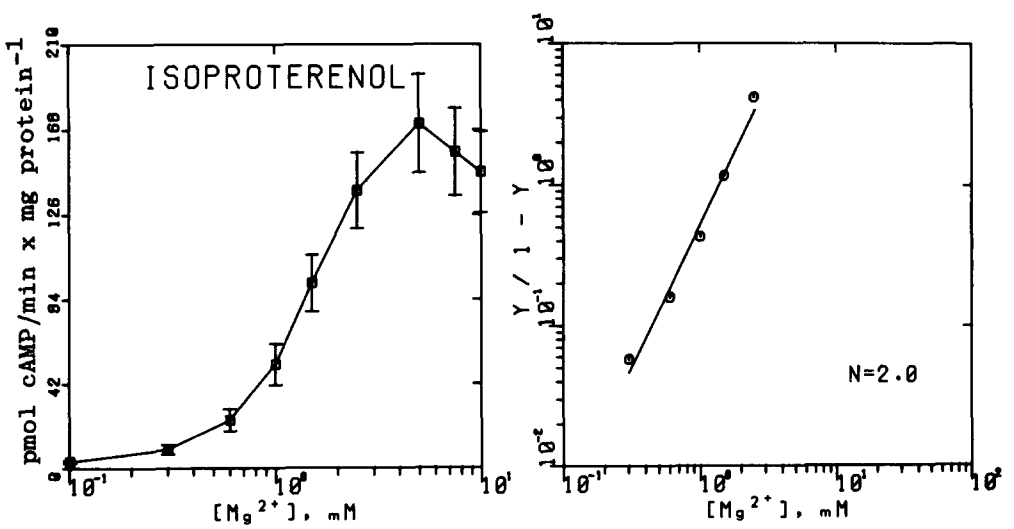

Fig. 3. The effect of 10 μM (±)-isoproterenol on Mg^{2+} activation of adenylate cyclase in granulocyte homogenate. Each point represents the mean ± S.D. of triplicate determinations from three experiments. The fractional activity (Y) and the Hill coefficient (N) are shown in the right panel.

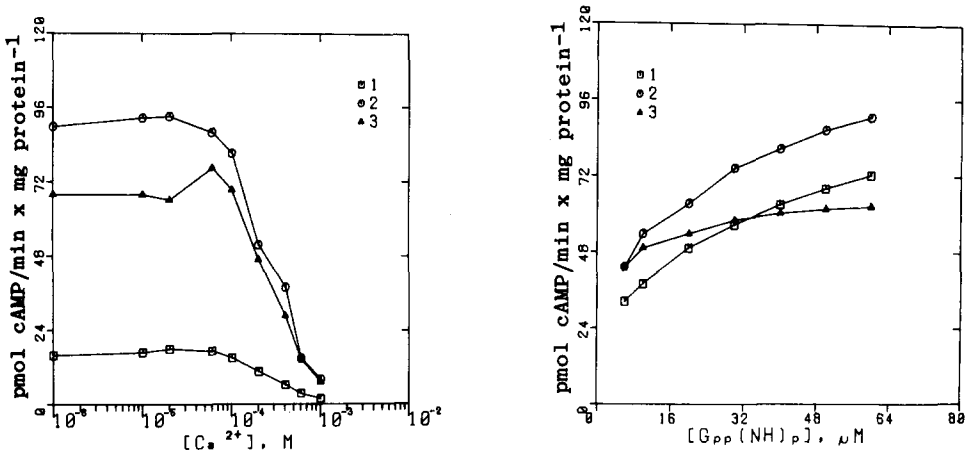

Fig. 4. The effect of graded Ca^{2+} concentration on adenylate cyclase activity in control (1), prostaglandin E_1 (2) and (±)-isoproterenol (3) treated granulocyte homogenates. The concentration of agonists was 10 μM. The results shown represent the mean ± of triplicate determinations from four experiments.

Fig. 5. The effect of 50 μM histamine (1), 10 μM (±)-isoproterenol (2) and 2 μM prostaglandin E_1 (3) on adenylate cyclase activity in granulocyte homogenate that was treated with increasing concentrations of Gpp(NH)p. Each value represents the mean of triplicate determinations from three experiments.

the adenylate cyclase activity in the cell homogenate was investigated. The dose-related effects of (±)-isoproterenol, (±)-noradrenalin, histamine and (—)-adrenalin are shown in Fig. 6 and the effect of prostaglandin E_1 in Fig. 7. The calculated Hill coefficients for (±)-isoproterenol, (±)-noradrenalin, histamine and (—)-adrenalin were 0.4, 0.4, 0.3, and 0.3 and 0.5 for prostaglandin E_1. The S_{50} values for five agonists were 1, 60, 2, 20 and 1 μM

Formation of cyclic AMP in whole granulocytes

Effect of prostaglandin E_1 and divalent cations. Table I shows the effect of

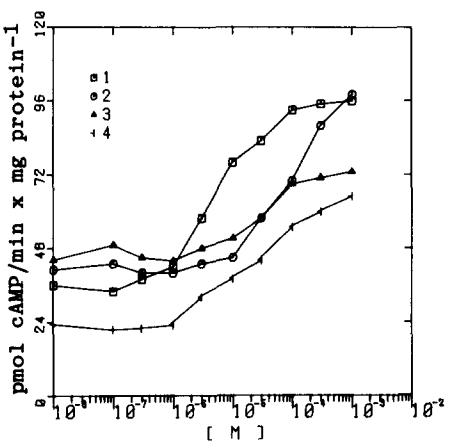

Fig. 6. The effect of (±)-isoproterenol (1), (±)-noradrenalin (2), histamine (3) and (—)-adrenalin (4) on adenylate cyclase activity in granulocyte homogenates. Each point represents the mean of triplicate determinations from six experiments.

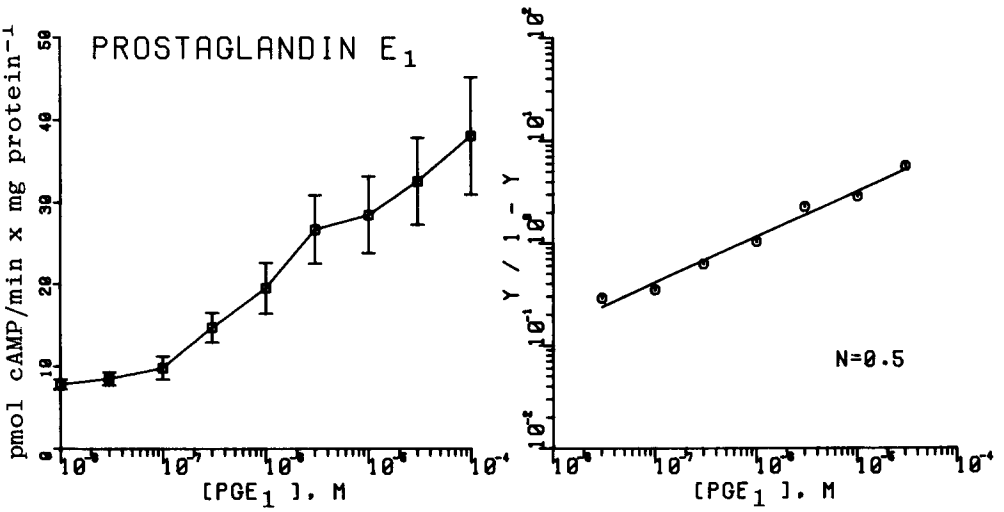

Fig. 7. The effect of prostaglandin E_1 on adenylate cyclase activity in granulocyte homogenates. Data points represent the mean ± S.D. of triplicate determinations from four experiments. The fractional activities (Y) and the Hill coefficient (N) for the prostaglandin E_1 activation of adenylate cyclase are shown in the right panel.

TABLE I

EFFECT OF DIVALENT CATIONS ON THE CYCLIC AMP FORMATION IN WHOLE CELLS

The granulocytes were incubated with or without 1 μM ionophore A23187, 0.3 μM prostaglandin E_1 and 5 mM Ca^{2+} or Mg^{2+} in the presence of 1 mM aminophylline for 10 min at 37°C. Because of the variability of the cyclic AMP levels found in granulocytes obtained from different blood donors, the statistical analysis was performed using Student's t-test for pair differences. The data in each control group (lines 1, 5, 9 and 13) were compared with the results of three experimental groups (1 vs. 2, 3, and 4; 5 vs. 6, 7, and 8; etc.) and the significant differences were calculated. The results represent mean ± S.E. for 10 assays. NS = not significant.

Line	Addition	Ca^{2+}	Mg^{2+}	pmol cyclic AMP/10^7 cells	$P <$
1	Nil	—	—	10.1 ± 0.63	—
2	Nil	+	—	17.1 ± 1.63	0.005
3	Nil	—	+	10.3 ± 0.73	NS
4	Nil	+	+	11.9 ± 0.69	0.05
5	A23187	—	—	10.4 ± 0.55	—
6	A23187	+	—	6.1 ± 0.69	0.001
7	A23187	—	+	8.9 ± 0.55	0.05
8	A23187	+	+	5.6 ± 0.53	0.001
9	Prostaglandin E_1	—	—	40.2 ± 2.01	—
10	Prostaglandin E_1	+	—	49.0 ± 3.57	0.05
11	Prostaglandin E_1	—	+	35.5 ± 1.79	0.005
12	Prostaglandin E_1	+	+	31.3 ± 1.39	0.001
13	A23187 + Prostaglandin E_1	—	—	28.9 ± 3.43	—
14	A23187 + Prostaglandin E_1	+	—	13.1 ± 2.68	0.01
15	A23187 + Prostaglandin E_1	—	+	47.0 ± 6.38	0.005
16	A23187 + Prostaglandin E_1	+	+	15.3 + 4.35	0.02

5 mM Ca^{2+}, 5 mM Mg^{2+}, 0.3 μM prostaglandin E_1 and 1 μM ionophore A23187 on the cyclic AMP formation in whole granulocytes. We have found that in the absence of Ca^{2+} and Mg^{2+} the values of cyclic AMP in control and prostaglandin E_1 treated granulocytes were 10.1 and 40.2 pmol cyclic AMP/10^7 cells. Addition of 5 mM Ca^{2+} increased the values to 17.1 and 49.0 pmol cyclic AMP/10^7 cells. By contrast, in the present of 1 μM A23187, calcium causes reduction of the cyclic AMP formation in the control and prostaglandin E_1 treated granulocytes to 6.1 and 13.1 pmol cyclic AMP/10^7 cells. Hence, the stimulatory or inhibitory effect of Ca^{2+} on the cyclic AMP formation in whole cells depends on its extracellular or intracellular location.

It is of interest that the stimulatory or inhibitory effect of Mg^{2+} on the cyclic AMP formation in whole granulocytes also depends on Mg^{2+} location, especially in the presence of prostaglandin E_1. Extracellular Mg^{2+} decreases the cyclic AMP formation in prostaglandin E_1-treated cells from 40.2 to 35.5 pmol cyclic AMP/10^7 cells. However, in the presence of ionophore A23187, magnesium increases the cyclic AMP level in prostaglandin E_1-treated granulocytes from 28.9 to 47.0 pmol cyclic AMP/10^7 cells. Thus extracellular Mg^{2+} inhibits and intracellular Mg^{2+} stimulates the cyclic AMP formation in whole prostaglandin E_1-treated granulocytes.

Discussion

The current concept regarding the adenylate cyclase suggests that the receptors and catalytic subunits of enzyme represent separate molecular entities containing several binding sites [25,26] for cations and other regulators, perhaps linked to the GTPase system [27]. Two groups of investigators have shown that guanyl nucleotides decrease the binding affinity of agonists to receptors of adenylate cyclase [8,19]. We have studied the formation of cyclic AMP in whole cells treated with or without agonists and Gpp(NH)p and have found [12] that Gpp(NH)p has no effect on cyclic AMP formation in whole cells. It does, however, significantly stimulate adenylate cyclase activity in the cell homogenate. The results presented here also indicate that the positive effectors have little or no effect on the Hill coefficient for the guanine nucleotide activation of adenylate cyclase. We conclude from these studies that agonists have no effect on the character of the guanine nucleotide binding that is directly related to the adenylate cyclase activation. Because the Hill coefficients for Mg^{2+} and Ca^{2+} adenylate cyclase activation were not affected by the agonists, it is possible that the rate of adenylate cyclase activation after the initial stimulation by the agonists acting extracellularly may be controlled by the level of intracellular effectors such a guanyl nucleotides and divalent cations. It is interesting to note that the Hill coefficients for the action of five agonists were repeatedly 0.5 or less. These results were obtained also by other investigators [19,28] and it suggests that the stimulatory effect of hormones on cyclic AMP production is not carried out by a cooperative interaction of the catalytic subunits of the adenylate cyclase.

By taking the advantage of the apparent differences in the mechanism of action of Ca^{2+} and Mg^{2+}, we can conclude that there are at least two Mg^{2+} binding sites in the adenylate cyclase complex. Ca^{2+} at 1 mM level decreases the

Hill coefficient for Mg^{2+} from 2 to about 1 that suggests that the mechanism of Ca^{2+} inhibition of the adenylate cyclase activity is related to the replacement of Mg^{2+} by Ca^{2+} at one of the intracellular Mg^{2+} binding sites. One of the Mg^{2+} binding sites is the site occupied by the substrate $MgATP^{2-}$. It is difficult to assume that Ca^{2+} would replace Mg^{2+} at this site as $CaATP^{2-}$ is not a substrate for the adenylate cyclase and the affinity constant at 5 mM Mg and 1 mM Ca^{2+} is shifted significantly toward $MgATP^{2-}$ formation. This fact indicates, as has been suggested by others [9,29,30], that there must be a second site that is occupied by Mg^{2+} independent of the $MgATP^{2-}$ catalytic site.

An important conclusion might be drawn from the comparison of cyclic AMP production in cell homogenate or membrane preparation with the whole cells. It is evident that the adenylate cyclase in cell homogenate can bind the ligands simultaneously at both extracellular and intracellular locations but the adenylate cyclase activity in whole cells is more sensitive to ectopically acting effectors, especially in short time incubation. However, the use of ionophores that bind Ca^{2+} and Mg^{2+} [31] can increase the influx of divalent cations into the cell and should allow to study their effects at both sides of the adenylate cyclase complex that is located in the cell plasma membrane. Our data presented in Table I suggest that there is a significant difference in the action of Mg^{2+} that is present in the extracellular or intracellular space. The results with the use of ionophore A23187 indicate that extracellular Mg^{2+} inhibits the cyclic AMP formation in the prostaglandin E_1-stimulated cells but potentiates the action of prostaglandin E_1 on cyclic AMP formation when its entry into the cells is facilitated by ionophore A23187. These results suggest that the Mg^{2+} site at which prostaglandin E_1 action is potentiated might be located on the intracellular portion of the prostaglandin E_1 receptor. Since Mg^{2+} has not increased the cyclic AMP in control cells, it would appear that Mg^{2+} directly enhances the prostaglandin E_1 action probably by the formation of high affinity complex between prostaglandin E_1 and the adenylate cyclase coupled receptors. Such data have been recently shown by Williams et al. [10].

The results in Table I also show that Ca^{2+} stimulates the cyclic AMP production when present extracellularly but has an inhibitory effect when its influx into the cell is facilitated by ionophore A23187. It would appear that Ca^{2+} increases the cyclic AMP formation in control and prostaglandin E_1-treated cells at the extracellular site of the adenylate cyclase complex. In contrast, intracellular Ca^{2+} inhibits the cyclic AMP production. The experimental results are not so clear when ionophore and prostaglandin E_1 are employed without divalent cation supplementation. The decreased cyclic AMP formation in cells treated by prostaglandin E_1 and ionophore (28.9 pmol cyclic AMP/10^7 cells) against the value found in prostaglandin E_1-treated cells (40.2 pmol cyclic AMP/10^7 cells) may be due to the leakage of Ca^{2+} and Mg^{2+} from the cells causing decreased prostaglandin E_1 stimulation.

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