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THE ROLE OF Mg^{2+} IN HORMONE STIMULATION OF RAT OSTEOSARCOMA ADENYLATE CYCLASE

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Summary

1. Mg^{2+} concentration dependence of adenylate cyclase activity, in a rat osteosarcoma cell line (ROS 2/3), exhibits two apparent affinities with K_m values of approx. 2 mM and 10 mM.

2. Only one Mg^{2+} affinity with a K_m value of around 1 mM was apparent at saturating concentrations of: (i) guanosine-5'-(β,γ-imido)triphosphate; (ii) parathyroid hormone and GTP; and (iii) (—)-isoproterenol and GTP.

3. Conversely, at saturating concentrations of Mg^{2+} (40 mM) only high hormone concentrations, acting on low affinity sites, stimulated adenylate cyclase.

4. At saturating concentrations of guanosine-5'-(β,γ-imido)triphosphate, hormone stimulation decreased with increasing Mg^{2+} concentrations and none was seen at 40 mM Mg^{2+}.

The findings suggest that hormone stimulation of adenylate cyclase is associated with Mg^{2+} activation of a 'high hormone affinity' responsive state dependent on triphosphoguanine nucleotide. The hormone effect on Mg^{2+} affinity fully accounts for hormone stimulation of adenylate cyclase at physiologically relevant concentrations.

Introduction

Divalent cations are closely involved in the activity of phosphohydrolases including adenylate cyclase. Mg^{2+} is a constituent of the substrate complex,

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Abbreviations: ACTH, adrenocorticotropin; Gpp(NH)p, guanosine-5'-(β,γ-imido)triphopshate.

$MgATP^{2-}$ and was shown to be a requisite activator of the enzyme in many tissues [1—7]. Guanine nucleotide and hormone stimulation of adenylate cyclase were reported to increase the enzyme affinity for Mg^{2+} activation in skeletal [8], cardiac [9,10] and smooth muscle [11] as well as in brain [12—14]. In several systems kinetic analysis revealed multiple affinities (site heterogeneity) for Mg^{2+} dependence of enzymatic activity [3,10,14,15]. Above observations were recently confirmed in rat osteosarcoma adenylate cyclase [16] which, in addition, has the following features. (i) Site heterogeneity for Gpp(NH)p, isoproterenol and bovine parathyroid hormone activation of adenylate cyclase. (ii) High affinity hormone sites, being (kinetically) associated with triphosphoguanine nucleotide-containing complexes. (iii) Competition of the two hormones for stimulation of high-affinity adenylate cyclase complexes. (iv) Additive low affinity hormone effects, probably due to independent interaction of bovine parathyroid hormone and isoproterenol with GDP-containing complexes [17]. These findings were consistent with a high hormone affinity-enzyme activity state produced by the concomitant presence of hormone and triphosphoguanine nucleotide. In this study we examined the role of Mg^{2+} in hormone activation of adenylate cyclase in the context of above features.

Materials and Methods

Tissue culture materials, radiochemicals and other chemicals were purchased as described in Ref. 17.

Determination of adenylate cyclase activity

Particulate fractions were prepared as described previously [17]. Adenylate cyclase activity was measured at 30°C according to Salomon et al. [18]. The standard incubation mixture contained 25 mM Tris-HCl (pH 7.8)/0.2 mM $MgATP^{2-}$/1 mM dithiothreitol/1 mM cyclic AMP/5 mM phosphocreatine/6.2 U phosphocreatine kinase and between $2 \cdot 10^6$—$3 \cdot 10^6$ cpm of [α-^{32}P]ATP. The concentrations of Mg^{2+} were calculated by solving the multiple equilibria equations describing the interaction of Mg^{2+} with the anionic species of ATP and EGTA [16]. EGTA concentration was 0.1 mM. The reaction was initiated by the addition of protein (5—10 μg) to yield a final volume of 100 μl. The assay was run for 7 min unless stated otherwise. The reaction was linear with protein concentrations in the range used, for at least 15 min. When Gpp(NH)p was present, the enzyme was preincubated with nucleotide for 5—7 min at 30°C. All measurements were done in triplicate and the mean coefficient of variation was 5%. Kinetic constants were computed by numerical curve fitting [19].

Results

The effects of bovine parathyroid hormone, (—)-isoproterenol and Gpp(NH)p on Mg^{2+} dependence of adenylate cyclase activity

As seen in Fig. 1, the Mg^{2+} dependence of adenylate cyclase activity at 5 μM GTP exhibited site heterogeneity reflected in the non-linear (concave downward) Likeweaver-Burk plot. Curve fitted Mg^{2+} apparent K_m values were around 10 mM (range among experiments 7.7—14.5 mM) for the low affinity

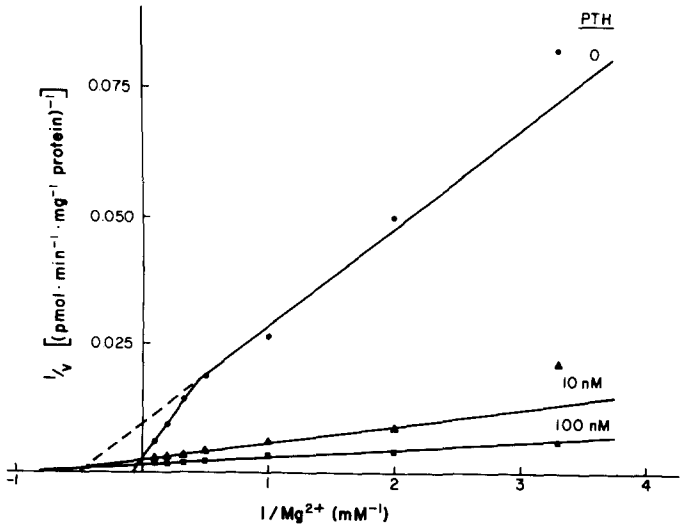

Fig. 1. The effect of bovine parathyroid hormone (PTH) on Mg^{2+} dependence of adenylate cyclase. Lineweaver-Burk plot. Adenylate cyclase was assayed on 8.7 μg protein for 10 min. GTP (5 μM) and bovine parathyroid hormone were present in the assay mixture when protein was added. Values are the means of triplicate determinations.

site and around 2 mM (range among experiments 1.3—3.4 mM) for the high affinity sites. The presence of bovine parathyroid hormone 'linearized' the Mg^{2+} dependence yielding apparent K_m values of 1.6 mM at 10 nM bovine parathyroid hormone and 1.1 mM at 100 nM bovine parathyroid hormone. Bovine parathyroid hormone at 10 nM thus increased the affinity for Mg^{2+} but had no effect on V, whereas 100 nM bovine parathyroid hormone increased both the enzyme V and Mg^{2+} affinity. (—)-Isoproterenol had the same effect. Fig. 2 shows the Mg^{2+} dependence of adenylate cyclase in the low affinity Mg^{2+} range (2—30 mM) in the presence and absence of 1 μM (—)-isoproterenol. This relatively low concentration of hormone increased the apparent Mg^{2+} affinity from a K_m of 14.5 mM to 1.3 mM without affecting V.

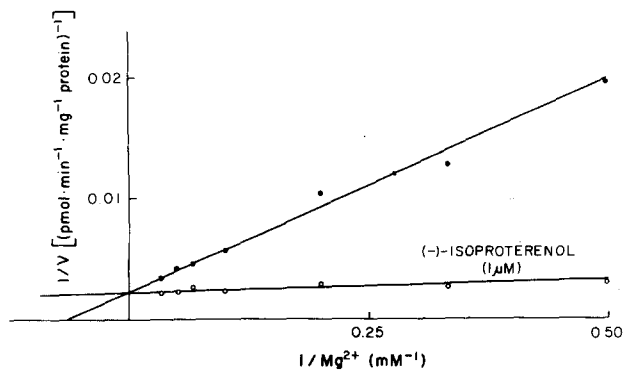

Fig. 2. The effect of (—)-isoproterenol on Mg^{2+} dependence of adenylate cyclase. Lineweaver-Burk plot. Adenylate cyclase was assayed on 7—8 μg protein for 7 min. GTP (5 μM) and (—)-isoproterenol were present in the assay mixture when protein was added. Values are the means of triplicate determinations.

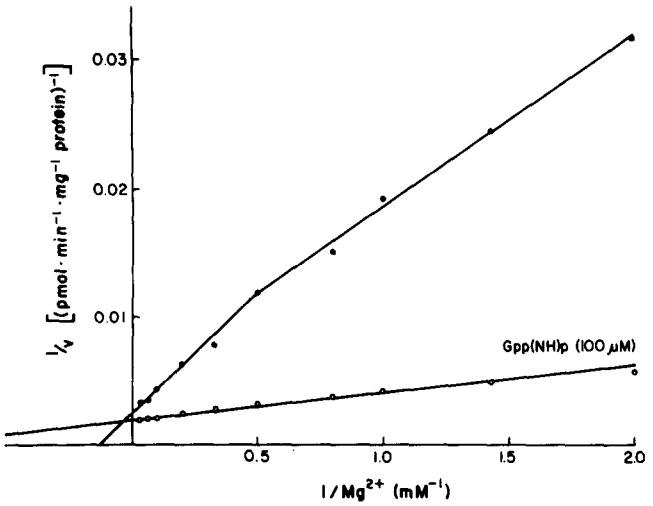

Fig. 3. The effect of Gpp(NH)p on Mg^{2+} dependence of adenylate cyclase. Lineweaver-Burk plot. Adenylate cyclase was assayed on 8 μg protein for 7 min after 5 min incubation with Gpp(NH)p. Values are the means of triplicate determinations.

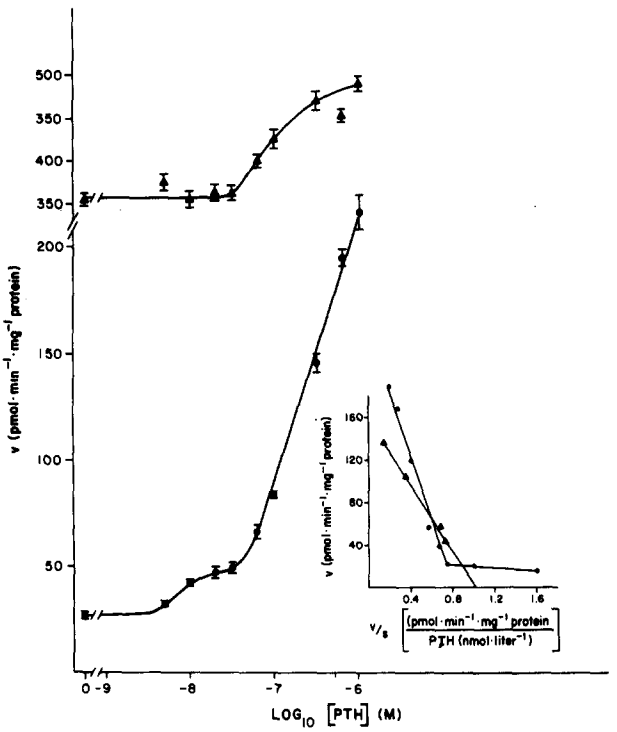

Fig. 4. The effect of Mg^{2+} on bovine parathyroid hormone stimulation of adenylate cyclase. Adenylate cyclase was assayed on 8 μg protein. Mg^{2+} concentrations were 1 mM (●——●) and 40 mM (▲——▲). GTP (5 μM) and the concentrations of bovine parathyroid hormone indicated in the graph were present in the assay mixture when protein was added. Values are the difference between means of triplicate determinations of hormone-stimulated minus basal activity. Insert: Eadie-Hofstee plot of the same data. PTH, bovine parathyroid hormone.

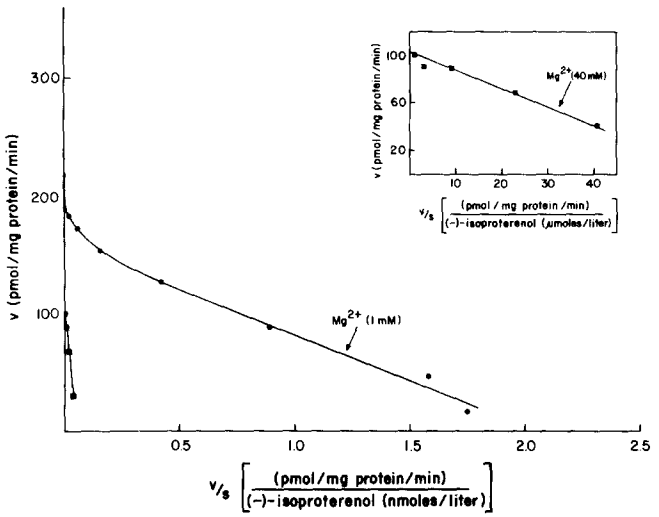

Fig. 5. The effect of Mg^{2+} on (—)-isoproterenol stimulation of adenylate cyclase. Eadie-Hofstee plot. Adenylate cyclase was assayed on 8 μg protein. Mg^{2+} concentrations were 1 mM (●———●) and 40 mM (■———■). GTP (5 μM) and various concentrations of (—)-isoproterenol were present in the assay mixture when protein was added. Values are the difference between means of triplicate determinations of enzyme activity in the presence of (—)-isoproterenol minus basal activity.

Gpp(NH)p, in the absence of hormones also linearized the Lineweaver-Burk Mg^{2+}-dependence plot as shown in Fig. 3. In the absence of nucleotide (no exogeneous GTP was added), the computed K_m values were 7.7 mM and 2.7 mM and in its presence 1.3 mM.

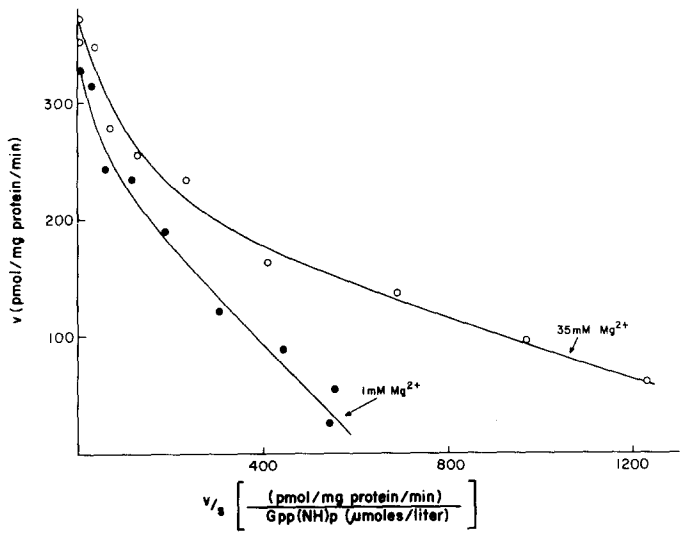

Fig. 6. The effect of Mg^{2+} on Gpp(NH)p stimulation of adenylate cyclase. Eadie-Hofstee plot. Adenylate cyclase was assayed on 9 μg protein for 7 min after 5 min incubation with the concentrations of Gpp(NH)p indicated in the graph. GTP (5 μM), 1 mM Mg^{2+} (●———●) and 35 mM Mg^{2+} (○———○) were present in the assay mixture when protein was added. Values are the difference between means of triplicate determinations of Gpp(NH)p stimulated minus basal activity.

GTP had a minor effect on Mg^{2+} affinity, only when it stimulated adenylate cyclase activity. Thus, K_m values of 10 and 5 mM were shifted to 7.8 and 3.4 mM. Although 'GTP-free' ATP was used in these experiments, the membranes were not cleaned of endogeneous GTP, which may explain the variability in GTP effects and in Mg^{2+} affinities of basal activities.

Effect of Mg^{2+} on PTH, (−)-isoproterenol and Gpp(NH)p stimulation of adenylate cyclase

Fig. 4 present bovine parathyroid hormone log-dose response curves for adenylate cyclase stimulation at low and high Mg^{2+} concentration. At 1 mM Mg^{2+}, bovine parathyroid hormone stimulation exhibited site heterogeneity (seen in Eadie-Hofstee insert) with apparent K_m of 7.9 and 330 nM. At 40 mM Mg^{2+}, only high bovine parathyroid hormone concentrations interacting with the low affinity sites were stimulatory (K_m 160 nM). Mg^{2+} had the same effect on (−)-isoproterenol adenylate cyclase stimulation as shown in the Eadie-Hofstee plots presented in Fig. 5. At 1 mM Mg^{2+}, isoproterenol stimulation exhibited site heterogeneity with apparent K_m values of 79 and 300 nM, whereas at 40 mM Mg^{2+} only high concentrations acting on low affinity sites were stimulatory.

Mg^{2+}, however, did not abolish (or mask) site heterogeneity for Gpp(NH)p stimulation, as shown in Fig. 6. The high affinity K_m decreased from 0.42 μM at 1 mM Mg^{2+} to 0.19 μM at 35 mM Mg^{2+}, but remained unchanged for the low affinity K_m of 1.0 μM and 1.1 μM, respectively.

As in the case of hormone stimulation the extent (-fold) stimulation by Gpp(NH)p was reduced at high Mg^{2+}. This is also seen in Table I which shows the effect of Mg^{2+} on combined Gpp(NH)p and hormone stimulation. Gpp(NH)p shifts the hormone stimulation to high affinity sites and under these conditions, no hormone stimulation occurs at 40 mM Mg^{2+}, and relatively less stimulation is seen at 5 mM Mg^{2+} as compared to 1 mM Mg^{2+}.

TABLE I

THE EFFECT OF Mg^{2+} ON HORMONE STIMULATION OF ADENYLATE CYCLASE IN THE PRESENCE OF Gpp(NH)p

Membranes were preincubated with Gpp(NH)p for 5 min at 30°C. Assays were started by adding assay mixture (at 30°C) containing the hormones and were run for 9 min. Values are means ±S.E. of triplicate determinations.

Gpp(NH)p	Mg^{2+} (mM)	Adenylate cyclase (pmol · min^{-1} · mg^{-1} protein)				
		Basal	Bovine parathyroid hormone		Isoproterenol	
			20 nM	300 nM	0.15 μM	5.0 μM
2 μM	1	105 ± 2	151 ± 6	302 ± 7	173 ± 14	255 ± 5
	5	211 ± 10	272 ± 7	378 ± 14	282 ± 2	344 ± 7
	40	405 ± 34	408 ± 13	405 ± 19	437 ± 3	396 ± 21
50 μM	1	127 ± 15	208 ± 4	317 ± 16	271 ± 1	355 ± 10
	5	263 ± 6	369 ± 10	462 ± 25	419 ± 11	525 ± 14
	40	458 ± 4	454 ± 17	476 ± 14	447 ± 16	486 ± 14

Discussion

This study documents site heterogeneity for Mg^{2+} activation of osteosarcoma-cell adenylate cyclase, kinetically resolvable into two apparent affinities with K_m values of around 2.0 and 10 mM. Adenylate cyclase stimulating hormones and Gpp(NH)p linearized the Lineweaver-Burk downward concave curve yielding one high affinity K_m for Mg^{2+} of around 1 mM. Hormone-dependent increases in Mg^{2+} affinity and non-hyperbolic Mg^{2+} dependence of adenylate cyclase, reflected in curvilinear double reciprocal plots or Hill slopes different from one, were previously observed in several tissues. In fat cells, Birnbaumer et al. [7] showed that ACTH increased the Mg^{2+} affinity for a 'regulatory' site approx. 6-fold. In liver, Londos and Preston [3] observed two apparent affinities with K_m values of approx. 0.9 and 6.0 mM, which shifted to the high affinity K_m in the presence of glucagon or GTP. Alvarez and Bruno [10] reported similar behaviour for cardiac adenylate cyclase where Mg^{2+} affinity increased and Hill plots approached unity in the presence of histamine, epinephrine and Gpp(NH)p. Narayanan and Sulakhe [9] showed in the same tissue that the K_m for Mg^{2+} shifted from 4 mM, under basal conditions, to 1.4 mM and 0.8 mM in the presence of Gpp(NH)p and isoproterenol with nucleotide, respectively. In skeletal muscle [8], isoproterenol with Gpp(NH)p, hormone alone, but not Gpp(NH)p alone, increased Mg^{2+} affinity; the approx. K_m values were 0.5 mM, 1.0 mM and 8 mM, respectively. In brain [12], histamine increased Mg^{2+} affinity for a 'regulatory' site from a K_m of 6 mM to approx. 2 mM. Similar observations were reported by Clement-Cormier et al. [13]. Our findings based on rigorous accounting for Mg^{2+} concentrations and the use of curve fitting for computation of kinetic constants [16] are in qualitative and quantitative agreement with above findings. The shift from low to high Mg^{2+} affinity may thus represent a mechanistic feature for hormone activation of adenylate cyclase.

The obvious question raised by this hypothesis is the relationship of Mg^{2+} to triphosphoguanine nucleotides which have been strongly implicated in hormone-adenylate cyclase coupling [20—23]. Comparison of the Mg^{2+} effects on hormone stimulation in the presence of GTP and Gpp(NH)p (Figs. 4 and 5 and Table I) offers some insight into this question. In the presence of GTP, there is an equilibrium between GTP and GDP-occupied nucleotide subunits ($N(GTP) \rightleftharpoons N(GDP)$). Under these conditions hormone stimulation of adenylate cyclase activity was still seen at saturating (35—40 mM) Mg^{2+} concentrations (Figs. 4 and 5). This stimulation required high hormone concentrations, which interact with kinetically distinguishable low affinity sites [17], presumably associated with the R (receptor) · N(GDP) · C (catalytic unit) complex. On the other hand, lower hormone concentrations interacting with high affinity sites, presumably R · N(GTP) · C complexes, were non-stimulatory at saturating Mg^{2+} concentrations, indicating that such stimulation would be totally accounted for by the association of Mg^{2+} with the enzyme complex. This is apparent from Fig. 4 where the double reciprocal Mg^{2+} dependence plot at 10 nM bovine parathyroid hormone intersects the basal activity on the ordinate, whereas the 100 nM bovine parathyroid hormone line shows a V effect when extrapolated to maximum Mg^{2+}. Identical observations were made for isoproterenol which competes with bovine parathyroid hormone for the activa-

tion of the high-hormone-affinity enzyme complex [17]. In the presence of Gpp(NH)p, which being non-hydrolyzable, shifts the equilibrium to *N*-(triphosphoguanine nucleotide) complexes and hormone activation to high affinity sites, hormone stimulation of adenykate cyclase was reduced with increasing Mg^{2+} concentrations (Table I) to the point where it was virtually absent at saturating Mg^{2+} concentrations. This substantiates the conclusions drawn above.

Mg^{2+} has also been shown to affect hormone binding [24,25] but these effects were of an enhancing nature and would not explain our data. The likely explanation is that the activation of the high-hormone affinity (N(GTP)) enzyme complex is due to Mg^{2+} binding, either as a result of the hormone-dependent rise in Mg^{2+} affinity or increased Mg^{2+} concentrations. This mechanism emphasizes the importance of (intracellular) Mg^{2+} in regulating the susceptibility of adenylate cyclase to hormone stimulation. Since Ca^{2+} is a competitive inhibitor of Mg^{2+} in this reaction [16], an increase in Ca^{2+} concentration would shut off enzyme activation. Ca^{2+} could rise, for example, as a result of a hormone-dependent increase in Ca^{2+} permeability. Interestingly, although Gpp(NH)p increased Mg^{2+} affinity by itself (in the absence of hormones), saturating Mg^{2+} concentrations did not abolish Gpp(NH)p enzyme stimulation or the apparent Gpp(NH)p site heterogeneity, pointing to mechanistic differences between hormone and nucleotide stimulation. GTP also increased Mg^{2+} affinity when it stimulated adenylate cyclase activity in the absence of hormones (data not shown). GTP stimulation varied, however, from preparation, possibly due to endogemous GTP or other constituents of the adenylate cyclase system, an observation which awaits further clarification.

The ROS 2/3 adenylate cyclase, thus, has the following Mg^{2+} related features: (i) Mg^{2+} is a requisite activator of adenylate cyclase [16]; (ii) the enzyme exhibits site heterogeneity with respect to Mg^{2+} with K_m values of approx. 2 and 10 mM, which are within the relevant 'physiological' range; (iii) Gpp(NH)p or hormones and GTP, shift the low affinity to the high one; (iv) this effect fully for hormone stimulation of adenylate cyclase via high-affinity, physiologically relevant, triphosphoguanine nucleotide-dependent sites; (v) Gpp(NH)p increases both Mg^{2+} affinity as well as enzyme velocity independent of Mg^{2+}.

These findings can easily be integrated into the current view on the proposed mechanisms for adenylate cyclase activation: thus, hormone promoted binding of Mg^{2+} could be necessary for GTP hydrolysis (or GDP release), occuring concomitantly with the adenylate cyclase catalytic step [23]; Mg^{2+} action is best located at the formation of the R · N(GTP) complex in the 'disaggregation-coupling' model [20] or the isomerization step in a two-state model [26—28]. On the other hand, Mg^{2+} may be required only for the adenylate cyclase catalytic step, in analogy to its proposed role in the sarcoplasmic reticulum ATPase [19] and become rate-limiting in the activated enzyme.

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