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Hormonal Inhibition of Adenylate Cyclase

A Crucial Role for Mg^{2+}

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SUMMARY

In several adenylate cyclase systems (anterior pituitary gland, human platelets, adipocytes, rat liver membranes), inhibitory hormones were shown to reduce basal adenylate cyclase activity by decreasing the "apparent affinity" of those systems for Mg^{2+} activation, without modifying the V_{max} of the reaction. In the absence of hormones, the Mg^{2+} dose-activation curves were monophasic, whereas in the presence of hormones a clear heterogeneity was revealed. Therefore, inhibitory hormones induced a right-hand shift in the Mg^{2+} dose-activation curve. This hormonal effect was concentration-dependent. In human platelets, the inhibition of prostaglandin E_1-stimulated adenylate cyclase by norepinephrine was also due to a decrease in the apparent affinity for Mg^{2+}. In anterior pituitary gland, when Mg^{2+} was substituted by Mn^{2+}, similar results were obtained. Thus, dopamine produced its inhibition by decreasing the apparent affinity for Mn^{2+} both under basal and vasoactive intestinal peptide-stimulated conditions. At Mg^{2+} or Mn^{2+} concentrations high enough to obtain saturation of the low apparent affinity state, hormone-induced inhibition was not observed. In anterior pituitary gland and in human platelet membranes, Na^+ was not required in order to observe adenylate cyclase inhibition by catecholamines. In adipocytes and rat liver membranes, however, Na^+ was required. In both systems, GTP was able to transform adenylate cyclase to a low Mg^{2+} apparent affinity state. Na^+ was able to reverse (in a dose-dependent manner) the system to a high Mg^{2+} apparent affinity state. Once in this state, hormones were shown to inhibit adenylate cyclase activity by reverting the enzyme to a low apparent affinity state for Mg^{2+}.

INTRODUCTION

More than 20 years after the discovery of cyclic AMP, we are beginning to understand the molecular mechanisms by which hormones and neurotransmitters stimulate adenylate cyclase. At least three proteins are involved: a receptor protein, a catalyst, and a distinct nucleotide-binding regulatory component (1) called *N* or N_s (2, 3), G/F (4), or G (5). This N_s protein is composed of two subunits, a *beta* subunit of 35,000 daltons, and an *alpha* subunit that shows size heterogeneity, being between 42,000 and 52,000 daltons in mass. The *alpha* subunit is ADP-ribosylated by cholera toxin and contains the guanine nucleotide-activating site (4, 6–9). It has been clearly demonstrated that N_s activation is the rate-limiting step in adenylate cyclase stimulation (10–13). Activation seems to be associated with dissociation of the *alpha* from the *beta* subunit (3, 7). Studies on the mechanism of N_s activation have provided evidence for a central role for Mg^{2+} (3, 7). Both the binding of guanine nucleotides and the kinetics of N_s activation are Mg^{2+}-dependent (3, 7). Earlier and more recent studies demonstrated that adenylate cyclase activity is dependent not only on the presence of the substrate ATP-Mg^{2+} but also on an excess of Mg^{2+}, acting at allosteric sites. There is evidence for the existence of at least two Mg^{2+} sites, one on the stimulatory regulatory protein (3, 7) and the other on the catalytic unit (14–17). It has been repeatedly shown that hormones activating adenylate cyclase increased the "apparent affinity" of the enzyme for Mg^{2+} (for review see ref. 16). Thus, such an increase may be the key mechanism by which hormones modulate the activation of N_s (3, 16). Iyengar and Birnbaumer (3) have therefore termed hormone occupation of receptor as a "Mg^{2+} switch."

In addition to receptors which induce adenylate cyclase activation, some receptors mediate adenylate cyclase inhibition. We are also beginning to understand the process of adenylate cyclase inhibition. A different nucleotide-binding protein, called N_i, is involved (2, 18, 19). N_i is also a protein composed of a *beta* subunit of 35,000 daltons and an *alpha*-subunit, which is 39,000–41,000 daltons in mass (20, 21). The *alpha* subunit of N_i is ADP-ribosylated by the toxin of *Bordetella pertussis* (20–22).

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In view of the importance of Mg^{2+} in adenylate cyclase stimulation, we have investigated in some detail the role of this ion in adenylate cyclase inhibition.

EXPERIMENTAL PROCEDURES

Membrane Preparation from Anterior Pituitary Lobe

Female Wistar rats (200–250 g) were killed by decapitation. Six anterior pituitary lobes were homogenized in a glass-Teflon Potter homogenizer in 1 ml of 1 mM Tris-maleate buffer (pH 7.2), 1 mM EGTA,[1] and 300 mM sucrose (TES medium). Two milliliters of the same medium without sucrose were added. Membranes were pelleted by centrifugation at 10,000 × *g* and resuspended in 1.5 ml of TES.

Membrane Preparation from Human Platelets

Platelet-rich plasma containing sodium citrate (0.5%) was obtained from the Montpellier Centre de Transfusion Sanguine. Platelet membranes were prepared according to the method of Insel *et al.* (23).

Preparation of Hamster Fat Cell Ghosts

Male golden hamsters (80–120 g) were killed by decapitation, and ghosts from epididymal fat cells were prepared as described by Jakobs and Aktories (24). Ghosts were kept at −70° in 1 mM $KHCO_3$.

Preparation of Liver Plasma Membranes

Membranes were prepared as previously described (25).

Adenylate Cyclase Assays

The incubation medium always contained 1–2 μCi of [α-^{32}P]ATP, 0.001 μCi of [^{3}H]cyclic AMP, 5 mM creatine phosphate, and creatine kinase (0.2 mg/ml) plus the specific components specified below. [α-^{32}P]cyclic AMP was purified as previously described (26).

Pituitary membranes. Pituitary membranes were added to the assay mixture (50 μl), which contained 50 mM Tris-maleate (pH 7.2), 1 mM cyclic AMP, ATP concentrations as specified in the figure legends, 0.01 mM GTP, 10 mM theophylline, and the Mg^{2+} concentrations required. The mixture was incubated for 10 min at 30°.

Platelet membranes. Platelet membranes were added to the assay mixture (50 μl), which contained 50 mM triethanolamine HCl (pH 7.4), 0.1 mM EGTA, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM cyclic AMP, 0.1 mM ATP, 0.01 mM GTP, 0.2% (w/v) bovine serum albumin, 5 μM propranolol, and the Mg^{2+} concentrations required. The mixture was incubated for 20 min at 30°.

Fat cell ghosts. Fat cell membranes were added to the assay mixture (50 μl), which contained 50 mM triethanolamine HCl (pH 7.4), 0.05 mM ATP, 0.01 mM GTP, 1 mM 3-isobutyl-1-methylxanthine, 5 mM dithiothreitol, 0.2% (w/v) bovine serum albumin, and the Mg^{2+} concentrations required. The mixture was incubated for 10 min at 25°.

Liver membranes. The adenylate cyclase assay was carried out as previously described (25).

Mg^{2+} and ATP Concentrations

The free Mg^{2+} concentrations were calculated as described in ref. 3 by using the formula

$$\text{Mg total} = \text{Mg-free}\left[1 + \frac{A\text{ total}}{(\text{Mg-free} + K_A)} + \frac{B\text{ total}}{(\text{Mg-free} + K_B)}\right]$$

in which *A* total and *B* total are the concentrations of added ATP and EDTA (when present), and K_A and K_B are the stability constants of Mg-ATP (60 μM) and Mg-EDTA (0.4 μM).

Concentrations of the substrate Mg-ATP were calculated as proposed by Garbers and Johnson (27) and were found to vary by less than 10% when free Mg^{2+} concentrations ranged from 0.5 to 50 mM. All experiments were performed at least twice, and values reported are the means of duplicates. The maximal difference between the two determinations never exceeded 5%.

Materials

[α-^{32}P]ATP and [^{3}H]cyclic AMP were purchased from New England Nuclear Corporation (Boston, Mass.). (Asn^1, Val^5)-Angiotensin II was obtained from UCB Bioproducts; other compounds were purchased from Sigma Chemical Company (St. Louis, Mo.).

RESULTS

Effects of DA and VIP on the apparent K_m for Mg^{2+} and Mn^{2+} in the anterior pituitary adenylate cyclase system. Anterior pituitary gland adenylate cyclase is inhibited by DA through a D_2-dopaminergic receptor (28). In a preliminary study, we have shown that DA decreases the apparent affinity for Mg^{2+} activation without affecting the maximal activation obtained (29). This was confirmed in the experiment illustrated in Fig. 1, which also shows that the right-hand shift of the Mg^{2+} activation curve is a function of DA concentration. The Hofstee plots of the data also indicate that, under basal conditions, the Mg^{2+} dose-activation curve contained only one component (apparent affinity 1 mM), whereas in the presence of DA two components were observed, one having roughly the initial apparent affinity (Component *1*, Fig. 1) and the second having a much lower affinity (Component *2*, Fig. 1).

Similar results were obtained at low and high ATP concentrations (0.06 and 0.6 mM ATP) (Fig. 1). At both ATP concentrations, DA inhibition was the result of a decrease in the apparent affinity for Mg^{2+} (Fig. 1). A slightly lower percentage of inhibition was observed at higher ATP concentrations. When Mg^{2+} was substituted by Mn^{2+}, DA also decreased both basal and VIP-stimulated adenylate cyclase activities by shifting the Mn^{2+} dose-activation curve to the right (Fig. 2). Therefore, at Mn^{2+} concentrations high enough to saturate the low apparent affinity component (1–1.5 mM), DA was unable to inhibit adenylate cyclase (Fig. 2). At those Mn^{2+} concentrations, VIP stimulation was unaffected (Fig. 2). Much higher concentrations were needed to suppress VIP stimulation (Fig. 2). In the anterior pituitary gland, DA inhibition of adenylate cyclase was obtained in the absence of added Na^+. Furthermore, increasing concentrations of Na^+ did not significantly increase the inhibitory effect of DA on basal or VIP-stimulated adenylate cyclase activities (Fig. 3).

Effects of NE, PGE_1, and PGE_1 plus NE on apparent K_m for Mg^{2+} in the human platelet adenylate cyclase system. In human platelets, Na^+ does not appear to be required for adenylate cyclase inhibition by hormone (30, 31). This finding is confirmed in results shown in Fig. 4. As reported by Steer and Wood (31), increasing Na^+ concentrations decreased the basal adenylate cyclase system to a level similar to the level obtained with NE alone in the absence of Na^+ (Fig. 4). Therefore, very little adenylate cyclase inhibition was obtained with NE in the presence of high concentrations of Na^+ (Fig. 4) (31).

In the absence of Na^+ (Fig. 5A), NE increased the Mg^{2+} concentration required to obtain half-maximal

[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DA, dopamine; VIP, vasoactive intestinal peptide; NE, norepinephrine; PGE_1, prostaglandin E_1.

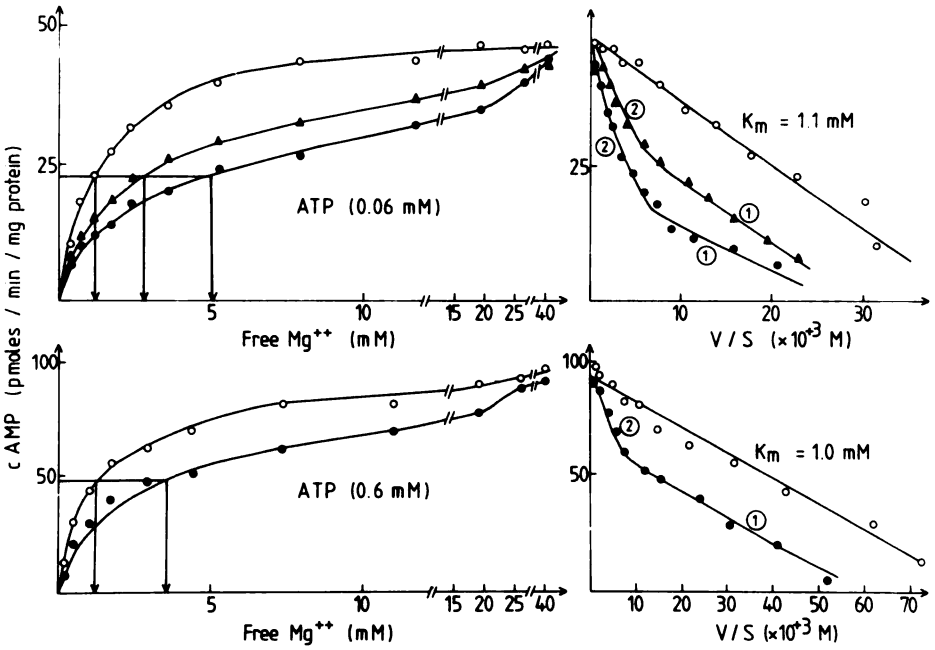

FIG. 1. *Basal and DA-inhibited adenylate cyclase activities as the function of free Mg^{2+} concentrations in anterior pituitary gland mmbranes* Left, direct representation; right, Hofstee plots. ATP concentrations are given on the figure. ○, Basal activities; ▲, 0.5 μM DA; ●, 10 μM DA.

stimulation. This right-hand shift of the Mg^{2+} dose-activation curve was NE concentration-dependent (Fig. 5A). Hofstee plots indicated that, whereas the basal dose-activation curve was composed of a simple hyperbolic component (apparent K_m = 3 mM), the addition of NE introduced heterogeneity (Fig. 5A). The plots obtained with increasing concentrations of NE suggest that, as the NE concentration increased, the proportion of the high-affinity component decreased, whereas the proportion of the low-affinity component increased (Fig. 5). PGE_1-stimulated adenylate cyclase was inhibited by NE, producing an increase in the free Mg^{2+} concentration needed to produce half-maximal enzyme activation (Fig. 5B).

The adipocyte adenylate cyclase system. The adipocyte adenylate cyclase system presents two characteristics which were not observed in the two preceding systems: (a) Na^+ is absolutely necessary to obtain hormonally induced adenylate cyclase inhibition (24, 30, 32). (b) GTP concentrations required to obtain adenylate cyclase inhibition by hormone lower the enzyme activity (2, 33). We therefore decided to investigate the effects of GTP, Na^+, and agonist on the Mg^{2+} affinity of the adipocyte system (Fig. 6). The first finding was that GTP alone inhibited the enzyme by lowering the apparent affinity for Mg^{2+}. In the presence of GTP, this apparent affinity decreased from 0.6 to 25 mM (Fig. 6).

The second surprise finding was that, by adding Na^+ in the presence of GTP, the system was returned to a high-affinity state (1 mM). This Na^+ effect was concentration-dependent (Fig. 7). In the presence of both GTP and Na^+, nicotinic acid was able to inhibit the system by again reducing the Mg^{2+} apparent affinity (Fig. 6). Similar results have been obtained by Jakobs and Aktories (24) using the same system. However, these authors did not interpret their data as modifications of Mg^{2+} affinity.

The liver membrane adenylate cyclase system. As in the adipocyte adenylate cyclase system, GTP inhibited the enzyme by lowering the apparent affinity for Mg^{2+}; Na^+ returned the system to a high Mg^{2+} apparent affinity (this apparent affinity was even higher in the presence of GTP + Na^+ than in the presence of Na^+ without GTP). Once in this high Mg^{2+} affinity state, epinephrine and angiotensin II decreased the adenylate cyclase by reducing the apparent affinity for Mg^{2+} to a level obtained with GTP alone (Fig. 8).

DISCUSSION

As already demonstrated for adenylate cyclase stimulation (3, 7, 13, 16) we present evidence for a crucial role for Mg^{2+} in adenylate cyclase inhibition. There seems to be an over-all symmetrical effect of stimulating and inhibiting hormones on Mg^{2+} affinity of the adenylate cyclase systems. Stimulating hormones increase the apparent affinity for Mg^{2+} (see Figs. 5 and 3, and ref. 16) and the V_{max} of the reaction. As shown in this report, inhibitory hormones decrease the apparent affinity for Mg^{2+} without significantly changing the V_{max} of the reaction. Jakobs *et al.* (34) have reported completely different results. In the human platelet adenylate cyclase system, they showed that *alpha*-adrenergic receptor-mediated inhibition was the result of a decrease in V_{max} with no reduction in the affinity for Mg^{2+} (34). The most likely explanation for this discrepancy in interpretation is that these authors studied the effect of NE on Mg^{2+} affinity of the platelet enzyme only at low Mg^{2+} concentrations (0.4–8 mM). Indeed, at these low Mg^{2+} concentrations, only the high Mg^{2+} affinity component was measured (Component *1* in Fig. 1). The low "Mg^{2+} affinity component" induced by NE was not detected (Com-

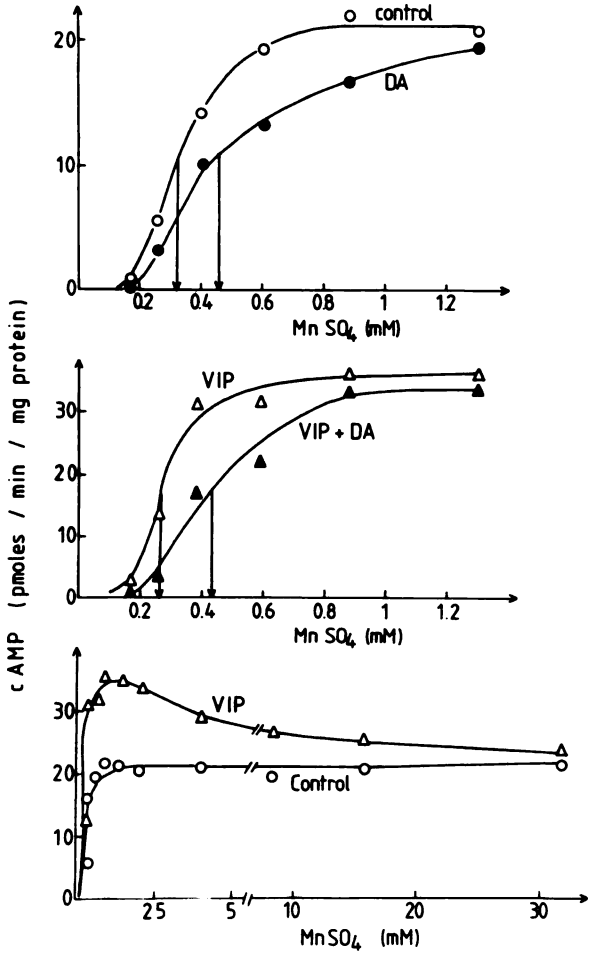

FIG. 2. *Basal, DA, VIP, and VIP plus DA adenylate cyclase activities as the function of total Mn^{2+} concentrations*

ATP concentration was 0.15 mM; VIP and DA concentrations were 0.1 μM and 10 μM, respectively.

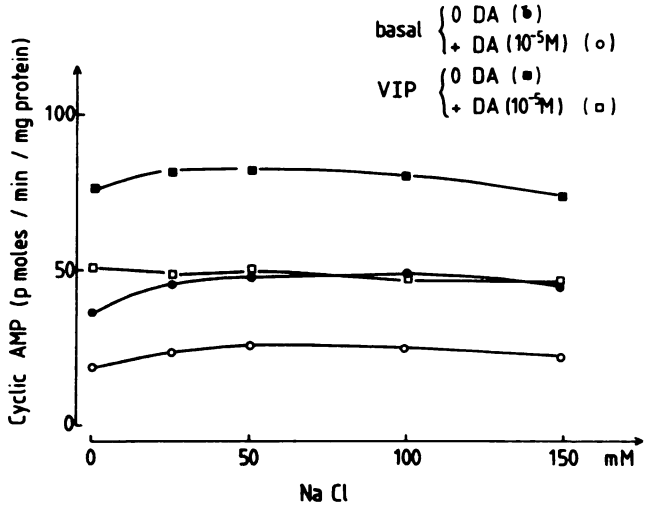

FIG. 3. *Effect of NaCl on the inhibition by DA of basal and VIP-stimulated adenylate cyclases in anterior pituitary gland membranes*

ATP concentration was 0.15 mM. Creatine phosphate-Tris was used instead of creatine phosphate Na^+. The VIP concentration was 0.1 μM.

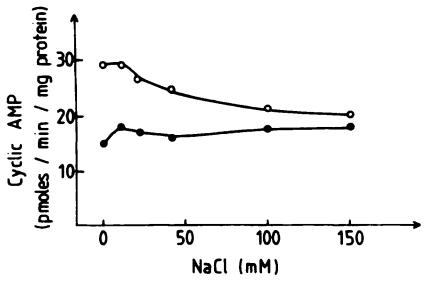

FIG. 4. *Effect of NaCl on basal and NE-inhibited adenylate cyclase activities in human platelet membranes*

Basal (O) and NE (10 μM)-inhibited (●) adenylate cyclase activities. Creatine phosphate-Tris was used instead of creatine phosphate Na^+.

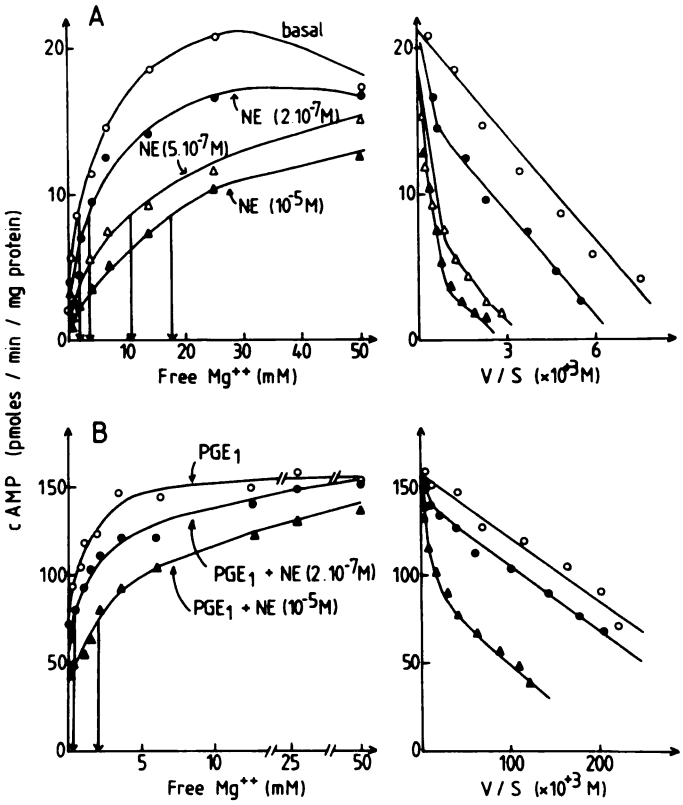

FIG. 5. *Effect of NE on basal and PGE_1-stimulated adenylate cyclase activities in the presence of different free Mg^{2+} concentrations in human platelet membranes*

A. Control conditions: direct representation (left) and Hofstee plots (right). B. PGE_1-stimulated adenylate cyclase activities: direct representation (left) and Hofstee plots (right). The PGE_1 concentration was 10 μM.

ponent 2 in Fig. 1). Therefore, these authors concluded an absence of modification of Mg^{2+} affinity during adenylate cyclase inhibition (34).

As pointed out by Cech and Maguire (15), Cech *et al.* (16), and Iyengar and Birnbaumer (3), the alterations in Mg^{2+} requirement may be of physiological relevance concerning hormone-induced adenylate cyclase activation and inhibition. Indeed, it appears that hormone regulation of the adenylate cyclase systems may not be the result of a modulation in N_s and N_i occupation by guanine nucleotides, as the intracellular concentrations of

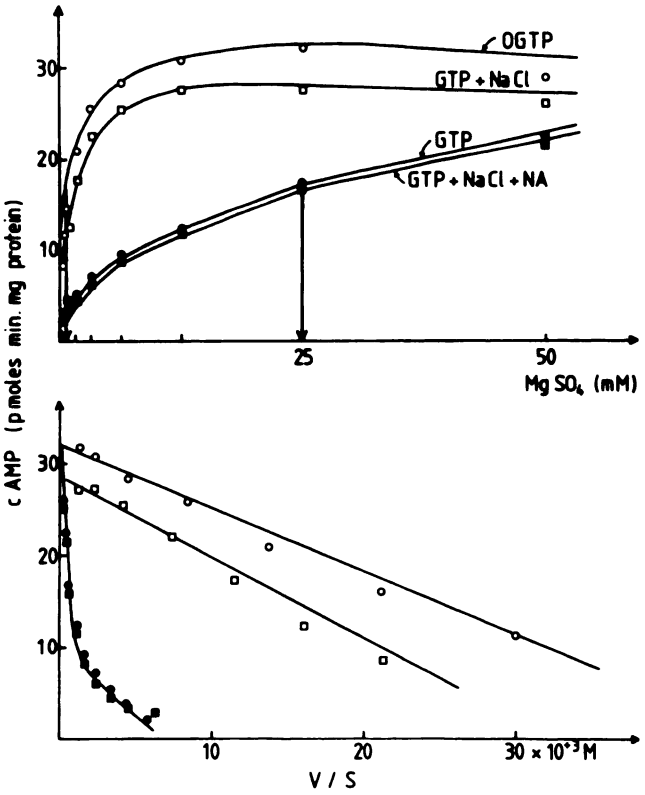

FIG. 6. *Effect of different experimental conditions on the apparent affinity of adipocyte membrane adenylate cyclase for* Mg^{2+}

Top, direct representation; bottom, Hofstee plots. GTP and NaCl concentrations were 10 μM and 150 mM, respectively.

these compounds are high enough (>100 μM) to largely saturate these nucleotide-binding sites.

In contrast, the "basal" K_m for free Mg^{2+} (1–3 mM) (see, for example, Figs. 1, 5, 6, and 8) is substantially higher than the intracellular concentration for free Mg^{2+}, which is generally about 0.3–1 mM, depending on the cell type (15, 35–37). Under these conditions, it is clear that a 10-fold decrease in the affinity for Mg^{2+} will result in a considerable reduction of adenylate cyclase activity in intact cells.

In the absence of hormone, the Mg^{2+} dose-activation curve was found to follow simple Michaelis-Menten kinetics in most of the systems tested in this study. Similar results have been reported in other adenylate cyclase systems (15–27). This apparent homogeneity may be surprising, as several authors have presented evidence suggesting that Mg^{2+} sites exist on both the catalytic unit and the GTP-binding proteins (at least on N_s) (14–17). Although speculative, it is possible that all of these sites have a similar apparent K_m for Mg^{2+} under basal conditions and that differential modifications of these affinities occur in the presence of hormones.

In addition to demonstrating that receptor-mediated inhibition by hormones may be considered analogous to those mechanisms activated by stimulatory hormones, i.e., "Mg^{2+} switches," our results may serve to reexamine two problems that have arisen from other adenylate cyclase inhibition studies. The first problem concerns the effect of Mn^{2+} on hormone-induced adenylate cyclase inhibition. Hoffman *et al.* (38) have shown that, in human platelets, relatively low concentrations of Mn^{2+} (1–2 mM) suppress *alpha*-adrenergic receptor-mediated adenylate cyclase inhibition, whereas much higher Mn^{2+} concentrations (1–10 mM) can suppress PGE_1 receptor-mediated adenylate cyclase stimulation. The uncoupling

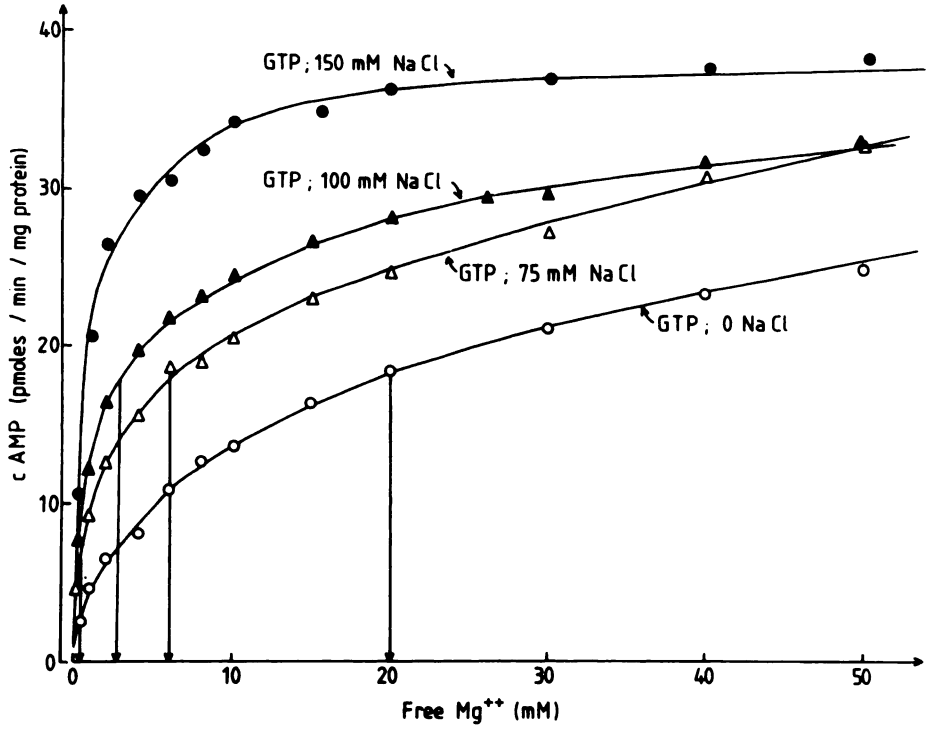

FIG. 7. *Effect of different* Na^+ *concentrations on the apparent affinity of the GTP-inhibited adenylate cyclase of adipocytes for* Mg^{2+}

GTP concentration was 10 μM.

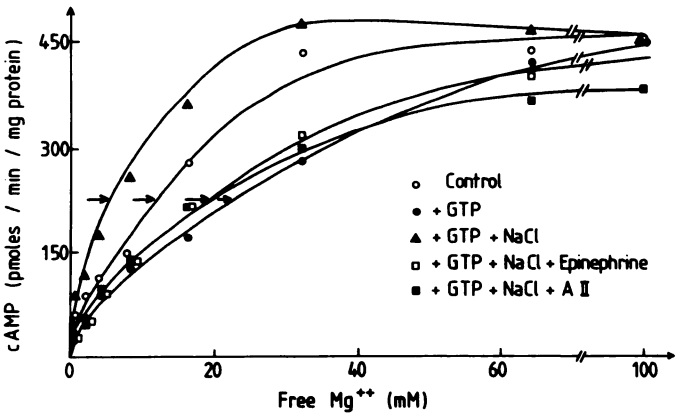

FIG. 8. *Effect of different experimental conditions on the apparent affinity of hepatocyte membrane adenylate cyclase for Mg^{2+}*

GTP, NaCl, epinephrine, and angiotensin II (*A II*) concentrations were 0.1 mM, 200 mM, 0.1 mM, and 10 μM, respectively.

effect of high Mn^{2+} concentrations on adenylate cyclase stimulation by hormones has also been reported in several other systems (16–39). We have found similar results in studies of pituitary gland membranes: VIP-stimulated adenylate cyclase was progressively attenuated when Mn^{2+} concentrations increased from 1 to 30 mM, whereas basal enzyme activity was unaffected (Fig. 2). Concerning the effect of low Mn^{2+} (1–2 mM) concentrations on receptor-mediated adenylate cyclase inhibition, our interpretation is slightly different from the one proposed by Hoffman *et al.* (38). If our suggestion that hormonally induced adenylate cyclase inhibition is due to a rightward shift of the divalent cation dose-response curve without modification of the V_{max} is correct, it may be expected that, at Mn^{2+} concentrations producing this V_{max}, the hormone is unable to exert its effect (Fig. 2).

The second problem concerns the possible role for Na^+ in hormone-dependent adenylate cyclase inhibition. In some, but not all, adenylate cyclase systems, Na^+ seems to be required in order to obtain an adenylate cyclase inhibition. Our findings suggest that in two Na^+-dependent systems, i.e., adipocyte and hepatocyte membranes, GTP inhibited the enzyme by shifting it to a low Mg^{2+} apparent affinity state (Figs. 6 and 8). Therefore, in this state, inhibitory hormones cannot produce their effects, which are to bring the system under this same low Mg^{2+} affinity state. In those systems, Na^+ appears to restore the system to its high Mg^{2+} affinity state (Figs. 6 and 7), this Na^+ effect being concentration-dependent (Fig. 7). The molecular mechanism of such an effect and its physiological relevance remain unclear. Once in a high Mg^{2+} apparent affinity state, hormones can produce their inhibition by switching the system back to a low apparent affinity state (Figs. 6 and 8).

In conclusion, it has been previously shown that the dissociation of the two N_s subunits is a Mg^{2+}-dependent process which appears to be the rate-limiting step in adenylate cyclase activation (7–10). Stimulatory hormones, by increasing the apparent affinity for Mg^{2+}, enhance the dissociation of N_s subunits and therefore adenylate cyclase activity (3). In this context, the kinetic experiments reported here showing that inhibitory hormones decrease the apparent affinity for Mg^{2+} of several adenylate cyclase systems may help to propose future molecular models of adenylate cyclase inhibition.

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