Magnesium ion exerts a central role in the regulation of inhibitory adenosine receptors

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Guanine nucleotides and Mg^{2+} differentially regulate agonist binding to adenosine (R_i) receptors in fat-cell plasma membranes. GTP alone decreases binding of the agonist ligand [3H]N⁶-cyclohexyladenosine (CHA) by increasing the dissociation constant (K_d). Mg^{2+} alone also decreases [3H]CHA binding, which is associated with a decrease in the number of receptors and in the dissociation constant. In the presence of Mg^{2+} , the effect of GTP is to increase [3H]CHA binding by increasing the total number of receptors. It thus appears that Mg^{2+} acts specifically at a bivalent-cation site which, with GTP, regulates agonist binding. This putative Mg site is highly sensitive to alkylating agents. Mild treatment with *N*-ethylmaleimide (NEM) abolishes the characteristic GTP effect on agonist binding in the presence of Mg^{2+} . In addition, the effect of Mg^{2+} alone is also eliminated. The effect of GTP alone is largely unaltered. Studies of the adenylate cyclase activity indicate that this NEM treatment also abolishes the inhibition of basal activity by adenosine analogues, whereas guanylyl imidodiphosphate inhibition of forskolin-stimulated activity is only slightly impaired at this NEM concentration. These observations indicate that a Mg^{2+} 'site' or 'component' is required for the integration of receptor (R_i) occupancy with regulation of catalytic activity (C). The regulatory role of Mg^{2+} is more demonstrable in receptor–GTP–regulatory-protein (R_i – N_i) interactions than in GTP–regulatory-protein–catalytic-unit (N_i –C) interactions.

The adenosine receptor associated with adipocyte adenylate cyclase is typical of those neurotransmitter receptors that inhibit the enzyme's activity in numerous tissues (Londos *et al.*, 1981). This receptor regulates enzyme activity through a GTP-regulatory-protein complex (N_i) that is distinct from that which mediates stimulation of the enzyme (N_s) (Jakobs, 1979; Rodbell, 1980; Limbird, 1981; Cooper, 1982; Hildebrandt *et al.*, 1983; Gilman, 1984). The GTP-regulatory complex modulates enzyme activity via a GTP-hydrolytic cycle involving heterotropic interactions with the

receptor and catalytic unit (Cassel & Selinger, 1976; Ross & Gilman, 1980; Aktories & Jakobs, 1981; Koski & Klee, 1981; Aktories *et al.*, 1982; Michel & Lefkowitz, 1982). Considerable progress has been made on characterizing the two types of GTP-regulatory complex. Both appear to comprise at least three subunits, one of which is unique and two that are common to both complexes. The unique subunit associated with N_i can be ADP-ribosylated by the toxin elicited by *Bordetella pertussis*, which is known as islet-activating protein (IAP) (Katada & Ui, 1982*a,b*; Gilman, 1984). At present, the most direct functional demonstration that the IAP substrate is a component of N_i that mediates hormone inhibition rests on the attenuation by IAP of hormonal inhibition and the reversal of this attenuation by the addition of exogenous (non-treated) IAP substrate (Katada & Ui, 1982*a,b*; Katada *et al.*, 1984). Such evidence clearly implicates the IAP substrate in an N_i com-

Abbreviations used: CHA, N⁶-cyclohexyladenosine; PIA, N⁶-phenylisopropyladenosine; p[NH]ppG, 5'-guanylyl imidodiphosphate; NEM, *N*-ethylmaleimide.

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plex, although it does not exclude the possibility that other subunits might be required for the promotion of hormonal inhibition. The most direct approach to enumerating the number of components of N_i would be the demonstration of interaction of resolved components (receptor and GTP-regulatory elements) in detergent solution. Although success has been achieved along these lines with the stimulatory β -adrenergic receptor and the purified N_s components in phospholipid micelles (Brandt *et al.*, 1983; Caron *et al.*, 1985), the same situation has not been described for inhibitory receptors. It is our contention that full reconstitution of N_i - R_i (receptor) interactions in solution might not be achieved until characterization of these interactions has been delineated in native membranes. The present report addresses a possible role for a bivalent-cation regulatory site that may be central to the integration of R_i - N_i and N_i -C (catalytic activity) communication.

Considerable precedent exists in the literature for a role for Mg^{2+} in inhibitory regulation. Previous studies have indicated that both Mg^{2+} and GTP can affect inhibitory receptor binding (Tsai & Lefkowitz, 1979; U'Prichard & Snyder, 1980; Yeung & Green, 1983). Mg^{2+} and p[NH]ppG also modulate the sedimentation characteristics of the IAP substrate on sucrose gradients (Codina *et al.*, 1983). It has also been known for a considerable time that bivalent cations, most effectively Mn^{2+} , but also Mg^{2+} at higher concentrations, uncouple hormonal inhibition of adenylate cyclase (Cooper *et al.*, 1979; Jakobs & Aktories, 1981; Hoffman *et al.*, 1981). Bockaert *et al.* (1984) have proposed that inhibitory ligands act to decrease the affinity of the catalytic unit for Mg^{2+} , whereas stimulatory ligands increase this affinity. It is unlikely that all of the reported effects of Mg^{2+} are mediated through one site. The present study was undertaken in an attempt at clarifying the role of Mg^{2+} in inhibitory receptor regulation. An attempt was made to distinguish the receptor-regulatory role of Mg^{2+} from that of GTP by the use of the alkylating agent NEM, which has proven useful in studies of stimulatory receptor systems (Schramm & Naim, 1970; Schramm, 1976). Our studies indicate that Mg^{2+} , in the presence of GTP, promotes a state of the adenosine R_i -receptor that is essential for receptor-mediated inhibition of adenylate cyclase activity. Achievement of the Mg^{2+} -induced state can be blocked by NEM treatment, although GTP continues to regulate binding. NEM treatment does not eliminate inhibition of catalytic activity by guanine nucleotides. However, it does eliminate receptor-mediated inhibition. It is therefore concluded that the Mg^{2+} site is essential to integrated R_i - N_i -C communication.

Methods

Materials

N^6 -Cyclohexyl[adenine-2,8- 3H]adenosine (13.5 Ci/mmol), [α - ^{32}P]ATP and cyclic [3H]AMP were from New England Nuclear. PIA and CHA were from Boehringer Mannheim. The sources of other materials and the preparation of fat-cell membranes were as previously described (Londos *et al.*, 1978; Cooper *et al.*, 1979). Briefly, the epididymal fat-pads from male (200g) Sprague-Dawley rats are excised and disaggregated with collagenase. The washed fat-cells are lysed and the plasma membranes are collected from a sucrose gradient.

Adenosine-receptor binding

The method used was based on that of Trost & Schwabe (1981). Incubations were performed under simulated adenylate cyclase assay conditions, with 4nM-[3H]CHA, 30mM-Tris/HCl, pH 7.4, 2mM-MgCl₂ (unless specified otherwise), 25 units of creatine kinase/ml, 1 mM-phosphocreatine (to maintain GTP concentrations), 1 unit of adenosine deaminase/ml and approx. 20 μ g of fat-cell membranes in a total volume of 100 μ l, for 120 min at 24°C. Adenosine deaminase was included in the incubations to metabolize any adenosine present in the membrane preparation. Preincubation with adenosine deaminase to metabolize endogenous adenosine (as described by Trost & Schwabe, 1981) did not offer any advantage over inclusion of the enzyme during the extended binding incubations employed in the present study. Incubations were terminated by addition of 4 ml of cold 30mM-Tris/HCl, pH 7.4, containing 2mM-MgCl₂ (except where MgCl₂ concentrations were varied in the incubation) and rapid filtration through 24mm Whatman glass-fibre discs (GF/C) at approx. 4 ml/s. Non-specific binding was determined in the presence of 100 μ M-PIA or -CHA. Binding to filters alone was about 0.5% of the total radioactivity (c.p.m.) added and accounted for almost all 'non-specific' binding. Maximum specific binding was 3-4% of the total radioactivity added. Binding parameters (K_d and B_{max} values) were determined by least-squares analysis of Scatchard plots. Computer analysis of the untransformed data with the program LIGAND (Munson & Rodbard, 1980) suggested that a two-site model was not significantly better than a one-site model. Data analysed with LIGAND with a one-site model gave similar values as when analysed by least-squares analysis; thus routinely all data were analysed by the latter procedure. Values are summarized as means $\pm$ S.E.M. (n), where n is the number of values determined in separate experiments.

Equilibrium binding was achieved at the [3H]CHA concentration used over the incubation

period of 120 min (results not shown). The relative binding affinities of PIA and 5'-N-ethylcarboxamido-adenosine observed in displacement experiments (results not shown) confirmed that the binding being measured was to an adenosine receptor of the R_i subtype, as would be expected from previous binding and activity studies (Londos *et al.*, 1980; Trost & Schwabe, 1981).

Adenylate cyclase assay

Adenylate cyclase was assayed at 24°C to facilitate expression of both stimulation and inhibition (Cooper *et al.*, 1979), with 1–5 µg of membrane preparation and [α -³²P]ATP (0.1 mM; 1 µCi), 0.1 mM-cyclic AMP, 30 mM-Tris/HCl, pH 7.4, 2 mM-MgCl₂, 2 mM-phosphocreatine, 25 units of creatine kinase/ml and 0.1 unit of adenosine deaminase/ml in an incubation volume of 100 µl for approx. 10–20 min as previously described (Londos *et al.*, 1978; Cooper *et al.*, 1979).

Membrane pretreatment with NEM

Fat-cell membranes (1 mg/ml) were incubated with an equal volume of various concentrations of NEM (as indicated) in 20 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-EDTA (Buffer A), for 15 min at 25°C. The reaction was stopped by the addition of 2 vol. of 2-mercaptoethanol, with a concentration equal to that of NEM in Buffer A, and kept on ice for 10 min. Under the conditions utilized, mercaptoethanol, up to the highest concentration studied, did not affect [³H]CHA binding substantially. The mixture was centrifuged for 10 min at 10000 rev./min (12000g, 4°C). After removal of the supernatant, the membranes were re-suspended in 20 mM-Tris/HCl buffer, pH 7.4. Control preparations were treated similarly, except that NEM (and mercaptoethanol) were replaced with Buffer A.

Results

A major purpose of the present study was to relate GTP regulation of receptor binding to GTP regulation of receptor-mediated inhibition of catalytic activity. Consequently, initial binding studies simulated adenylate cyclase assay conditions, so that comparisons could be made readily. Thus in Fig. 1(a) the effect of GTP on the displacement of [³H]CHA by unlabelled CHA was examined in the presence of 2 mM-MgCl₂. The data demonstrate that GTP markedly increased [³H]CHA binding. A Scatchard plot of these data (Fig. 1b) reveals that the affinity for the receptor was unaltered by GTP. However, the number of binding sites increased from approx. 1.1 to 2.6 pmol/mg of protein. The K_d and B_{max} calculated from three such experiments are shown in Table 1. Although there were some

Fig. 1. CHA binding to fat-cell membranes in the absence (○) or presence (●) of 0.1 mM-GTP in the presence of 2 mM-MgCl₂.

(a) [³H]CHA (3.52 nM) binding was measured in the presence of the indicated concentrations of unlabelled CHA. (b) Scatchard analysis of the data.

daily variations in the K_d and B_{max} values, GTP consistently increased B_{max} approx. 2-fold, without appreciably affecting K_d .

Since bivalent cations have been reported to affect ligand binding to receptors coupled to adenylate cyclase, and the experiments shown above were done under standard conditions (which included 2 mM-MgCl₂), it was decided to determine the influence of Mg²⁺ on the GTP effect. In the absence of Mg²⁺, increasing GTP concentrations decrease agonist binding to the receptors (Fig. 2). This decreased binding is caused by a 2-fold increase in K_d , without alteration in the number of receptors. The K_d and B_{max} values calculated from two such experiments were 2.72 ± 0.09 nM and 2.23 ± 0.17 pmol/mg of protein, respectively, in the absence of GTP. In the presence of GTP, these values were 5.67 ± 0.02 nM and 2.03 ± 0.01 pmol/mg of protein respectively (cf. Table 2). By contrast, in

Table 1. *Affinity constants and maximal binding capacities of adipocyte plasma membranes for [³H]CHA assayed in the absence or presence of GTP*

K_d and B_{max} values are determined from Scatchard plots. The experiments were done with a fixed concentration of [³H]CHA and various amounts of unlabelled CHA. The incubations were carried out in duplicate (Expt. 3) or triplicate (Expts. 1 and 2) in the absence or presence of 0.1 mM-GTP. All incubations included 2 mM-MgCl₂. The K_d values of control and the '+ GTP' group were not significantly different. However, the increments in B_{max} on addition of GTP were highly significantly different ($P < 0.01$).

Expt. no.	Control		+ GTP		$\frac{B_{max.} (+GTP)}{B_{max.} (control)}$ (%)
	K_d (nM)	$B_{max.}$ (pmol/mg)	K_d (nM)	$B_{max.}$ (pmol/mg)	
1	6.23	1.30	4.29	2.69	207
2	5.36	1.96	5.19	4.42	226
3	4.30	0.76	3.04	1.42	188

Table 2. *Summary of K_d and B_{max} values for [³H]CHA binding to control and NEM-treated adipocyte membranes*
Results for no pretreatment are means $\pm$ S.E.M.; the numbers of separate experiments are shown in parentheses. Results for 50 μ M-NEM pretreatment are means from a single experiment in triplicate.

Pretreatment	Addition	K_d (nM)	$B_{max.}$ (pmol/mg)
None	Control	2.87 ± 0.16 (3)	2.31 ± 0.11 (3)
	GTP	5.67 ± 0.02 (2)	2.03 ± 0.01 (2)
	Mg ²⁺	5.74 ± 0.60 (4)	1.31 ± 0.25 (4)
	Mg ²⁺ and GTP	4.17 ± 0.62 (3)	2.84 ± 0.87 (3)
50 μ M-NEM	Control	5.63	1.09
	GTP	9.84	1.04
	Mg ²⁺	5.44	0.95
	Mg ²⁺ and GTP	7.41	0.98

Fig. 2. *Effects of GTP on [³H]CHA binding to fat-cell membranes in the absence (○) or presence (●) of 2 mM-MgCl₂*

Non-specific binding in the absence (□) or presence (■) of MgCl₂ is indicated. Non-specific binding is defined as the binding in the presence of 0.1 mM-L-PIA.

the presence of 2 mM-Mg²⁺, increasing GTP concentrations increased [³H]CHA binding to a maximum at 0.1 mM; higher concentrations of GTP decreased binding (Fig. 2). These results demonstrate that the GTP effect can be modified by Mg²⁺ and vice versa. It is also worth noting that these GTP concentrations are approximately 10-fold less

potent than those that promote adenosine-receptor-mediated inhibition of adenylate cyclase (Cooper *et al.*, 1979). The non-hydrolysable GTP analogue, p[NH]ppG, shares the effects of GTP on binding, with the same concentration-dependency (results not shown).

The dose-dependency of the Mg²⁺ effect in the absence or presence of GTP was examined in Fig. 3(a). Increasing Mg²⁺ concentrations steadily decreased [³H]CHA binding in the absence of GTP. This decrease in binding is due to a marked decrease in the number of binding sites and a 2-fold increase in K_d , as revealed by Scatchard analysis (Fig. 3b). In the presence of GTP, Mg²⁺ exerted the opposite effect, i.e. binding steadily increased, reaching a maximum at 1 mM (as shown in Fig. 1, this is due to an increase in $B_{max.}$). Ca²⁺ and Mn²⁺ give the same qualitative results as Mg²⁺, but with different concentration-dependencies (S.-M. H. Yeung & D. M. F. Cooper, unpublished work).

Thus an intimate relationship is suggested between a GTP-regulatory site and a Mg²⁺-regulatory site. The following experiments sought to distinguish the processes regulated by these two sites.

High-affinity binding can be perturbed by reagents that perturb the coupling of the receptor

Fig. 3. Effects of Mg²⁺ on [³H]CHA binding to fat-cell membranes

(a) Dose-response curves of MgCl₂ effect on [³H]CHA binding in the absence (O) or presence (●) of 0.1 mM-GTP. (b) Scatchard plots of [³H]CHA binding in the absence (O) or presence (●) of 2 mM-MgCl₂.

Fig. 4. Effect of NEM on [³H]CHA binding to fat-cell membranes

Adipocyte membranes were divided into batches and incubated with equal volumes of buffer alone or with NEM to the final concentration indicated. The incubation was performed for 15 min at 25°C. The reaction was stopped by the addition of 2 vol. of 2-mercaptoethanol in a 2-fold excess of the NEM concentration. The mixture was centrifuged for 10 min at 12000g, 4°C. The pellet was resuspended in 20 mM-Tris/HCl buffer, pH 7.4, and [³H]CHA binding was measured in the absence (a) or presence (b) of 2 mM-MgCl₂ in the absence (O) or presence (●) of 0.1 mM-GTP.

to adenylate cyclase. Treatment with thiol reagents, such as NEM, has been reported to decrease high-affinity [³H]CHA binding (Yeung & Green, 1983, 1984). Thus the effect of NEM was examined on the interaction between bivalent cations and guanine nucleotides in regulating this binding. In the absence of Mg²⁺, NEM decreased specific [³H]CHA binding in a dose-dependent manner; the effect reached a plateau at approx. 0.1 mM, whether in the presence or absence of GTP (Fig. 4a). However, in the presence of Mg²⁺, when GTP was not added, NEM affected [³H]CHA binding minimally (Fig. 4b). GTP increased the binding about 2-fold in the presence of Mg²⁺ (as

shown in Fig. 1), but this effect was steadily decreased by increasing NEM concentrations. It is noteworthy that at 50 μM-NEM the GTP effect on binding was no longer detectable. However, as the NEM concentration was increased above 50 μM, GTP behaved as it did in the absence of Mg²⁺, i.e. it decreased binding. This decrease is modest, but is consistently observed. The inability of GTP to increase the binding after NEM treatment suggests that the Mg²⁺ effect was eliminated.

CHA-binding isotherms were compared in control and NEM-treated preparations, in the absence of Mg²⁺. Analysis of the data in Scatchard plots showed that NEM treatment decreased the

number of receptors, with a slight increase in K_d . GTP had no significant effect on the B_{max} in the 50 μ M-NEM-treated preparations, although it did shift the K_d . These results are summarized in Table 2.

CHA-binding isotherms were examined after NEM treatment in the presence of 2 mM-Mg²⁺. In the absence of GTP, the isotherms were very similar in the control or 50 μ M-NEM-treated preparations. However, the mild treatment with NEM abolished the effect of GTP, and the isotherm resembled that in the absence of GTP (results not shown). Scatchard plots of these data are shown in Fig. 5. The data show that the GTP effect was eliminated with 50 μ M-NEM treatment (Fig. 5b), i.e. the Mg²⁺-dependent increase in the B_{max} owing to GTP, which was characteristic of control preparations, was not apparent. After treatment with higher NEM concentrations, an effect of GTP was still present. Fig. 5(c) shows the Scatchard plots of CHA binding in high (2.5 mM)-NEM-treated preparations in the presence of 2 mM-Mg²⁺. The K_d in the absence of GTP was 7.8 nM, and increased to 13.4 nM in the presence of GTP,

whereas the B_{max} was unaffected. Thus, after 2.5 mM-NEM treatment, the system behaved as it would in the absence of Mg²⁺. Consequently, it appears that the Mg²⁺ site is the target of NEM action. Treatment with much higher (approx. 20 mM) NEM concentrations was required to eliminate the GTP effect (results not shown).

The adenylate cyclase activity is also sensitive to thiol reagents (Schramm & Naim, 1970; Ross & Gilman, 1980). However, the sensitivity of the adipocyte enzyme to low NEM concentrations has not previously been examined. The effect of NEM treatment on the enzyme activity under various conditions is examined in Table 3. Forskolin stimulated the enzyme activity about 10-fold, and p[NH]ppG inhibited the forskolin-stimulated activity by about 68%. In 50 μ M-NEM-treated preparations, the p[NH]ppG inhibition of forskolin-stimulated activity was only slightly impaired. In contrast, this treatment abolished PIA inhibition of catalytic activity. These findings suggest that low NEM concentrations selectively disrupt receptor-N_i interactions, as assayed by PIA inhibition, without affecting N_i-C interactions, as

Fig. 5. Scatchard plots of [³H]CHA binding to fat-cell membranes in the absence (○) or presence (●) of 0.1 mM-GTP. MgCl₂ (2 mM) was included in the binding assay. The adipocyte membranes were divided into three equal portions and treated with buffer alone (a) or final concentrations of NEM of 50 μ M (b) or 2.5 mM (c). The procedure for NEM treatment was as described as in the legend to Fig. 4. K_d and B_{max} values are expressed in nM and pmol/mg respectively.

Table 3. *Effect of NEM treatment of adipocyte adenylate cyclase*

Fat-cell membranes were treated with or without 50 μ M-NEM. Activities were determined in the presence of 100 μ M-forskolin with or without 1 μ M-p[NH]ppG, or in the presence of 10 μ M-GTP with or without 1 μ M-PIA, as indicated.

Membranes ...	Control		50 μ M-NEM-treated	
	Activity (pmol/10min per mg)	Inhibition (%)	Activity (pmol/10min per mg)	Inhibition (%)
Forskolin	2128		1490	
Forskolin + p[NH]ppG	697	68	725	52
GTP	121		167	
GTP + PIA	59	52	168	0

assayed by p[NH]ppG inhibition of forskolin-stimulated activity (Table 3).

Discussion

A basic finding of this study is that the binding of the agonist [³H]CHA to fat-cell membrane adenosine R₁-receptors is regulated by guanine nucleotides and Mg²⁺ in an interactive manner. Either agent alone decreases binding, whereas addition of either agent in the presence of the other results in an approx. 2-fold increase in binding. Similarities and dissimilarities are noted between this and previous studies of inhibitory receptor binding. Trost & Schwabe (1981) noted that L-PIA bound to a single class of binding sites in fat-cell membranes. The K_d and B_{max} values determined in the present study are similar to those previously reported by those authors (Trost & Schwabe, 1981). The GTP-mediated decrease in binding in the absence of bivalent cations is also similar to the effect noted in the study of guinea-pig and bovine brain adenosine R₁-receptors (Goodman *et al.*, 1982). However, the results differ when the effects of bivalent cations are considered in these other systems. In adipocyte membranes, Mg²⁺ decreases receptor binding, mainly as a consequence of decreasing receptor number; in brain membranes, Mg²⁺ increases receptor binding as a consequence of increasing receptor affinity (Goodman *et al.*, 1982). In a study of the hippocampal adenosine R₁-receptor, Yeung & Green (1983) observed that addition of guanine nucleotides, in the presence of bivalent cation, decreased receptor agonist binding by decreasing both receptor number and affinity, the converse to the results that are encountered in adipocyte membranes.

No immediate explanation suggests itself as to why the adenosine receptor from central sources and adipocytes should differ in some of its regulatory properties. That the effects of Mg²⁺ seen with adipocytes are not peculiar to peripheral tissues is apparent from studies performed with the α_2 -adrenergic receptor of platelet and brain cortex. In

rat and calf brain cortex, Mg²⁺ alone decreases binding to α_2 -adrenergic receptors; in the presence of GTP, Mg²⁺ progressively increases α_2 -adrenergic-receptor binding, a situation that is directly analogous to that observed with adipocyte adenosine receptors (U'Prichard & Snyder, 1980). However, in platelet membranes, Mg²⁺ alone increases α_2 -adrenergic-receptor binding, an effect that is reversed by GTP (J. Mitrius & D. U'Prichard, unpublished work). This latter behaviour is analogous to the regulation of adenosine receptor binding in the brain. It is to be hoped that further study of the adenosine receptor from both sources will reveal the basis for the differing effects of Mg²⁺, since the consequence for the occupancy of the receptor is the same in both systems, i.e. the inhibition of adenylate cyclase.

The adenosine receptor is not unique among inhibitory fat-cell receptors. A GTP-promoted increase in inhibitory agonist binding to adipocyte membranes has previously been reported (Grandt *et al.*, 1982). In this case guanine nucleotides stimulated prostaglandin E₂ binding to its receptor. This binding study was conducted in the presence of 2mM-MgCl₂, and thus the GTP-promoted increases in binding would be absolutely dependent on the presence of the bivalent cation, as predicted from the present study. Univalent cations at high (50mM) concentrations mimicked the effect of the guanine nucleotides (in the presence of MgCl₂). We have observed a similar enhancement of adenosine receptor binding by Na⁺, in the presence of MgCl₂, which was additive with the effects of low concentrations of guanine nucleotides (results not shown). It thus appears that univalent cations and guanine nucleotides have similar effects. However, the bivalent cations dictate the type of effect (increase or decrease binding) elicited by these effectors.

As a step towards unravelling the interaction of the bivalent cation and GTP on the adipocyte adenosine receptor, the effects of the alkylating agent *N*-ethylmaleimide were investigated. The results indicate that treatment with low concentra-

tions of NEM abolished the characteristic effect of Mg^{2+} ions, without affecting the action of GTP. The consequence of this loss of the Mg^{2+} effect was that the effect of GTP was altered from that of increasing overall binding to decreasing binding, in the presence of the cation. This transition in the effect of GTP resulted in a crossover point in the profile of NEM concentration versus GTP regulation of binding, such that at intermediate NEM concentrations no effect of GTP was detectable (Fig. 4). Although NEM has been investigated with regard to its effects on GTP regulation of receptor binding in a number of inhibitory systems, the interaction of the alkylating agent with Mg^{2+} regulatory sites has not previously been examined. In the anterior pituitary and cardiac tissue, GTP decreased the affinity for D_2 -dopaminergic (Kilpatrick *et al.*, 1982) and muscarinic cholinergic agonists (Harden *et al.*, 1982), respectively; treatment with NEM also decreased receptor affinity and abolished the GTP effect. In the present study, when binding was examined in the absence of Mg^{2+} , the effect of GTP was largely abolished at low concentrations of NEM, as in the previous studies. However, at higher NEM concentrations, an effect was still detectable.

The consequence of the loss of the Mg^{2+} regulation was striking for the regulation of cyclase activity. Treatment with low NEM concentrations abolished adenosine-receptor-mediated inhibition of catalytic activity, whereas p[NH]ppG inhibition of forskolin-stimulated activity was only marginally decreased. This suggests that the putative Mg^{2+} site is vital for the productive interaction between receptor and guanine nucleotide regulatory complex, but not for the interaction of the inhibitory GTP regulatory complex with the catalytic unit of adenylate cyclase. This finding is in apparent contradiction with recent studies on S49 mouse lymphoma *cyc⁻* cells. Hildebrandt & Birnbaumer (1983) and Jakobs *et al.* (1983), using *cyc⁻* cells, showed that there is an absolute requirement for micromolar concentrations of Mg^{2+} for activation of N_i . However, in intact systems where both N_s and N_i are present, no such Mg^{2+} requirement for activation of N_i was found (Jakobs *et al.*, 1981). Since the adipocyte adenylate cyclase system is also intact, comprising both N_s and N_i circuits, there is no conflict between our results and those on the *cyc⁻* system. It is also possible that a separate Mg^{2+} site exists in N_i , which is not sensitive to alkylating agents and which is absolutely required for N_i -C interactions. Harden *et al.* (1982) have previously shown that low concentrations of NEM (approx. 0.1 mM) eliminate oxotremorine inhibition of cardiac cyclase activity without affecting basal or isoprenaline-stimulated activity.

The results presented in this study show that guanine nucleotide regulation of receptor-binding and guanine-nucleotide-mediated inhibition of adenylate cyclase can both be observed when receptor-mediated inhibition of activity has been eliminated by NEM treatment. These results indicate that the properties of the GTP-regulatory component mediating inhibition of adenylate cyclase differ depending on whether receptor or catalytic-unit regulation is measured. Our studies indicate that a Mg^{2+} -regulatory site associated with receptor regulation may provide the basis for such differences.

The present results do not allow determination of the molecular events underlying these interactions. One possibility involves monomer-dimer transitions. In such a scheme, in the absence of both bivalent cations and guanine nucleotides, the adenosine R_i -receptors exist as monomers of receptor and GTP regulatory protein. Occupation by Mg^{2+} of a distinct allosteric site, a putative ' Mg^{2+} ' site, heterotropically linked to the GTP site in the regulatory protein, induces the dimerization of receptor molecules. In this case only one binding site is available to adenosine analogues, and binding to the second site is allosterically inhibited. Occupancy by GTP of the GTP site in the regulatory protein produces a conformational change of the receptor molecules, so that the dimers dissociate to monomers, permitting binding of one adenosine analogue molecule per monomer. An alternative hypothesis would be that Mg^{2+} enables some other unidentified component(s) of the adenylate cyclase system to couple to the receptors, thereby lowering the affinity of some CHA-binding sites so markedly that they can no longer be detected by the filtration-binding assay. Such an effect would result in an apparent B_{max} change. Although the present data cannot discriminate between these possibilities, the former is attractive because of the, possibly coincidental, 2-fold changes in receptor number and because the existence of receptors in different oligomeric forms is not without precedent. It has been reported that several oligomeric forms of the acetylcholine receptor occur. Avissars *et al.* (1983), using photo-affinity probes, showed that muscarinic cholinergic receptors exist in two forms: (1) an 80000-Da dimer composed of two 40000-Da subunits, joined via a covalent bond, and (2) a tetramer (160000 Da) formed by two such dimers. These represent low- and high-affinity states of the receptor, respectively, and there is rapid and reversible interconversion between these states. Chang & Bock (1977) demonstrated by sucrose-density-gradient analysis that the nicotinic cholinergic receptor of *Torpedo* occurs as a dimer, which can form monomers by the breakage of disulphide

bonds. Irradiation-analysis studies by Venter & Fraser (1984) indicate that adrenergic receptors may also occur as dimers in intact membranes. Thus it is not too provocative to propose that the R_i-adenosine receptors might exist in monomeric and dimeric forms, and that the interconversion between these forms is regulated by guanine nucleotides and bivalent cations, which may involve disulphide linkages.

A study by Gill & Meren (1983) of the GTP regulatory complex mediating the stimulation of adenylate cyclase in pigeon erythrocytes indicated two GTP-binding sites, one mediating stimulation of the enzyme and the other mediating ADP-ribosylation by cholera toxin. Since the purified GTP-binding subunit of N_s contains only one binding site for GTP per molecule (Gilman, 1984), Gill & Meren (1983) suggested that equivalent GTP-binding sites in a dimer could display different properties through allosteric interactions. This suggestion, taken with the proposal advanced in the present paper, might suggest that receptors and GTP-regulatory proteins could be assembled as (RN) dimers. A similar proposal had been raised previously by Rodbell and colleagues with regard to the glucagon receptor (Schlegel *et al.*, 1979; Rodbell, 1980).

Further experimentation, involving radiation-inactivation analysis of intact membrane receptors and sucrose-density-gradient analysis of solubilized receptors, will be required to obtain clearer insights into the molecular basis for these Mg²⁺-based transitions. Whatever the precise mechanism turns out to be, the present data clearly indicate that an interaction between GTP and Mg²⁺ is required for the promotion of receptor-mediated inhibition of adenylate cyclase, which is not required for the regulation by the inhibitory GTP-regulatory complex of either receptor binding or catalytic activity.

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