# 2′-DEOXYADENOSINE FUNCTIONALLY UNCOUPLES ADENYLATE CYCLASE FROM THE GUANYL NUCLEOTIDE SUBUNIT WITHOUT ALTERING SIMULTANEOUS GppNHp OCCUPANCY

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## 1. Introduction

Adenosine can effect adenylate cyclase in an inhibitory or excitatory manner. These interactions are mediated by two distinct classes of sites:

(i) 'R sites' which are sensitive to nM levels of adenosine and which can be blocked by methyl xanthines. R sites will mediate activation of adenylate cyclase in certain tissues [1–5] but will mediate inhibition of adenylate cyclase in other tissues [1,6,17];

(ii) 'P sites' which inhibit adenylate cyclase at μM levels of adenosine and which are insensitive to methyl xanthines [1,8].

The distinction between the two classes of sites was made in [9] on the basis of structural requirements for conservation of the ribose moiety of adenosine (R sites) or the purine moiety of adenosine (P sites).

2′-Deoxyadenosine is a potent probe of P sites and will inhibit adenylate cyclase in the presence of GppNHp [9] a non-hydrolysable analogue of GTP which usually maintains a high level of adenylate cyclase activity [10]. It is not clear whether GppNHp is displaced by 2′-deoxyadenosine.

This work describes experiments with 2′-deoxyadenosine and membranes derived from the neuroblastoma × glioma hybrid cell line NG 108-15 which show that the expression of the interaction between the guanyl nucleoside subunit and adenylate cyclase is prevented by 2′-deoxyadenosine although the guanyl nucleoside subunit remains fully occupied by GppNHp.

## 2. Experimental

NG 108-15 cells were a gift from M. Nirenberg to D. E. Koshland jr. Cells were grown in 150 $cm^2$ T flasks in 5% $CO_2$ and 95% air as in [11]. Confluent cultures of cells were harvested by shaking and washing twice in $D_1$ harvest buffer essentially as in [12] except that the homogenization buffer consisted of 5 mM Tris–HCl (pH 7.4) and 1 mM $MgCl_2$. Membranes were further purified on a sucrose gradient [13] and were collected and stored at 70°C in 1 ml aliquots in a buffer of 25% sucrose, 50 mM Tris–HCl (pH 7.4) and 1 mM $MgCl_2$.

Prior to use, membranes were thawed rapidly, washed in 30 vol. 50 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$ and 1 mM EDTA (TME)′ and resuspended in the same buffer to ~1 mg protein/ml. Protein concentration was measured as in [14] using bovine serum albumin as standard.

The adenylate cyclase assay mixture, at 37°C, contained, in 150 μl, 50–70 μg membrane protein, 1 mM cAMP, 10 mM $MgCl_2$, 0.5 mM isobutylmethyl xanthine, 20 mM disodium creatine phosphate, 10 units creatine kinase and 1 mM disodium [α-$^{32}$P]-ATP (~10 cpm/pmol) and additional ligands as described in figure legends. cAMP was measured according to [15].

For preactivation, which separates the activation step from the cyclase assay [16], membranes were incubated with 0.1 mM GppNHp and 0.1 mM adenosine for 10 min at 37°C and washed to a $10^4$ dilution by repeated centrifugations at 38 000 × *g* for 10 min in TME (pH 8.0).

For reversion experiments [16,17], which follow

the decay of the preactivated adenylate cyclase to basal activity levels, the preactivated and washed membranes were incubated with various ligands and at certain times 70 μl aliquots were removed into 40 μl ice-cold 6.7 mM caffeine and 6.7 μM naloxone. After all aliquots were collected the assay cocktail was added, tubes were transfered to 37°C and cAMP was accumulated for 20 min.

[α-$^{32}$P]ATP and c[$^{3}$H]AMP were from New England Nuclear, USA, GppNHp from Boehringer, ATP from Pierce Chemicals, 2′-deoxyadenosine from P and L Chemicals, cAMP, caffeine, adenosine and regenerating system from Sigma and isobutyl methyl xanthine from Aldrich.

## 3. Results

The adenylate cyclase prepared from neuroblastoma × glioma hybrid cell line NG 108-15 is activated by 50 μM adenosine. This activation is clearly inhibitable by 2′-deoxyadenosine and an R site antagonist, caffeine, to the same extent (fig.1a).

When GppNHp is added a 5-fold activation of adenylate cyclase is observed (fig.1b). The entry of GppNHp to its site of action is fascile and rapid. It occurs independently of adenosine or caffeine and with no discernable lag time. Under these experimental conditions 2′-deoxyadenosine is completely inhibitory and adenylate cyclase remains almost at basal levels of activity.

2′-Deoxyadenosine could prevent GppNHp from occupying the active site either by blocking the entry to the site or by causing the removal of GppNHp. Alternatively it could prevent the information encoded in the GppNHp subunit (N) from being expressed as an activation of the catalytic moiety of adenylate cyclase (AC).

To test these alternatives, the adenylate cyclase was preactivated by GppNHp, washed extensively to a $10^4$ dilution to remove all free exchangeable GppNHp and exposed to various ligands to induce reversal to basal activity levels (fig.2). In turkey erythrocytes [16–18] these experimental conditions first cause GppNHp to occupy N quasi-irreversibly and subsequently GppNHp is dissociated from N [18] through an interaction with receptors which are thought to fascilitate GTP–GDP exchange [19].

In NG 108-15 membranes the GppNHp induced activity remains stable to extensive washing at 0°C

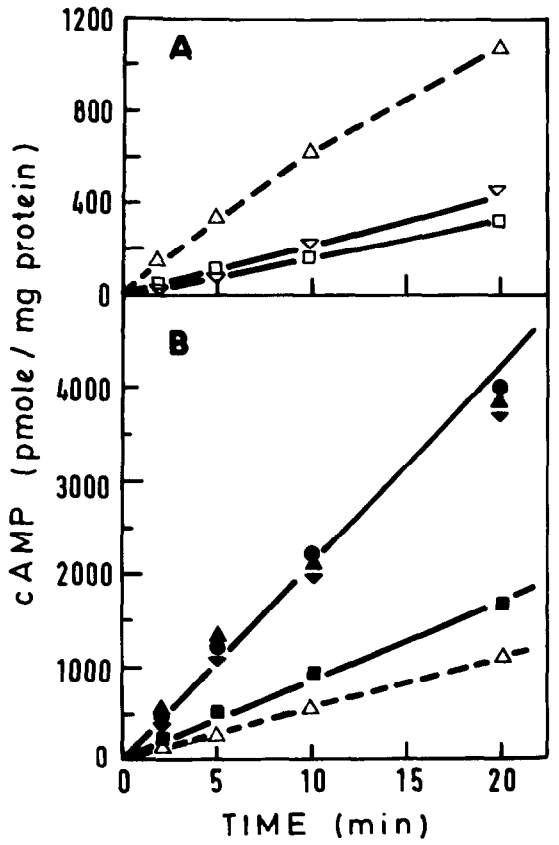


Fig.1a. The activation of NG 108-15 membranal adenylate cyclase by 50 μM adenosine (△) and the inhibition of the adenosine response by 16.3 mM caffeine (▽) or 0.4 mM 2′-deoxyadenosine (□) was followed for 20 min at 37°C. At the times indicated 150 μl aliquots were removed into 100 μl 2% SDS. Fig.1b cAMP accumulation in the presence of 50 μM GppNHp: 50 μM GppNHp alone (●); 50 μM GppNHp and 50 μM adenosine (▲); 50 μM GppNHp and 50 μM adenosine and 16.3 mM caffeine (▼); or 50 μM GppNHp, 50 μM adenosine and 0.4 mM 2′-deoxyadenosine (■). 50 μM adenosine level (△) is inserted for comparison. Sampling was as in fig.1a.

and subsequent incubation at 37°C in the absence of added ligands. Adenosine at 50 μM induces very slight reversion. GTP (50 μM) is less effective than the combined effect of adenosine and GTP added simultaneously. 2′-Deoxyadenosine reduces the level of activation to below the final level obtained with adenosine and GTP very rapidly such that inhibition is complete in <30 s. Reversion by adenosine and GTP is much slower. Caffeine seems to activate the reversion as well.

2′-Deoxyadenosine inhibition of membranes

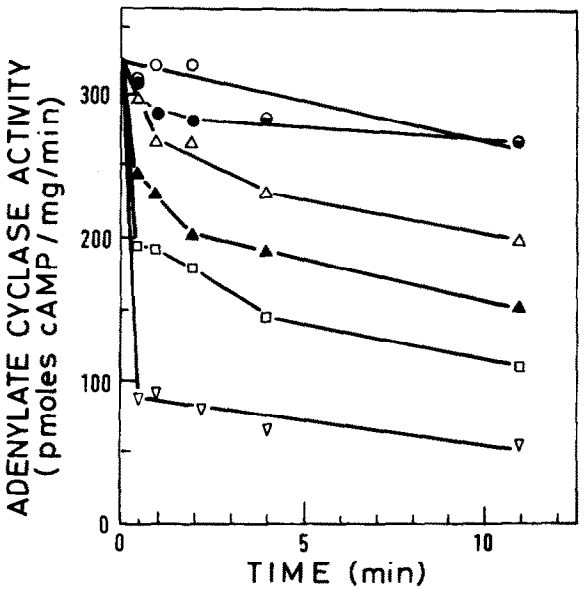


Fig.2. Reversion of GppNHp and adenosine-preactivated membranes as induced by the following ligands: none (○); 50 μM adenosine (●); 50 μM GTP (△); 50 μM adenosine and 50 μM GTP (▲); 50 μM adenosine, 50 μM GTP and 16 mM caffeine (□); 50 μM adenosine, 50 μM GTP and 0.4 mM 2′-deoxyadenosine (▽). All the assay tubes contained 6.7 mM caffeine and 6.7 μM naloxone.

preactivated by GppNHp and washed extensively is easily reversed by washing out 2′-deoxyadenosine (table 1) in cold TME and GppNHp resumes its full activity with no discernable lag time. Thus, it seems that the only effect of 2′-deoxyadenosine is to prevent the functional guanyl nucleoside subunit from expressing an activatory interaction with the catalytic moiety.

## 4. Discussion

The adenylate cyclase of NG 108-15 cell line is very similar to other membranal systems of neural origin [4,20], where N is also easily accessible to guanyl nucleotides in native washed membranes. But once GppNHp occupies the active site it is not easily removed.

The adenosine induced activity is entirely caffeine sensitive and is ~20% of the GppNHp activity levels indicating, by analogy to [19], that GTP–GDP exchange is probably the rate limiting step of the catalytic activation–inactivation cycle.

The GppNHp activated state is very slowly reversible but can be enhanced by GTP and more so by adenosine and GTP. GTP could cause enhanced reversal by two mechanisms depending on the state of N to AC coupling:

(i) If all N is coupled to AC in the presence of GppNHp then only an allosteric second site for GTP in addition to the GppNHp site would account for an enhancement of a monomolecular dissociation step such as that of GppNHp from the complex N · GppNHp.

Table 1
The effect of 2′-deoxyadenosine on the stability of the GppNHp · N complex

| | Additions to preactivated and washed membranes | Concentration of additions in assay | Net adenylate cyclase act. (pmol cAMP . $mg^{-1}$ . $min^{-1}$) |
|---|---|---|---|
| I | none | – | 307 ± 10 |
| II | 0.4 mM 2′-deoxyadenosine | 0.2 mM | 130 ± 9 |
| III | 0.4 mM 2′-deoxyadenosine | 0.2 mM | 104 ± 2 |
| | 50 μM adenosine | 25 μM | |
| | 50 μM GTP | 25 μM | |
| IV | 0.4 mM 2′-deoxyadenosine and washed | None | 280 ± 2 |

NG 108-15 membranes were activated for 10 min at 37°C with 0.1 mM GppNHp and 0.1 mM adenosine, washed to a $10^4$-fold dilution in ice-cold TME and incubated in the presence (II,III) or absence (I) of 0.4 mM 2′-deoxyadenosine for 10 min at 37°C. 2′-Deoxyadenosine was added alone (II) or in addition to 50 μM adenosine and 50 μM GTP (III). Only tube II containing 2′-deoxyadenosine was washed in 30-fold vol. TME, drained, and the pellet was resuspended in TME (IV). cAMP was accumulated in all the tubes for 15 min at 37°C. The activity levels are net after subtraction of the basal level of activity. Assays were performed in duplicate

(ii) If, on the other hand, all N is not completely occupied by GppNHp at saturation, an entire N · GTP complex could exchange with N · GppNHp.

Carefully designed reversal experiments will distinguish between these two alternatives.

2′-Deoxyadenosine inhibits adenylate cyclase activity under all 3 experimental conditions tested. But it does not cause permanent reversal of the GppNHp-activated adenylate cyclase as do GTP and adenosine [17]. Thus the effect of 2′-deoxyadenosine is expressed directly at the level of catalysis, at AC.

We now possess highly specific quantitative modulators of the specific concentrations of the 3 major components of the adenylate cyclase regulatory system: the receptor, which can be titrated by its agonist, the guanyl nucleoside subunit whose concentration can be limited by GppNHp, and the catalytic moiety whose concentration can be effectively reduced by 'R site' agonists without effecting N.

Using 2′-deoxyadenosine, it is possible to design novel kinetic experiments which can test whether the guanyl-nucleoside subunit is permanently coupled to the catalytic moiety.

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