

2',3'-Dialdehyde of GTP blocks regulatory functions of adenylate cyclase N_s protein

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Preincubation of bovine caudate nucleus membranes with the 2',3'-dialdehyde of GTP (oGTP) reduces adenylate cyclase activation by guanylyl imidodiphosphate (GppNHp) in a time-dependent fashion. A slower rate of inhibition is observed if membranes are treated with both GTP and oGTP. The efficacy of oGTP action is enhanced by raising the Mg^{2+} concentration. Reduction of adenylate cyclase sensitivity to GppNHp is followed by an irreversible decrease of enzyme stimulation by forskolin. Addition of a Lubrol soluble preparation from guinea pig lung membranes to oGTP-treated caudate nucleus membranes causes restoration of the adenylate cyclase sensitivity to GppNHp. These data suggest that oGTP blocks the GTP-binding site of the adenylate cyclase system localized on the N_s protein. Such modification leads to the elimination of the N_s -mediated regulation of the enzyme.

GTP 2',3'-dialdehyde Adenylate cyclase N_s protein Caudate nucleus Forskolin Reconstitution

1. INTRODUCTION

It is well established that activation of adenylate cyclase by hormones requires guanine nucleotides. Under physiological conditions, this requirement is fulfilled by GTP. Normal functions of the guanine nucleotide-binding protein (N_s) are necessary for GTP-dependent adenylate cyclase activation. N_s is distinct from both the catalytic component and the hormone receptor of adenylate cyclase. Thus far N_s has been purified to homogeneity from several sources [1,2]. The major advances in this field have been made by investigators through the use of cyc⁻S49 lymphoma cell membranes, a test system for N_s . In this cell clone, hormones and guanine nucleotides do not activate adenylate cyclase due to the deficiency in the α subunit of N_s [3]. Addition of detergent extracts containing N_s to such membranes leads to a reconstitution of the adenylate cyclase complex which closely resembles those prepared from wild-type S49 cells [1,2].

To date, to obtain plasma membranes with inac-

tivated N_s but containing an active catalytic component has been difficult. The catalytic subunit is more thermolabile, and its reactive groups are more sensitive to such reagents as *N*-ethylmaleimide [4], butanedione [5] and phenylglyoxal [6]. The affinity modification method seems to be a useful approach allowing selective inactivation of N_s in plasma membranes. It was shown earlier that azide derivatives of guanine nucleotides irreversibly block some N_s -mediated functions [7-9]. However, the possibility of restoration of regulatory properties in such membrane adenylate cyclase complex after reconstitution with native N_s was not discussed in the literature. For this reason periodate-oxidized guanosine triphosphate was synthesized and used in this study.

2. EXPERIMENTAL

2.1. Preparation of membranes

Bovine caudate nuclei were stored at -70°C . The tissue was thawed and homogenized in 10 vols

of solution containing 10 mM Tris-HCl, pH 8.0, 0.32 M sucrose and 1 mM dithiothreitol. After centrifugation of the homogenate at $10\,000\times g$ for 5 min, the pellet was resuspended in the same volume of 50 mM sodium borate, pH 8.0, and centrifuged in a similar way. Crude plasma membranes were resuspended and washed twice with the buffer and then frozen and stored at -70°C .

2.2. Treatment of membranes by oGTP

Preincubation was carried out at 30°C in a medium containing 50 mM sodium borate, pH 8.0, 1 mM ATP, crude membrane fraction (0.3 mg protein per ml), bovine serum albumin (2.5 mg per ml), MgCl_2 and oGTP as indicated. In control samples, oGTP was omitted. At defined time intervals aliquots were centrifuged at $10\,000\times g$ for 5 min, the pellets were washed once in 50 mM sodium borate, pH 8.0, suspended in a small volume of the same buffer and adenylate cyclase activity was determined.

For reconstitution experiments, membranes preincubated with oGTP were treated with glycerol and sucrose as in [10] and suspended in 50 mM Tris-HCl, pH 7.6.

2.3. Preparation of N_s from guinea pig lungs

The animals were killed by decapitation, and the lungs were sliced and homogenized in 5 vols of 10 mM Tris-HCl, pH 7.6, 0.25 M sucrose and 0.5 mM EGTA in a Potter homogenizer. The homogenate was filtered through 2 layers of cheesecloth and centrifuged at $800\times g$ for 5 min. The supernatant was centrifuged at $40\,000\times g$ for 10 min. The pellet was suspended in 10 vols of 10 mM Tris-HCl, pH 7.6, and 0.5 mM EGTA and recentrifuged. The membranes obtained were resuspended in 0.5 vol. of 50 mM Tris-HCl, pH 7.6; then Lubrol PX was added to a final concentration 0.7%. The mixture was stirred for 40 min and centrifuged at $100\,000\times g$ for 40 min. The supernatant fraction was frozen and stored at -70°C .

2.4. Reconstitution experiments

A standard reaction mixture for studying the interaction between soluble N_s and caudate nucleus membranes contained in $100\ \mu\text{l}$: 50 mM Tris-HCl, pH 7.6, 5 mM MgCl_2 , 0.1 mM GppNHp, 0.09% Lubrol PX, membranes treated with oGTP (50 μg protein) and N_s extract (0–55 μg protein). The con-

stant concentration of Lubrol PX in the reaction mixture was achieved by addition of heat-inactivated preparation of N_s (100°C , 30 s). After incubation at 30°C for 30 min, 1 ml of 50 mM Tris-HCl, pH 7.6, was added, the mixture was centrifuged at $10\,000\times g$ for 10 min, and adenylate cyclase activity in the pellet was determined.

2.5. Preparation of oGTP

Periodate oxidation of guanosine 5'-triphosphate was performed by the method of Easterbrook-Smith et al. [11]. Immediately after the termination of the reaction the solution was applied to a Sephadex G-10 column ($3\times 150\text{ cm}$) at 4°C , and eluted with distilled water. The fractions containing the nucleotide were tested for iodate as described by Collier and Nishimura [12]. Only iodate-free fractions were pooled, evaporated and stored at -20°C .

Adenylate cyclase activity was assayed by the method of Salomon et al. [13], using [α - ^{32}P]ATP (Amersham) as described in [14].

Protein was determined by the Lowry method.

3. RESULTS

Among the guanine nucleotides, one of the most potent adenylate cyclase activators is the non-hydrolysable GTP analog, guanylyl imidodiphosphate (GppNHp). On the other hand, GTP does not cause sufficient stimulation of the enzyme activity in brain tissue [15]. oGTP does not possess stimulatory properties either, so the effect of the analog can be determined by decreasing the GppNHp-induced activation.

When bovine caudate nuclei membranes were incubated with 0.5 mM oGTP, an irreversible decrease of the GppNHp-induced adenylate cyclase activation was observed. After membrane treatment with both 0.5 mM oGTP and 2 mM GTP, the rate of inhibition by dialdehyde analog was slower (fig.1).

The efficacy of oGTP action increased with rise in magnesium concentration (fig.2). The value of half-maximal inhibition time was decreased from 102 min in the absence of MgCl_2 to 79 and 27 min in the presence of 5 and 40 mM MgCl_2 , respectively. Increased magnesium caused a fall in stimulation by GppNHp in control samples; this effect, however, was not time-dependent. These results

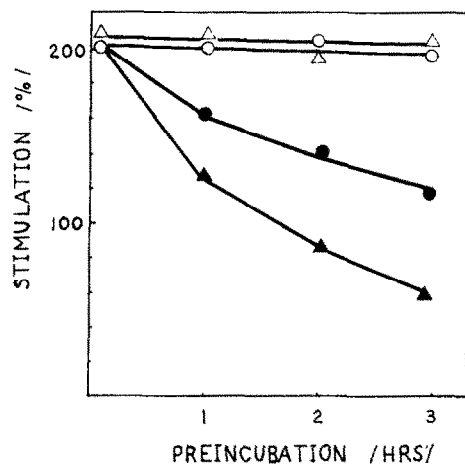


Fig. 1. Time course of oGTP action on adenylate cyclase stimulation by GppNHp. The membranes were preincubated with 0.5 mM oGTP (▲), 0.5 mM oGTP + 2 mM GTP (●), 2 mM GTP (△) and in the absence of guanine nucleotides (○) in media containing 5 mM $MgCl_2$. At the indicated time intervals, adenylate cyclase activation by 0.1 mM GppNHp was determined. The basal activity changed from 85 to 50 pmol cAMP/min per mg protein after 3 h preincubation.

can be explained by the partial extraction of N_s or other adenylate cyclase components from the membrane by increasing ionic strength.

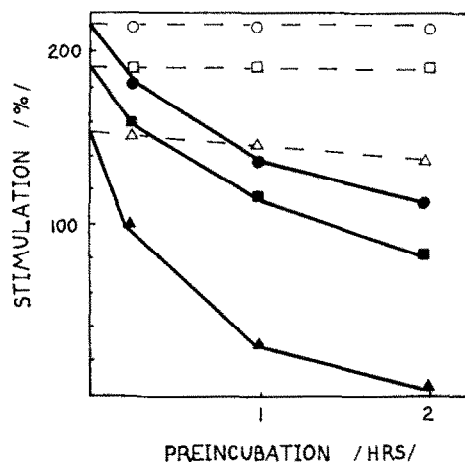


Fig. 2. Effect of Mg^{2+} on the time course of N_s inactivation by oGTP. The membranes were preincubated with 0.5 mM oGTP (●, ▲, ■) and without the nucleotide (○, △, □) in media devoid of $MgCl_2$ (○, ●) and containing 5 mM (□, ■) and 40 mM $MgCl_2$ (△, ▲). Adenylate cyclase activation was determined with 0.1 mM GppNHp.

Forskolin is a well known stimulator of adenylate cyclase [16]. Under the conditions used for membrane treatment with oGTP, the degree of adenylate cyclase stimulation by forskolin was 2-times greater than GppNHp (fig. 3). As for the GppNHp-induced enzyme stimulation, oGTP caused a time-dependent decrease of adenylate cyclase sensitivity to forskolin. It is noteworthy that the rates of both processes were equal.

As follows from the results obtained, the membrane treatment with oGTP abolishes the regulatory effect of guanine nucleotides on adenylate cyclase. We considered the feasibility of restoration of the enzyme sensitivity to GppNHp in such membranes after addition of native N_s . As a source of N_s a Lubrol extract from guinea pig lung plasma membranes was used. This preparation revealed negligible adenylate cyclase activity even in the presence of GppNHp; therefore no correction for an additional catalytic activity was necessary in the reconstitution experiments. As expected, the appearance and increase in adenylate cyclase stimulation by GppNHp depended on the amount of add-

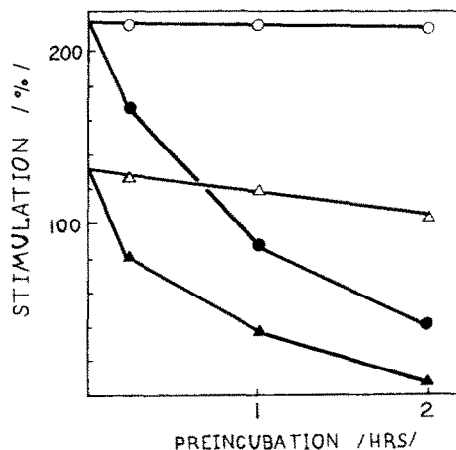


Fig. 3. Time course of oGTP action on adenylate cyclase stimulation by forskolin. The membranes were preincubated with 0.3 mM oGTP (●, ▲) and without the nucleotide (○, △) in media containing 40 mM $MgCl_2$. At the indicated time intervals adenylate cyclase activation was determined with 0.1 mM GppNHp (△, ▲) and 0.1 mM forskolin (○, ●). Forskolin was dissolved in dimethyl sulfoxide and added to the incubation mixture to a final concentration of the solvent 5%. The same amounts of dimethyl sulfoxide were added to control samples.

ed lung extract (fig.4). Thus, the results indicate that oGTP-treated membranes can be used as a test system for N_s .

4. DISCUSSION

Nucleotide dialdehyde derivatives are successfully used for the study of nucleotide-binding sites of various enzymes. It is supposed that such compounds interact with protein lysine residues. Our experiments demonstrate oGTP-induced irreversible blocking of adenylate cyclase regulation by guanine nucleotides. The finding that guinea pig lung extract completely restores the enzyme sensitivity to GppNHp provides evidence for the inactivation of membrane-bound N_s (fig.4). Protection by GTP from irreversible inhibition was interpreted as representing specific interaction of oGTP with the GTP-binding site of N_s (fig.1). Addition of sodium borohydride was not required for the oGTP action. Such treatment is necessary for stabilization of a Schiff base formed during the reaction of aldehyde with the amino group. However, it has been shown that oATP interacts with lysine of the phosphofructokinase active site

to form a product which has a morpholine structure, rather than a Schiff base [17]. Another possibility was considered in the study of mitochondrial ATPase inhibition by oATP [18]. It was proposed that an elimination reaction occurs which results in the liberation of the triphosphate group and formation of a compound containing an adenine and a conjugated aldehyde group. This could form a very stable Schiff base with amino groups. This is the reason why NaBH_4 is not necessary for irreversible inhibition.

The rate of N_s inactivation by oGTP is enhanced by Mg^{2+} (fig.2). These data are in agreement with the model proposed on a magnesium-potentiated slow dissociation of the $\alpha\beta$ dimer of N_s [19]. According to the model, the liberated α subunit having a GTP-binding site shows a higher affinity for guanine nucleotides as compared to the $\alpha\beta$ complex. Hence, the rate-limiting step in the oGTP-induced N_s inactivation can be $\alpha\beta$ dissociation.

The rates of N_s inactivation and of the decrease in the forskolin-mediated stimulation of adenylate cyclase are apparently equal (fig.3). This suggests that the effect of forskolin may require a functional N_s . Inactivation of N_s by GTP- γ -azidoanilide also caused a decrease of the adenylate cyclase sensitivity to forskolin [9]. However, the azide component is not selective with respect to the modifying group. Besides membrane proteins GTP- γ -azidoanilide is incorporated into membrane lipids [7] which may influence the effects observed. Due to the selectivity of the aldehyde group, the use of oGTP for inactivation of the membrane-bound N_s is preferable.

The reconstitution of the adenylate cyclase sensitivity to guanine nucleotides in the membranes devoid of N_s but containing a functional catalytic component is well established [4,10]. After oGTP-induced inactivation of N_s , the latter is still found in the membranes. Exogenous N_s caused restoration of the adenylate cyclase sensitivity to GppNHp in such membranes (fig.4). These results suggest that there is no tight binding between the catalytic subunit and the oGTP-inactivated N_s , which might lead to its complete replacement from the adenylate cyclase complex by the native regulatory component.

Our results indicate that oGTP can be a useful tool in the study of adenylate cyclase regulation via the N_s protein.

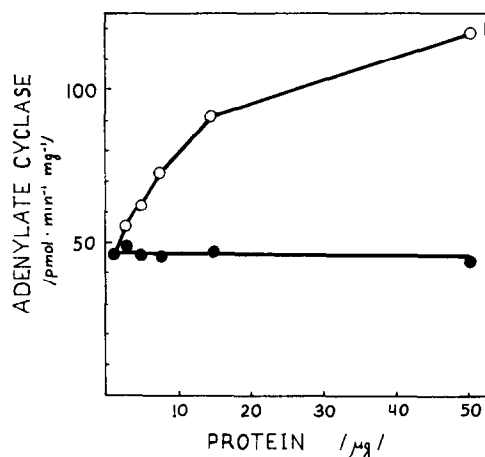


Fig.4. Reconstitution of adenylate cyclase sensitivity to GppNHp. Bovine caudate nucleus membranes were treated with 0.5 mM oGTP and 40 mM MgCl_2 for 2 h. Fixed amounts of Lubrol extract from guinea pig lung membranes were added and the adenylate cyclase activity was assayed with 0.1 mM GppNHp (○) and without the stimulator (●).

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