Treatment of oocyte membranes with the 2′, 3′-dialdehyde of guanosine triphosphate reduces progesterone inhibition of adenylyl cyclase

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Treatment of *Xenopus laevis* membranes with the 2′,3′-dialdehyde of GTP (dial GTP) drastically inhibits their adenylyl cyclase activity. Optimal inhibition is obtained by treatment with 1 mM dial GTP for 1h at 32°C. Using guanyl-5′-yl imidodiphosphate, F^-, forskolin and Mn^{2+} as activators of the enzyme it can be concluded that dial GTP preferentially reacts with the stimulatory subunit (N_s) and slightly with the catalytic subunit. Dial GTP treatment greatly reduces the inhibition of adenylyl cyclase by progesterone. Pure exogenous N_s stimulates the enzyme but does not restore progesterone inhibition. Treatment with dial [α-^{32}P]GTP labels several membrane proteins some of which have similar M_r to N_s and N_i.

Adenylyl cyclase *GTP 2′,3′-dialdehyde* *Progesterone inhibition* *Stimulatory subunit N_s* *G-protein*

1. INTRODUCTION

Guanine nucleotides play an important role in the hormonal regulation of membrane-bound adenylyl cyclase. The evidence gathered in recent years indicates that two guanine nucleotide binding proteins are involved in modulating the activity of the catalytic subunit of the enzyme. Stimulatory hormones appear to activate a 43-kDa membrane protein (N_s) which binds GTP or GDP and which can be ADP-ribosylated by cholera toxin. Most inhibitory hormones, on the other hand, seem to function through a similar GTP/GDP binding protein that can be ADP-ribosylated by pertussis toxin and has an $M_r = 41\,000$ (reviews [1,2]).

The adenylyl cyclase present in *Xenopus laevis* oocytes has been shown to contain both N_s and N_i type subunits [3,4], but is unusual on two counts: it is the only known adenylyl cyclase regulated by a steroid hormone, since it is inhibited by physiological concentrations of progesterone [5–7]; and its inhibition does not seem to be mediated by N_i [3,4]. The inhibition requires the presence of guanine nucleotides [7] and the hormone affects the kinetics of the activation of the enzyme by Gpp(NH)p [8], a known activator of N_s. On this basis, our current hypothesis proposes that progesterone inhibition may interfere with the N_s activation of the catalytic subunit of the enzyme.

We have now studied the effect of treatment of the membrane-bound adenylyl cyclase with the 2′,3′-dialdehyde of GTP (dial GTP) prepared by periodate oxidation of GTP. Dial ATP and dial GTP have been used to react with and inhibit enzymes that have high affinity for the corresponding nucleotide triphosphates and which have reac-

Abbreviations: N_s, stimulatory guanine nucleotide binding component that regulates adenylyl cyclases; N_i, inhibitory guanine nucleotide binding component that regulates adenylyl cyclases; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; App(NH)p, adenyl-5′-yl imidodiphosphate; dial GTP or dial ATP, the 2′,3′ dialdehyde of the nucleotide triphosphate

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tive amino groups in the vicinity of the binding sites [9,10]. The Schiff base that forms between the protein amino group and one of the carbonyls of the 2′,3′-dialdehyde of the nucleotide triphosphate can be stabilized by borohydride reduction. Dial ATP followed by reduction with $NaCNBH_3$ has been demonstrated to inhibit irreversibly the catalytic subunit of the enzyme [11].

The results presented below show that reaction of the membranes with dial GTP preferentially inactivates the N_s protein that mediates guanine nucleotide and fluoride stimulation of adenylyl cyclase. The catalytic subunit, as measured with forskolin and Mn^{2+}, is slightly affected by dial GTP. The reaction of the membrane with the dial GTP almost completely eliminates the inhibition of the adenylyl cyclase by progesterone. This inhibition by the hormone cannot be restored by additional exogenous N_s. Using radioactive dial GTP it is possible to label several membrane proteins, some of which have similar M_r to N_s and N_i.

2. MATERIALS AND METHODS

2.1. *Preparation and assay of adenylyl cyclase from* X. laevis *oocytes*

The isolation of *X. laevis* ovarian oocytes and the preparation of the membrane fractions that contain the major adenylyl cyclase activity were carried out exactly as described [3]. The activity of the adenylyl cyclase was assayed by the procedure of Rodbell [12], using the method of Solomon et al. [13] to purify the [α-^{32}P]cAMP synthesized. Other details have been published [8].

2.2. *Preparation of the 2′,3′-dialdehyde of GTP*

The synthesis of the 2′,3′-dialdehyde of GTP was done by the method of Easterbrook-Smith et al. [9], using periodate to oxidize the 2′- and 3′- of the ribose of the GTP and excess ethylene glycol to stop the reaction. The dial GTP was separated from the other reaction products by gel filtration on Sephadex G-10 and its purity was controlled by thin-layer chromatography on polyethyleneimine plates developed with 1.2 M LiCl. Dial [α-^{32}P]GTP was synthesized by first preparing [α-^{32}P]GTP using the procedure of Walseth and Johnson [14] and subsequently going through the oxidation and purification steps described above.

2.3. *Treatment of oocyte membranes with dial GTP*

The membrane preparations with adenylyl cyclase activity described above (15–25 mg/ml) that had been previously washed by resedimenting 3 times in an Eppendorf microfuge for 10 min in a buffer containing 50 mM sodium phosphate buffer (pH 8.0), 1 mM dithiothreitol and 1 mM EDTA were incubated for 1 h with 1 mM dial GTP in a buffer containing 50 mM sodium phosphate buffer (pH 8.0), 1 mM dithiothreitol, 0.3 mM EDTA, 5% glycerol, 5 mM $MgCl_2$, 4 mM creatine phosphate, 0.4 mg/ml creatine kinase and 5 mM App(NH)p. The reaction was stopped by addition of a few crystals of $NaBH_4$ and a 5-fold dilution with 50 mM Hepes, pH 8.0. The membranes were isolated again by centrifugation in an Eppendorf microfuge for 10 min and washed 3 times by resedimenting in the same buffer. Since dial ATP has been shown to inactivate the catalytic subunit of adenylyl cyclase [11], it seemed possible that dial GTP could also be reacting with the nucleotide triphosphate site of the catalytic subunit. To prevent this, the treatment of the membranes was carried out routinely in the presence of 5 mM App(NH)p, which should protect ATP sites and has the advantage of being resistant to ATPases present in the membranes.

2.4. *Other techniques and sources of materials*

SDS-polyacrylamide gels and autoradiography were carried out as described by Laemmli [15] and previously detailed [3]. [α-^{32}P]ATP was prepared as described by Walseth and Johnson [14] using ^{32}P purchased from the Chilean Atomic Energy Commission. [^{3}H]cAMP was purchased from New England Nuclear. Progesterone and forskolin were obtained from Calbiochem. Gpp(NH)p, ATP, GTP and cAMP were from Sigma. Pure human erythrocyte N_s was obtained as described by Codina et al. [16] and was a kind gift from Dr Birnbaumer.

3. RESULTS

3.1. *The effect of dial GTP treatment of oocyte membranes on the activity of adenylyl cyclase*

Fig.1A shows the effect of pretreating oocyte membranes with different concentrations of dial GTP on the activity of the adenylyl cyclase measured in the presence or absence of Gpp(NH)p. It

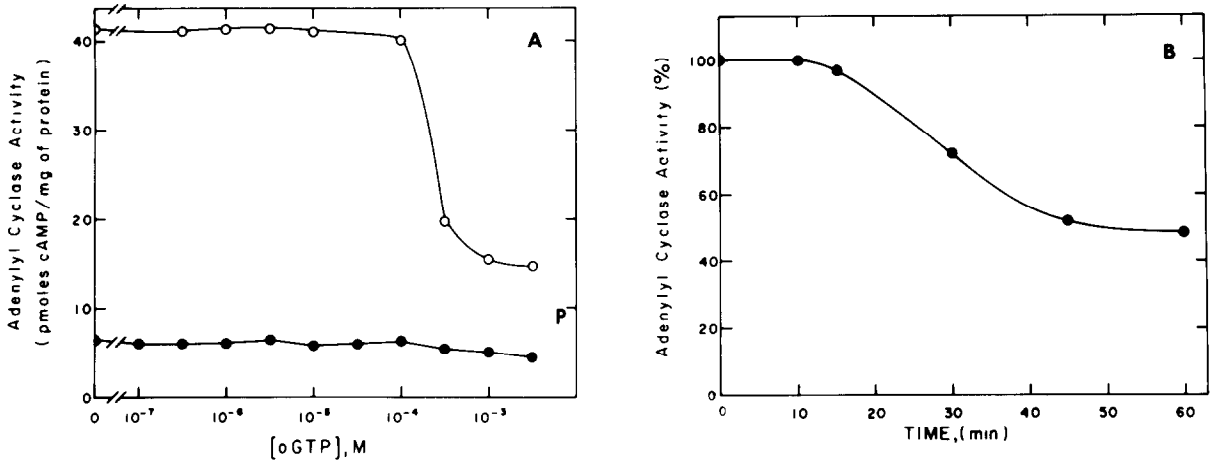


Fig.1. Inhibition of adenylyl cyclase by treatment of oocyte membranes with dial GTP. (A) Membranes were treated with the concentrations specified of dial GTP for 1 h by the procedure detailed in section 2. Subsequently the adenylyl cyclase activity was measured in the presence of 25 μM Gpp(NH)p (○—○) or without this guanine nucleotide (●—●) using 120 μg membrane protein as described in section 2. (B) Treatment of the membranes with 1 mM dial GTP was carried out at 32°C for different periods of time and the adenylyl cyclase activity was measured in the presence of 25 μM Gpp(NH)p. The control value of 0 time treatment, which was taken as 100% activity, was 22 pmol cAMP produced/mg membrane protein.

is clear that concentrations of dial GTP above 0.1 mM greatly inhibit the activation of the enzyme caused by GPP(NH)P without affecting the basal activity of the enzyme. The activation of adenylyl cyclase by Gpp(NH)p is mediated by N_s. Fig.1B shows that the inhibition of N_s by treatment of the membranes with dial GTP is a time-dependent process that approaches completion after 60 min incubation. Similar results were obtained using the 2′,3′-dialdehyde of GDP (not shown). The specificity of the inhibition caused by dial GTP was determined by measuring the activity of the membranes in the presence of several activators of adenylyl cyclase, as shown in table 1. In the control tubes the membranes were pre-incubated in the presence of an equal concentration of unmodified GTP. It has been shown [17] that GTP has a negligible effect on the oocyte adenylyl cyclase and that only the non-hydrolyzable analogues of GTP are able to stimulate the enzyme. It can be seen in table 1 that the most pronounced inhibition caused by the dial GTP involves Gpp(NH)p (79%) and fluoride (62%), both of which are known to act through N_s. It is also important to note that the presence of an equal amount of unmodified GTP during the treatment significantly reduces the inhibition caused by the dial GTP on the enzyme activated by Gpp(NH)p. Forskolin, a diterpene drug which is known to activate mainly the catalytic subunit, is also inhibited, but to a lesser degree than the N_s activators. Mn^{2+}, which is known to function exclusively through activation of the catalytic subunit, is not significantly inhibited and is therefore similar to the basal activity.

3.2. *Effect of dial GTP on the inhibition of the adenylyl cyclase by progesterone*

The oocyte adenylyl cyclase that is stimulated by guanine nucleotides can be strongly inhibited by micromolar concentrations of progesterone. The data presented in table 2 show that dial GTP treatment of the membranes, in addition to greatly reducing the activation of Gpp(NH)p, almost completely eliminates the inhibition caused by progesterone. Since the evidence indicated that the dial GTP was preferentially inactivating the N_s subunit of the membrane, an experiment was performed to determine the effect of adding increasing concentrations of exogenous untreated pure N_s from human erythrocytes (fig.2). The addition of exogenous N_s to the membranes treated with the dial GTP (dashed lines) in the presence of Gpp(NH)p increases the activity 3-fold. However, progesterone inhibits the adenylyl cyclase activity

Table 1

Effect of dial GTP treatment of membranes on the stimulation of adenylyl cyclase by different activators

Nucleotide used to treat membranes	Activator present in assay	Adenylyl cyclase activity (pmol cAMP/mg protein)	% inhibition caused by dial GTP
None	none	4.3	–
GTP	none	4.8	–
Dial GTP	none	4.6	4
GTP	Gpp(NH)p	85.1	–
Dial GTP	Gpp(NH)p	18.0	79
Dial GTP + GTP	Gpp(NH)p	40.0	53
GTP	forskolin	110.0	–
Dial GTP	forskolin	67.4	39
GTP	fluoride	78.7	–
Dial GTP	fluoride	30.3	62
GTP	Mn^{2+}	22.0	–
Dial GTP	Mn^{2+}	20.2	10

Treatment of the membranes with dial GTP or with GTP in the control tubes was carried out as described in section 2, always using 1 mM of each nucleotide. Gpp(NH)p was used at 15 μM, forskolin at 150 μM, NaF at 10 mM. In the experiments with Mn^{2+}, the $MgCl_2$ present in the normal assay mixture was replaced by 10 mM $MnCl_2$. The assay of adenylyl cyclase was performed as in [8], using 100 μg membrane proteins and incubating for 30 min at 32°C. All results presented are the averages of triplicate determinations. The % inhibition due to dial GTP treatment was calculated using as reference the value obtained with the same activator with membranes treated with GTP

only slightly even in the presence of the added untreated N_s. Exogenous erythrocyte N_s can also stimulate the adenylyl cyclase of the untreated membranes. In this case, the inhibition by progesterone is maintained at a constant absolute value when the N_s is limiting as seen by the fact

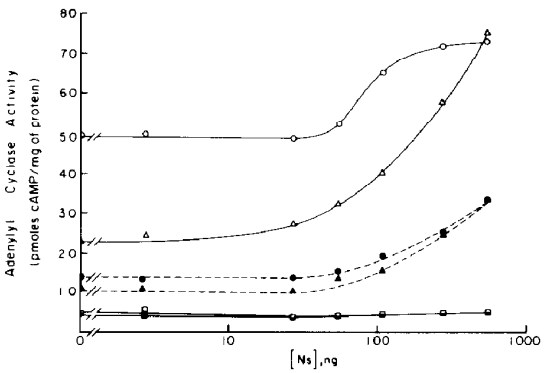


Fig.2. Effect of addition of exogenous N_s on the adenylyl cyclase activity of control and dial GTP treated membranes with and without progesterone. Pure human erythrocyte N_s was added to membranes that had been pre-treated with 1 mM dial GTP (▲,●,■) or with 1 mM GTP (△,○,□) and re-isolated, and incubated for 30 min at 4°C in the same buffer used for membrane isolation. The adenylyl cyclase activity was measured with addition of 15 μM Gpp(NH)p, in the presence (△,▲) or absence (○,●) of 5 μM progesterone. All the assays contained 75.0 μg membrane protein and the incubations (35 min) were carried out and processed as described in section 2. The squares at the bottom indicate the basal activity of the enzyme measured in the absence of Gpp(NH)p.

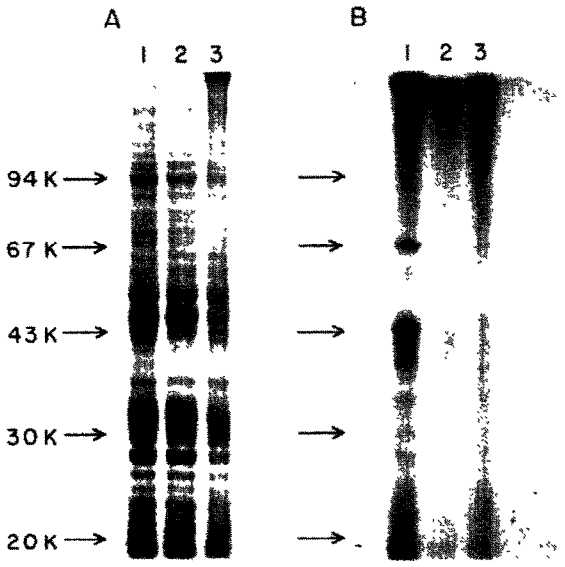
Table 2

Effect of dial GTP treatment on the inhibition of progesterone of adenylyl cyclase stimulated by Gpp(NH)p

Nucleotide used to treat membranes	Additions to cyclase assay	Adenylyl cyclase activity (pmol cAMP/mg protein)	% inhibition by progesterone
GTP	none	3.7	–
	Gpp(NH)p	62.8	–
	Gpp(NH)p + progesterone	29.4	53
Dial GTP	none	4.0	–
	Gpp(NH)p	16.0	–
	Gpp(NH)p + progesterone	14.6	9

The treatment of the membranes using 1 mM GTP or 1 mM dial GTP was as described in section 2. The assay of the adenylyl cyclase was carried out as detailed [8], using 100 μg membrane proteins and 30 min incubation at 32°C and where specified, 15 μM Gpp(NH)p and 5 μM progesterone. The % inhibition due to progesterone was calculated using as reference the value obtained with the respective membranes and Gpp(NH)p

that the curves with and without the hormone rise in parallel. At the highest concentration of exogenous N_3, when this protein is apparently in excess, the inhibition by progesterone is eliminated.



3.3. *Labelling of membrane proteins with radioactive dial GTP*

If the dial GTP is reacting to form covalent bonds with membrane proteins, it should be possible to label the membrane proteins that interact with the guanine nucleotide by using a radioactive derivative. The membrane preparations have a large number of proteins that can be separated by SDS electrophoresis and subsequently stained (fig.3A). Fig.3B shows the autoradiograph of these oocyte membrane proteins labelled by treatment with dial [α-^{32}P]GTP and subsequently separated

Fig.3. Labelling of membrane proteins with dial [α-^{32}P]GTP. Membrane proteins (0.3 mg) were incubated with dial [α-^{32}P]GTP under different conditions. Subsequently the membrane proteins were fractionated on an SDS-polyacrylamide gel electrophoresis. Channel 1 was loaded with membranes treated with 200 μM dial [α-^{32}P]GTP; in channel 2, 10 mM GTP was present in addition to the same amount of dial [α-^{32}P]GTP and in channel 3, the membrane proteins had been reacted with non-radioactive 1 mM dial GTP for 60 min and washed by sedimentation previous to the treatment with the dial [α-^{32}P]GTP. (A) Membrane proteins stained with Coomassie blue, (B) autoradiography of the same gel.

by SDS gel electrophoresis. It is evident that several proteins are labelled by this procedure (channel 1) and that the labelling can be prevented by carrying out the reaction in the presence of excess non-radioactive GTP (channel 2). Labelling can also be prevented by previously treating the membranes with non-radioactive dial GTP (channel 3). The most prominent bands are in the M_r range of 65 000 and of 40 000–44 000. These latter proteins are important since they approximately coincide with the M_r values of N_s and N_i of oocytes [3,4].

4. DISCUSSION

The results presented above demonstrate that treatment of *X. laevis* oocyte membranes with the 2′,3′-dialdehyde of GTP can affect the activity of the adenylyl cyclase present in those membranes. The dial GTP clearly acts preferentially on the guanine nucleotide-binding site of protein N_s as evidenced by the fact that GTP protects from inactivation and that the most pronounced inhibition is obtained with Gpp(NH)p and F^-, which act through N_s.

The dial GTP treatment also diminishes forskolin activation, albeit considerably less than the activation obtained with Gpp(NH)p and F^-. This result is interesting in the light of the fact that forskolin is supposed to act mainly on the catalytic subunit of adenylyl cyclase. It can be contrasted with the finding that Mn^{2+} activation, which is also thought to act exclusively on the catalytic subunit, is only slightly inhibited by the dial GTP. This apparent discrepancy could be resolved if N_s were to participate in the activation of the catalytic subunit by forskolin. A similar explanation was proposed by Wong and Martin [18], who obtained identical results using GTP-γ-azidoanilide on a rat liver adenylate cyclase. Other work supporting the partial involvement of N_s in forskolin activation of adenylyl cyclase has also appeared [19–21].

The fact that added exogenous pure N_s can restore a large part of the activity lost by dial GTP treatment of the membranes (fig.3) also strongly supports the idea that this treatment preferentially inactivates the endogenous N_s.

The most interesting finding of this work is that the treatment of the membranes with the dial GTP practically eliminates the inhibition of the adenylyl cyclase by progesterone. This finding is very different from that obtained with phenothiazines, which affect the general membrane properties [22], or with proteolytic treatment of the membranes [4], both of which cause a drastic decrease of the activity of the enzyme, but do not reduce the percent inhibition by progesterone. This finding lends support to our hypothesis that N_s is involved in the mechanism of action of progesterone inhibition of oocyte adenylate cyclase. It also indicates that the fraction of N_s that most readily reacts with the dial GTP is also the fraction of that subunit that is affected by progesterone inhibition.

Another important conclusion is that, while exogenous N_s can activate the catalytic subunit of the enzyme, the added protein cannot act as a transducer of the hormonal effect. The hormone and its receptor must be somehow coupled to the endogenous N_s is a manner that cannot be achieved by the exogenous component under the conditions tested. Pertinent to these observations are the findings of Arad et al. [23], who have reported evidence to show that the components of the adenylyl cyclase are interacting closely in the membrane. The results obtained with the untreated membranes also showed that when an excess exogenous N_s is added, and presumably the catalytic subunit is limiting, the endogenous N_s that is susceptible to progesterone inhibition can be competed out.

The use of dial GTP to label guanine nucleotide binding proteins may be useful in identifying 'G-proteins' and in studying their active residues and sites. In addition to the adenylyl cyclase regulatory proteins, other GTP and GDP binding proteins play key roles in protein synthesis, light transduction in the retina, tubulin polymerization [24,25], and even in the mechanism of oncogenesis involving the ras oncogene [26].

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