THE ACTIVATION OF ADENYLATE CYCLASE BY 1-EPINEPHRINE AND GUANYLYLIMIDODIPHOSPHATE AND ITS REVERSAL BY 1-EPINEPHRINE AND GTP

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1. Introduction

GTP and more so its non-hydrolyzable analog GppNHp were found to be positive allosteric effectors of hormone activated adenylate cyclases in numerous systems [1–3]. Among the hormone activated adenylate cyclases which are synergistically activated by hormone and GppNHp is the catecholamine dependent adenylate cyclase of nucleated erythrocytes [4–8]. Detailed kinetic analysis performed on the synergistic activation of turkey erythrocyte adenylate cyclase has demonstrated [7] that the hormone and GppNHp act in a synergistic fashion to bring about the formation of a permanently-active enzyme species. The activation process can be described according to the following scheme:

$$R \cdot E \cdot \text{GppNHp} + H \underset{k_2}{\overset{k_1}{\rightleftharpoons}} H \cdot R \cdot \text{GppNHp} \xrightarrow{k_3} H \cdot R \cdot E' \cdot \text{GppNHp} \quad (1)$$

where R is the hormone receptor, E the enzyme, H the hormone, E' the permanently active species of the enzyme and k_3 the rate constant characterizing the conversion of the inactive enzyme species to its active form E'. The E'-species is insensitive to β-adrenergic blockers such as propranolol and is permanently active [4–7]. This rate constant, k_3, was measured for the turkey erythrocyte catecholamine dependent adenylate cyclase and was found to be $k_3 = 0.7\ \text{min}^{-1}$ [7]. The specific activity of the enzyme in the presence of 1-epinephrine and GppNHp is 8-fold higher than in the presence of GTP and 1-epinephrine [6,7]. In view of the finding that turkey erythrocyte membranes possess a specific β-receptor-dependent GTPase [9] a general scheme describing both the activation of the adenylate cyclase by hormone and GTP and the termination of the hormonal signal was derived [10]. This general scheme can be summarized in eq.2:

$$R \cdot E \cdot \text{GTP} + \text{H} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} H \cdot R \cdot E \cdot \text{GTP} \xrightarrow{k_3} H \cdot R \cdot E' \cdot \text{GTP} \xrightarrow{k_4} H \cdot R \cdot E + \text{GDP} + \text{P}_\text{i} \quad (2)$$

where k_3 describes the rate of enzyme activation and k_4 describes the rate of enzyme deactivation via the hormone-dependent GTPase step. From eq.2 it can be derived [10] that in the presence of GTP the steady-state concentration of E' is given by:

$$E' = \frac{E_o}{1 + k_4/k_3} \quad (3)$$

where E_o is the total enzyme concentration. It follows that E' never exceeds 10–15% of E_o since k_4 was found to be $k_4 = 4.0$–$6.0\ \text{min}^{-1}$ [9]. When GppNHp is used instead of GTP, $k_4 = 0$ and E' equals E_o and thus the specific activity measured in the presence of GppNHp is 7–10-fold higher than in the presence of GTP [7,10]. To test this hypothesis a diagnostic experiment was designed. The experiment consists of the following: activation of the adenylate cyclase

Abbreviations: GppNHp guanylylimidodiphosphate, cAMP adenosine 3',5'phosphate

by 1-epinephrine + GppNHp to its E'-state (eq.1). Then adding GTP which should replace GppNHp in the $H \cdot R \cdot E'$ · GppNHp species to form the $H \cdot R \cdot E' \cdot$ GTP species. The latter species should then revert to $H \cdot R \cdot E$ according to eq.2 and the low steady-state level of E' (eq.3) should be re-established. The net result of such an experiment would be the reversion of the permanently active state of the enzyme to its low, steady-state level of activity, in a hormone-dependent fashion. Such an experiment is the subject of this communication. Preliminary results from our laboratory already indicated [7] that ATP and 1-epinephrine are capable of reverting the permanently active enzyme to its low activity form.

2. Materials and methods

Turkey erythrocyte membranes were prepared as described earlier [11]. Adenylate cyclase assay was performed as described by Salomon et al. [12]. All chemicals were of the highest analytical purity available and all solutions were prepared using Corning double distilled water.

3. Results

3.1. *The reversal of the GppNHp + 1-epinephrine active-state by GTP*

The kinetics of reversal of the permanently active state induced by 1-epinephrine and GppNHp is shown in fig.1. In this experiment the GppNHp + epine-

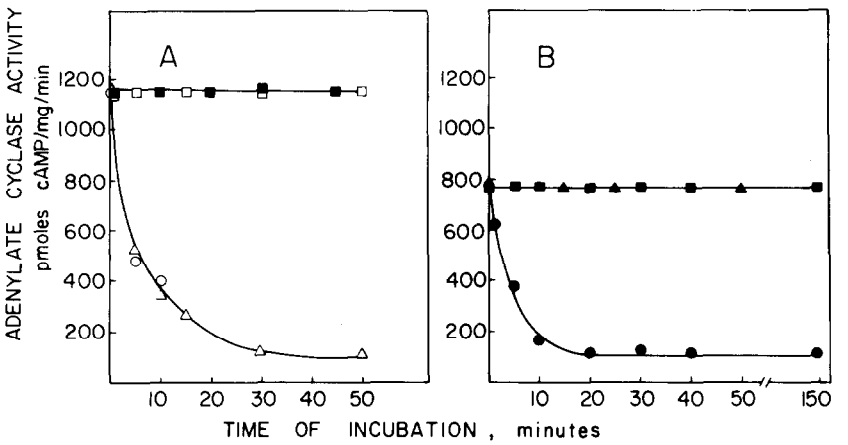

Fig.1. The reversal of the permanently active cyclase to its inactive form by 1-epinephrine and GTP and by 1-epinephrine and ATP. (A) Turkey erythrocyte membranes (1.8 mg/ml) were preactivated by 0.1 mM 1-epinephrine and 0.05 mM GppNHp in the presence of 0.05 M Tris–HCl, pH 7.4, containing 1.0 mM EDTA and 2.0 mM $MgCl_2$ in final vol. 10 ml for 40 min at 37°C, conditions sufficient to bring about full activation of the enzyme [7]. At the end of the incubation the incubation mixture was centrifuged at 20 000 × g for 15 min. Then the pellet was diluted into 10 ml 0.05 M Tris–HCl containing 1 mM EDTA and 3 mM $MgCl_2$ and centrifuged at 20 000 × g for 15 min. The pellet was resuspended in the above buffer and recentrifuged. The process was repeated four times. Then the pellet was resuspended in 4.0 ml 0.05 M Tris–HCl buffer, pH 7.4, containing 1.0 mM EDTA, 4.0 mM $MgCl_2$ GTP, phosphoenolpyruvate (0.5 mg/ml) pyruvate kinase (20 units/ml), 5.0 mM KCl and 0.1 mM 1-epinephrine in the absence or in the presence of 2×10^{-5} M DL-propranolol. At specified times the reversal process was stopped by withdrawing samples into an 0.05 M Tris–HCl buffer, pH 7.4, containing DL-propranolol to yield a final concentration of 5.0×10^{-6} M. The samples were washed four-times in Tris–$MgCl_2$–EDTA buffer containing 5.0×10^{-6} M DL-propranolol and then assayed for adenylate cyclase activity in the presence of 5×10^{-6} M DL-propranolol. The enzyme preparation used in this experiment exhibited a specific activity of 80 ± 5 pmol cAMP/mg/min in the presence of 1.0×10^{-4} M 1-epinephrine and in the absence of GppNHp. (○-○-○) 1.0×10^{-5} M GTP + 1.0×10^{-4} M 1-epinephrine. (△-△-△) 5×10^{-4} M GTP + 1.0×10^{-4} M 1-epinephrine. (□-□-□) 5×10^{-4} M GTP + 1.0×10^{-4} M 1-epinephrine + 2×10^{-5} M DL-propranolol. (■-■-■) 5×10^{-4} M GTP alone. (B) Experimental details are identical to those described in A except that ATP rather than GTP was used as the reversing ligand. In this case 1 mM ATP and an ATP regenerating system consisting of 4.9 mg/ml creatine phosphate and 0.22 mg/ml creatine phosphokinase was used instead of GTP and GTP regenerating system. All other details in B are identical to those described in A. (●-●-●) 1 mM ATP + 0.1 mM 1-epinephrine. (■-■-■) 1 mM ATP + 0.1 mM 1-epinephrine + 2.0×10^{-5} M DL-propranolol. (▲-▲-▲) 1 mM ATP alone.

Table 1
Reversal of the active-state to its inactive-state by purine nucleotides

Ligands added	Adenylate cyclase activity (pmol cAMP/mg/min)
None	1100 ± 110
1 mM AMP	1080 ± 110
1 mM AMP + 0.1 mM 1-epinephrine	915 ± 60
1 mM GMP	950 ± 95
1 mM GMP + 0.1 mM 1-epinephrine	582 ± 40
0.1 mM ADP	900 ± 80
1 mM ADP	880 ± 70
0.1 mM ADP + 0.1 mM 1-epinephrine	785 ± 60
1 mM GDP	950 ± 60
0.1 mM GDP + 0.1 mM 1-epinephrine	310 ± 40
0.1 mM 1-epinephrine alone	1190 ± 130
1 mM ATP alone	1210 ± 100
1 mM ATP + 0.1 mM 1-epinephrine	200 ± 30
0.5 mM GTP alone	1150 ± 120
0.5 mM GTP + 0.1 mM 1-epinephrine	130 ± 10

Experimental conditions are identical to those described in the legend to fig.1. The incubation time was 40 min at 37°C. When GDP, ADP, GMP and AMP were tested as reversing ligands, no regenerating systems were added to avoid the formation of the triphosphates. All assays were performed in triplicates. The results are presented as values ± SEM.

phrine activated enzyme was incubated in the presence of saturating 1-epinephrine and saturating GTP plus GTP regenerating system. Neither GTP alone nor 1-epinephrine alone cause any reversal (fig.1, table 1). For the GTP experiments phosphoenolpyruvate plus pyruvate kinase was found to be a more effective GTP-regenerating system than phosphocreatine plus creatine kinase. The reversal kinetics were plotted on a semilog plot (fig.2). From fig.2 it can be seen that the process of reversal by GTP in the presence of hormone is a first-order process with a half-life of $t_1/_2$ = 4.9 min and one can calculate a first-order rate constant of $k_{reversion}$ = 0.12 min^{-1}. This value probably corresponds to the rate of the GppNHp dissociation from the active $H \cdot R \cdot E^*$ · GppNHp complex (see Discussion). The β-adrenergic blocker propranolol inhibits this reversal effect (fig.1A).

3.2. *The reversal of the GppNHp + 1-epinephrine active-state by ATP*

ATP in the presence of 1-epinephrine is also capable of reverting the permanently active-state of

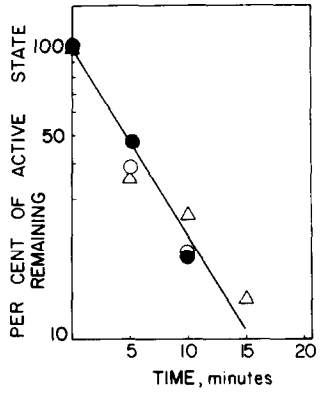

Fig.2. The first-order nature of the reversal process. Data of fig.1A and fig.1B are plotted on a semilogarithmic plot. The half-life of the activated state of the enzyme is $t_1/_2$ = 4.9 min which corresponds to a first-order rate constant k = 0.12 min^{-1}. (○-○-○) 1.0 × 10^{-5} M GTP + 1.0 × 10^{-4} M 1-epinephrine. (△-△-△) 5 × 10^{-4} GTP + 1.0 × 10^{-4} M 1-epinephrine. (●-●-●) 1 × 10^{-3} M ATP + 1.0 × 10^{-4} M 1-epinephrine.

the enzyme to its low activity form (fig.1B, table 1). ATP alone or 1-epinephrine alone are incapable of inducing such an effect (fig.1B, table 1). The concentrations of ATP required to induce the reversal effect are two orders of magnitude higher than the concentrations of GTP required to induce the same effect. As in the case of the GTP plus epinephrine induced effect the half-life of the reversal kinetics is $t_{1/2}$ = 4.9 min which is similar to the value observed for the GTP plus hormone-induced effect. As in the case of GTP, propranolol was found to inhibit the reversal reaction. Preliminary experiments [7] have already indicated to us that ATP in the presence of a hormone can revert the GppNHp plus hormone activated state to its lower activity form.

3.3. *The effects of ADP, GDP, AMP and GMP on the reversal reaction*

When enzyme pre-activated by 1-epinephrine plus GppNHp is incubated in the presence of 1 mM ADP or 1 mM GDP in the presence of 1-epinephrine, the enzyme is also partially reverted to its low activity level (table 1). GMP was found to be much less effective than the purine nucleoside disphosphates GDP and ADP (table 1). AMP was found to be completely ineffective to bring about the reversal of the activated-state (table 1). All the experiments using the purine nucleoside diphosphates and nucleoside monophosphates were performed in the absence of a regenerating system in order to avoid the formation of nucleoside triphosphates.

3.4. *The concerted nature of the GTP and hormone action*

When the highly active enzyme is incubated first with GTP (or ATP) and then with hormone or vice versa, no reversal occurs. Thus, both ligands must occupy their respective sites simultaneously in order for the reversal process to occur.

4. Discussion

4.1. *The nature of the reversal process*

The 1-epinephrine dependent adenylate cyclase of turkey erythrocyte can be activated to its permanently active state, E', by 1-epinephrine and GppNHp according to eq.1. In the presence of excess GTP and hormone the enzyme can be induced to revert to its characteristic lower activity form. This phenomenon probably involves the displacement of GppNHp from its regulatory site by GTP which subsequently is hydrolyzed to GDP and P_i, provided the β-adrenergic receptor is occupied by a β-agonist such as 1-epinephrine. This process can be summarized in eq.4:

$$H \cdot R \cdot E' \cdot \mathrm{GppNHp} \underset{+\mathrm{GppNHp}}{\overset{-\mathrm{GppNHp}}{\rightleftharpoons}} H \cdot R \cdot E' \underset{-\mathrm{GTP}}{\overset{+\mathrm{GTP}}{\rightleftharpoons}}$$

$$H \cdot R \cdot E' \cdot \mathrm{GTP} \longrightarrow H \cdot R \cdot E \cdot \mathrm{GDP} + P_i \longrightarrow$$

$$H \cdot R \cdot E + \mathrm{GDP} \qquad (4)$$

Thus, in the presence of excess GTP the characteristic steady-state level of active enzyme in the absence of GppNHp is re-established. This steady-state level is characterized by eq.3. The process of reversion in the presence of excess GTP is a first-order process as revealed by fig.2. It can be calculated that the rate constant characterizing the process is $k = 0.12\ \mathrm{min}^{-1}$. The subsequent step, namely the hormone-dependent GTPase step is fast (k_4 = 4.0–6.0 min^{-1} in eq.2) as was found by Cassel and Selinger [9] and therefore is not rate-limiting in the overall reversal process. Sequential exposure of the active enzyme to GTP and hormone or vice versa does not bring about the reversal process. Thus, the process of GppNHp dissociation from the GTP regulatory-site requires the presence of hormone. DL-Propranolol inhibits the conversion E'–E (fig.1) which again demonstrates that both the hormone and the purine nucleotide must bind simultaneously to induce reversion. One may also conclude that the affinity of GppNHp within the $H \cdot R \cdot E' \cdot$ GppNHp species is much lower than within the $R \cdot E' \cdot$ GppNHp species, since the sequential exposure of the activated enzyme to GTP and then to hormone does not lead to a reversion. The reversal of the highly active form of the enzyme to its low activity form can also be monitored using [^{3}H]GppNHp. Cassel and Selinger [13] were indeed able recently to demonstrate that L-isoproterenol and GTP induce the release of 1.0–2.0 pmol/mg membrane [^{3}H]GppNHp. Since turkey erythrocyte membranes possess 1.0–2.0 pmol/mg β-adrenergic receptor [14,15] one may conclude that the stoi-

chiometry of hormone-receptor to GTP regulatory-sites is 1.0–2.0.

4.2. *ATP as a reversing ligand*

ATP acts in a similar fashion to GTP (fig.1B, fig.2 and table 1) but is effective at concentrations two orders of magnitude higher than the concentration of GTP required to bring about the same effect. This may be due to the fact that all ATP preparations used are contaminated with GTP [16] and it is the latter which induces the reversal effects. It is still, however, possible that the effect is that of ATP which exhibits a much lower affinity to the GTP regulatory-site as compared to GTP.

4.3. *Purine nucleoside diphosphates and monophosphates as reversing ligands*

GDP and ADP at high concentrations in the presence of 1-epinephrine can induce slowly the process of reversion (table 1). GMP in the presence of hormone was also found to induce slowly the conversion of the highly active form of the enzyme to its inactive form whereas AMP was completely ineffective (table 1). The fact that GDP and even GMP can induce the reversal of the highly active form of the enzyme to its low-activity form in the presence of hormone indicates that the GTPase step is not essential for the reversal step per se. The action of GDP or GMP as reversing ligands can be described by eq.5:

$$H\cdot R\cdot E'\cdot \mathrm{GppNHp} \underset{+\mathrm{GppNHp}}{\overset{-\mathrm{GppNHp}}{\rightleftharpoons}} H\cdot R\cdot E'$$

$$\underset{-\mathrm{GDP}}{\overset{+\mathrm{GDP}}{\rightleftharpoons}} H\cdot R\cdot E'\cdot \mathrm{GDP} \rightarrow H\cdot R\cdot E + \mathrm{GDP} \qquad (5)$$

Thus, the conversion of E' to E does not require the GTPase step. The reversal process induced by GDP or GMP is slower than in the presence of GTP since probably the conversion of $H\cdot R\cdot E'\cdot \mathrm{GDP}$ to $H\cdot R\cdot E\cdot \mathrm{GDP}$ is slow and becomes rate-limiting.

5. Conclusions

The permanently active state of turkey erythrocyte adenylate cyclase induced by 1-epinephrine and GppNHp can be reverted to its low-activity form, characteristic to that in the absence of GppNHp, by GTP and 1-epinephrine. The process or reversal requires the simultaneous presence of GTP and a β-agonist and is blocked by the β-adrenergic blocker propranolol. ATP, GDP and GMP can mimic the effect of GTP but less effectively. The process of reversal in the presence of saturating hormone and GTP is first-order with a rate constant of $k = 0.12$ min^{-1}. This rate constant probably reflects the rate of GppNHp dissociation from the adenylate cyclase complex. The reversal process by GTP can be summarized by eq.6:

$$H\cdot R\cdot E'\cdot \mathrm{GppNHp} \rightleftharpoons H\cdot R\cdot E' \overset{\mathrm{GTP}}{\rightleftharpoons} H\cdot R\cdot E'\cdot$$

$$\mathrm{GTP} \overset{k'_4}{\longrightarrow} H\cdot R\cdot E\cdot \mathrm{GTP} \overset{k_4}{\longrightarrow} H\cdot R\cdot E + \mathrm{GDP} + P_i \qquad (6)$$

where k'_4 is fast compared to k_4 (the GTPase-step). The value of k'_4 is probably lower when nucleoside diphosphates and monophosphates are used. The fact that GTP is the most effective ligand inducing the reversion process indicates that it is probably the physiological ligand which controls the hormone activation of adenylate cyclase.

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