

SLOW GDP DISSOCIATION FROM THE GUANYL NUCLEOTIDE-BINDING SITE OF TURKEY ERYTHROCYTE MEMBRANES AS THE LIMITING STEP IN THE ACTIVATION OF ADENYLATE CYCLASE BY β -ADRENERGIC AGONISTS

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1. Introduction

Different biochemical entities taking place in the hormone-induced adenylylase activation have been experimentally identified. The β -adrenergic-sensitive adenylylase system would consist of specific β -adrenergic receptors capable of binding β -agonists, guanylyl nucleotide binding proteins [1] exhibiting GTPase activity [2] and activable adenylylase molecules. The turkey erythrocyte adenylylase system differs from other systems in several respects. For instance nonhydrolyzable analog of GTP as guanosine-5'-(β,γ -imino)triphosphate Gpp(NH)p, cannot activate rapidly a membrane preparation of turkey erythrocytes in the absence of β -agonist [3] and once the enzyme has been activated by isoproterenol and Gpp(NH)p, excess of hormone and Gpp(NH)p can be removed by washing while the adenylylase remains fully activated [4]. These two observations suggest that guanylyl nucleotides remain tightly bound to the specific binding protein in the absence of hormone [4]. The binding of a β -agonist would induce the opening of the guanylyl nucleotide binding protein allowing the binding of GTP or GTP analog which produces the activation of adenylylase [5]. The hydrolysis of bound GTP would revert the system to the inactive state.

The activation of the turkey erythrocyte adenylylase by β -agonist and Gpp(NH)p presents a lag period [6] which is a consequence of the existence of a limiting step. It has been proposed [7] and then observed [8] that the limiting step could be the GDP

dissociation from the binding protein.

Another possible mechanism accounting for a rate limiting step has been described by the collision coupling model [9]. The main hypothesis of this model is that the binding of the hormone-receptor complex with the adenylylase system (either the guanylyl nucleotide binding protein or the enzyme) would be rate limiting. This hypothesis is not contradictory with the apparent slow GDP dissociation as the binding of the receptor with the guanylyl nucleotide binding protein would precede and delay the GDP dissociation. However this argument was not developed by these authors. Theoretical analysis suggests that the collision coupling hypothesis is not valid as the binding of the receptor with the binding protein would be much too fast to account for the slow adenylylase activation [10]. This result prompted us to propose the alternative hypothesis [10]: the limiting step would be at the very level of the dissociation of GDP molecules from the guanylyl nucleotide binding protein.

The aim of this paper was to test experimentally this hypothesis by comparing the kinetics of adenylylase activation by stimulating, respectively, 'GDP-loaded' membranes and 'GDP-depleted' membranes. If our hypothesis is valid, the lag period should decrease as the occupancy level of the sites by GDP is decreasing whereas no effect should be observed if the collision coupling hypothesis applies. We report here experimental evidence that GDP dissociation would be rate limiting and that native turkey erythrocyte membranes would be GDP saturated.

2. Materials and methods

[α - 32 P]ATP and cyclic [3 H]AMP were obtained from the Radiochemical Centre, Amersham, Gpp(NH)p, GTP, ATP, creatine kinase and phospho-creatine from Boehringer and cyclic AMP from Sigma. Turkey erythrocytes membranes were prepared and adenylate cyclase was assayed as in [11,12]. The reaction mixture (150 μ Ci final vol./test tube), at 37°C contained 50 mM Tris-HCl (pH 7.4), 5 mM MgSO₄, 1 mM EDTA, 0.4 mg/ml theophylline, 20 mM creatine phosphate, 25 U/ml creatine kinase and 0.35 mg protein/ml erythrocyte membranes. The reaction was initiated by the addition of a solution containing 1 mM [α - 32 P]ATP (~10 cpm/pmol), 50 μ M isoproterenol, 0.1 mM ascorbic acid and 0.1 mM Gpp(NH)p. The reaction was stopped by adding 100 μ l of a solution at 0°C containing 50 mM ATP and 10 mM cyclic [3 H]AMP (~20 000 cpm/test tube) and the test tube was transferred to an ice bath. Cyclic AMP was then isolated, in one step, by elution on dry neutral alumina columns (5 cm long) [13]. The first 3 ml eluate were discarded and the next 3 ml eluate were counted for 3 H and 32 P activities. The recovery of cyclic AMP was ~70% and ATP contamination was \leq 0.01%. Proteins were measured according to [14] using bovine serum albumin as standard.

3. Results

Figure 1 shows the accumulation of cyclic AMP of native membranes stimulated by 50 μ M isoproterenol and 0.1 mM Gpp(NH)p. The time-course exhibits a lag period corresponding to the slow adenylate cyclase activation. The simultaneous addition of 10 μ M propranolol with isoproterenol and Gpp(NH)p completely blocked cyclic AMP synthesis as shown after 30 min incubation suggesting that adenylate cyclase was stimulated through the β -receptors.

In order to compare the kinetics of adenylate cyclase stimulation by isoproterenol and Gpp(NH)p obtained, respectively, with GDP-loaded membranes and GDP-depleted membranes, two different types of preincubation have been used. GDP-loaded membranes were obtained by preincubating the membranes for 30 min at 37°C with 50 μ M isoproterenol,

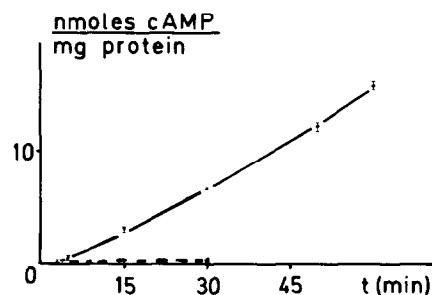


Fig.1. Time course of cyclic AMP production by turkey erythrocyte membranes stimulated at zero time by 50 μ M isoproterenol and 0.1 mM Gpp(NH)p in the absence (—) or in the presence of 10 μ M 1-propranolol (---). Experimental results are represented by means and ranges of triplicates.

0.1 mM ascorbic acid, 5 mM MgSO₄ and 0.1 mM GTP. In such conditions, GTP can easily bind to the binding protein [15] and is then hydrolyzed. GDP-depleted membranes were obtained by preincubating the membranes for 30 min at 37°C with 50 μ M isoproterenol, 0.1 mM ascorbic acid and 5 mM EDTA. The presence of EDTA is necessary for chelating Mg²⁺ which would delay the dissociation of GDP in the absence of other guanyl nucleotides. These preincubation conditions ensure a significant release of GDP from the specific binding sites [8]. After the preincubation both types of membranes were washed twice with 50 mM, Tris-HCl (pH 7.4) at 0°C and then assayed for adenylate cyclase activity as above. Table 1 shows that the accumulation of cyclic AMP for GDP-depleted membranes is significantly faster than for GDP-loaded membranes suggesting that the

Table 1
Cyclic AMP accumulation due to the stimulation by isoproterenol and Gpp(NH)p of GDP-loaded membranes and GDP-depleted membranes

Time (min)	GDP-loaded membranes	GDP-depleted membranes	Level of significance
	(pmol Cyclic AMP/mg protein)		
3	370 \pm 88	542 \pm 73	n.s.
5	801 \pm 154	1230 \pm 57	2 P < 0.02
30	9500 \pm 380	12 177 \pm 614	2 P < 0.01
50	16 851 \pm 450	21 847 \pm 1103	2 P < 0.01
60	22 542 \pm 2317	27 288 \pm 883	2 P < 0.05

The results are expressed as the mean of triplicates \pm 1 SD

limiting step is thus the GDP dissociation. The amount of cyclic AMP accumulated between $t = 50$ min and $t = 60$ min is essentially the same for both types of membranes indicating that the maximal stationary adenylate cyclase activity is not affected by the respective preincubation conditions. The only effect of the respective occupancy levels of the membranes by GDP is reflected by the rate of adenylate cyclase activation and thus the apparent lag period.

In order to estimate whether guanyl nucleotide binding proteins of native membranes are saturated in GDP or not, we compared the kinetics of adenylate cyclase activation obtained, respectively, with GDP-loaded membranes prepared as before and with native membranes which have been preincubated with isoproterenol and 5 mM MgSO_4 . The preincubation time was 10 min which is long enough for loading the GDP-loaded membranes in such conditions [8]. The presence of MgSO_4 and the absence of other nucleotides in the preincubation prevented a significant GDP depletion of the native membranes [8]. Table 2 shows that there is no significant difference between the time courses of cyclic AMP accumulation for GDP-loaded membranes and native membranes. Thus the native membranes appear to be saturated in GDP. This is in agreement with the fact that for native membranes adenylate cyclase activation by β -adrenergic agonists consistently exhibited a lag period [9].

4. Discussion and conclusion

The hypothesis has been proposed [7] that GDP

Table 2
Cyclic AMP accumulation due to the stimulation by isoproterenol and Gpp(NH)p of GDP-loaded membranes and native membranes

Time (min)	GDP-loaded membranes (pmol Cyclic AMP/mg protein)	Native membranes
3	779 \pm 463	206 \pm 67
5	523 \pm 59	440 \pm 197
30	6861 \pm 512	6775 \pm 134
50	12 686 \pm 678	12 281 \pm 444
60	16 460 \pm 1731	16 031 \pm 395

The results are expressed as the mean of triplicates \pm 1 SD

dissociation from the guanyl nucleotide binding protein could control the rate of adenylate cyclase activation. It has been shown [8] that in fact apparent GDP dissociation is induced by β -agonist binding and is as slow as the adenylate cyclase activation in the case of the turkey erythrocyte membranes. However, this observation is not a proof that the GDP dissociation is the rate limiting step since any other event taking place before the GDP dissociation and being rate limiting would provoke an apparent slow GDP dissociation. For instance the collision coupling model proposed in [9] could account for the observed slow GDP dissociation as the association of the hormone-receptor complex with the adenylate cyclase system which would be rate limiting in this model would precede the opening of the guanyl nucleotide binding site and the consequent release of GDP. However this model has been refuted on the basis of theoretical considerations [10]. It was thus of special interest to verify if the hypothesis concerning the rate limiting nature of the GDP dissociation is valid.

The experimental results presented above suggest that the rate of adenylate cyclase activation is inversely related to the occupancy level of the guanyl nucleotide binding protein by GDP and that the GDP dissociation would be rate limiting. Although we have not directly measured the content of bound GDP after the membrane preincubation, we can expect that the membranes treatment leads to GDP-loaded or GDP-depleted membranes depending on the type of preincubation. Indeed, the preincubations have been carried out according to the experimental conditions in [8] for inducing binding or release of GDP in the turkey erythrocyte membranes.

Our results suggest that the guanyl nucleotide binding sites of the native membranes are fully occupied by GDP since the rate of adenylate cyclase activation for the native membranes is not significantly different from the rate observed with GDP-loaded membranes.

It would be of interest to speculate on the biochemical consequences of the existence of a tight binding of guanyl nucleotide in the turkey erythrocyte system:

1. The lag period in the adenylate cyclase activation in the presence of Gpp(NH)p and β -agonist is particular to the turkey erythrocyte. It is possible

that native membranes of other systems do not exhibit such a GDP tight binding and thus the guanyl nucleotide binding proteins would be rapidly available for the adenylate cyclase activation.

2. Turkey erythrocyte adenylate cyclase does not exhibit any significant basal activity. If the model in [5] actually describes this system it is clear that the tight binding of GDP prevents the rapid association of GTP in the absence of β -agonist. The balance between GTP association rate (turn-on process) and the rate of GTP hydrolysis (turn-off process) is highly favourable to the inactive state of the adenylate cyclase. In other systems, a more rapid dissociation of GDP would allow a significant formation of active adenylate cyclase in the basal conditions.
3. The addition of Gpp(NH)p alone cannot rapidly activate turkey erythrocyte adenylate cyclase because of the persistent binding of GDP which prevents the association of Gpp(NH)p. In other tissues the binding site would be readily available even in the absence of β -agonist.
4. Turkey erythrocyte membranes do not display any effect of GTP on the affinity of β -agonist binding [16]. It is well known that in most cells the addition of GTP reduces the affinity of β -agonist binding to membranes preparations [17]. In the case of turkey erythrocyte membranes the tight binding of GDP would not allow the observation of a possible effect of GTP on the affinity of β -agonist binding.

If these arguments are valid, it should be possible to show that the GDP depletion of the turkey erythrocyte membranes would allow a Gpp(NH)p induced activation of adenylate cyclase in the absence of β -agonist, a GTP effect on the binding affinity of the β -agonist and a decrease in the lag period for the adenylate cyclase activation by Gpp(NH)p and β -agonist. The last point has been observed here. Experiments are in progress for demonstrating the two first points.

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