Determination of the Turn-off Reaction for the Hormone-activated Adenylate Cyclase*

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Previous work suggested that hormonal activation of adenylate cyclase involves the introduction of GTP to the regulatory site, and subsequent hydrolysis of the bound GTP terminates the activation. In many tissues the turn-off GTPase reaction cannot be readily measured because of a high background of nonspecific GTP hydrolysis. To circumvent this problem a general assay for the turn-off reaction has now been developed. The adenylate cyclase is first activated by hormone and GTP and the introduction of GTP is then stopped either by addition of an excess of guanosine 5'-O-(2-thiodiphosphate) (GDPβS) or by addition of a receptor blocking agent. The decay of adenylate cyclase activity brought on by these inhibitors is used to calculate the rate constant of the turn-off reaction. In turkey erythrocyte and rat parotid membranes the rate constant of the decay process as determined with GDPβS is similar to that determined with the β-adrenergic blocker propranolol. The rate constants (min⁻¹ at 30°C) for various adenylate cyclase preparations are 10 for turkey erythrocyte, 7.5 for rat parotid, and 6.2 for the rat liver enzyme. The finding of similar rate constants in the various preparations indicates that GTP hydrolysis at the regulatory site is a general mechanism for terminating the activation of adenylate cyclase.

Our studies on the turkey erythrocyte adenylate cyclase have shown that the activation of this enzyme is turned off by the hydrolysis of GTP at the regulatory site and that the hormone activates the adenylate cyclase by facilitating the replacement of bound GDP by GTP (1-3). The finding that cholera toxin activates the adenylate cyclase by causing an inhibition of the GTPase reaction (2) demonstrated that adenylate cyclase activity can be modulated by agents that affect the turn-off reaction. For this reason a general method for the determination of the off-rate for the hormone-activated adenylate cyclase is of considerable interest. Unfortunately, so far, direct measurement of GTPase activity at the regulatory site could be performed only on avian erythrocyte membranes. In other cell membranes this activity is obscured by potent enzymes which rapidly degrade GTP and are not related to the adenylate cyclase system (1, 4).

We have previously described a method for determination of the turn-off reaction, based on measurement of the decay of the hormone plus GTP-activated adenylate cyclase upon blocking of the β-adrenergic receptor with propranolol (5). It was assumed that addition of propranolol stops the catechol-

amine-induced introduction of GTP to the regulatory site, and thus the decay of adenylate cyclase activity reflects the rate of hydrolysis of the prebound GTP. In the present study GDPβS was used to determine the turn off reaction. This metabolically stable analog of GDP competitively inhibits the stimulation of adenylate cyclase activity by GTP (6). Therefore, addition of GDPβS to adenylate cyclase which had been activated by hormone and GTP stops the introduction of GTP to the regulatory site. This method which is not dependent on blockage of the receptor can be applied to a variety of adenylate cyclase systems, including those for which hormone antagonists are not available.

MATERIALS AND METHODS

GDPβS was prepared essentially as described for the preparation of adenosine 5'-O-(2-thiodiphosphate) (7). Turkey erythrocyte (6), rat liver (8), and rat parotid (9) membranes were prepared as described. Protein was determined by the method of Lowry et al. (10) using bovine serum albumin as standard.

The decay of adenylate cyclase activity upon addition of inhibitors was assayed essentially as previously described (5). The reaction mixture in a final volume of 1 ml contained 0.5 to 1 μM GTP, an appropriate hormone (either L-epinephrine or glucagon), 0.3 mM ATP (Sigma No. 2383), 0.1 mg/ml of pyruvate kinase, 10 mM phosphoenolpyruvate (potassium salt), 1 mM cAMP, 6 mM MgCl₂, 0.2 mM ethylene glycol bis(β-aminoethyl ether) N,N',N'-tetraacetic acid, 2 mM β-mercaptoethanol, 0.5 mg/ml of bovine serum albumin, and 50 mM Mops, pH 7.4. Membranes were added to the reaction mixture and preincubated for 2 min at 30°C. At zero time, the formation of [γ³²P]cAMP was initiated by the addition of 100 μl of a solution containing the tracer [α³²P]ATP (10⁶ cpm, 5 nmol) with or without GDPβS (0.5 μmol) and propranolol (50 nmol) as indicated in individual experiments. At various times the reaction was stopped by transferring 100-μl aliquots into 100 μl of a solution containing 2% sodium dodecyl sulfate and 40 mM ATP. [³²P]cAMP was determined by the method of Salomon et al. (11).

RESULTS

To study the turn-off reaction, adenylate cyclase was first activated by incubation in a reaction mixture containing hormone and GTP. Activation was then stopped by the addition of GDPβS or propranolol, each added together with the tracer [α³²P]ATP which initiates the production of [³²P]cAMP. The addition of the inhibitors resulted in a rapid decay of the adenylate cyclase activity (Figs. 1 to 3). Since cAMP formation almost ceases after a few seconds, high membrane concentration and [α³²P]ATP with high specific radioactivity had to be employed. In order to spare the costly [α³²P]ATP as much as possible, the concentration of the membranes was increased to the maximal value in which adenylate cyclase activity in a system which did not receive an inhibitor, was still linear during the assay period (see Figs. 1 to 3). The amount of

* The abbreviations used are: GDPβS, guanosine 5'-O-(2-thiodiphosphate); Mops, 3-(N-morpholino)propanesulfonic acid.

9835
Turkey erythrocyte membranes

Epi + GTP

* No inhibitor
  propranolol
  GDPβS
  GDPβS + propranolol

Cyclic AMP (pmol/mg protein)

5 10 15 20 25 30 35 40 45

TIME (sec)

Fig. 1. Decay of the epinephrine plus GTP-stimulated adenylate cyclase activity of turkey erythrocyte membranes upon addition of inhibitors. The decay measurements were performed as described under "Materials and Methods" using 5 mg of turkey erythrocyte membranes preincubated in the presence of 50 μM l-epinephrine (Epi) and 0.5 μM GTP. Accumulation of cAMP is plotted as a function of time from the addition of [α-32P]ATP with or without inhibitors as indicated. The experiment was repeated five times and the given koff value is the mean ± S.D.

[32P]cAMP formed was determined by the method of Salomon et al. (11), which gives very low reagent blanks (5 to 10 cpm/106 cpm of [α-32P]ATP). This reagent blank was less than 5% of the amount of cAMP formed during the decay process (cf. Figs. 1 to 3).

In the present study we employed GDPβS, an analog of GDP that competitively inhibits the stimulation of adenylate cyclase by GTP (6). In contrast to GDP, the analog is essentially not phosphorylated by the nucleoside triphosphate regeneration system present in the adenylate cyclase assay, nor is it degraded by the membrane (6). The addition of GDPβS to turkey erythrocyte adenylate cyclase, preactivated by epinephrine plus GTP, resulted in a decay of adenylate cyclase activity which followed a time course that was similar to that obtained upon blocking the β-adrenergic receptor by propranolol. Moreover, the effect of GDPβS and propranolol was not additive, and the addition of the two inhibitors together resulted in a decay curve which was similar to that obtained with each inhibitor alone (Fig. 1).

To calculate the rate constant of the decay (koff) an extrapolation procedure was used, as this analysis of rapid decay processes is more accurate than the semilogarithmic plot. As previously shown (5), the amount of cAMP formed, from zero to infinite time, by adenylate cyclase undergoing a first-order decay process is:

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cAMP_\infty = \frac{V_0}{k_{\text{off}}} \]

where \(V_0\) = activity at zero time.

The amount of cAMP formed at time \(t\) by an enzyme which did not receive an inhibitor and thus reveals a constant activity, is cAMP \((t) = V_0 \times t\). At \(t = \frac{1}{k_{\text{off}}}\), cAMP \((t) = cAMP_\infty\). Thus, the extrapolation of the decay curve and the curve of the uninhibited enzyme intersect at a time point equivalent to the reciprocal of the rate constant. This extrapolation gave a \(k_{\text{off}}\) value of 10 ± 2 min\(^{-1}\) at 30°C for the hormone plus GTP-stimulated adenylate cyclase of turkey erythrocyte membranes (Fig. 1). As expected the \(k_{\text{off}}\) obtained in this study at 30°C is smaller than the previously reported value of 15 min\(^{-1}\) at 37°C (5).

In the catecholamine-sensitive adenylate cyclase of rat parotid membranes, addition of either propranolol or GDPβS to an epinephrine plus GTP-activated adenylate cyclase did not completely inhibit the enzyme’s activity (Fig. 2). This is
because the parotid enzyme reveals basal activity in the absence of hormone, and because GDPβS acts in this system as a weak partial agonist of GTP (6). As might be expected, addition of GDPβS and propranolol together resulted in an almost complete inhibition of adenylate cyclase activity. Decay experiments in which GDPβS was added either alone or together with propranolol gave a similar koff value of 7.5 ± 0.5 min⁻¹ at 30°C. However, the decay obtained with propranolol (koff = 5.3 ± 0.5) was somewhat slower than that obtained from the experiments in which GDPβS was used (Fig. 2). It should be pointed out that both in the turkey erythrocyte and rat parotid systems, the koff values were not affected by varying the concentration of GDPβS (0.2 to 0.5 mM) or propranolol (10 to 50 μM).

In the glucagon-sensitive adenylate cyclase system of rat liver membranes, for which a hormone antagonist is not readily available, the turn-off reaction was determined with the aid of GDPβS. Addition of GDPβS to the glucagon plus GTP-activated adenylate cyclase of rat liver results in an inhibition of enzyme activity by about 70% at pH 7.4 (Fig 3 (left) and Ref. 6). We have observed that the extent of inhibition of the liver adenylate cyclase activity by GDPβS increases as the pH of the assay is decreased. Therefore the decay experiment was carried out also at pH 7.0, where the extent of inhibition was 85% (see Fig 3, right). This change in the pH had little effect on the rate constants of the decay processes, which were 6.2 ± 0.3 and 7.2 ± 0.6 min⁻¹ at pH 7.4 and 7.0, respectively (Fig. 3).

Treatment with cholera toxin and NAD stabilizes the adenylate cyclase-GTP complex due to an inhibition of the GTPase activity at the regulatory site (2, 5, 12-14). Thus, the decay of the hormone plus GTP-stimulated adenylate cyclase activity becomes much slower after toxin treatment. In toxin-treated turkey erythrocyte membranes the addition of GDPβS to the epinephrine plus GTP-activated enzyme resulted in an almost complete inhibition of activity (Fig. 4). The addition of propranolol did not completely inhibit the enzyme. This is because toxin-treated adenylate cyclase reveals significant basal activity in the presence of GTP (2, 5). Extrapolation of the decay curve obtained upon the addition of propranolol and GDPβS, either separately or together, gave a similar koff value of 0.55 min⁻¹ at 30°C. Thus the toxin inhibited the turn-off reaction by 95% (cf. Figs. 1 and 4).

**DISCUSSION**

We have previously demonstrated that the off-rate of turkey erythrocyte adenylate cyclase is in good agreement with the catecholamine-stimulated GTPase activity in this membrane (5). The results reported in this communication show that the off-rate of the hormone plus GTP-stimulated adenylate cyclase is rather similar in membranes of various tissues. The turn-off reaction was assayed with either a receptor blocking agent or with GDPβS. Clearly, addition of the competitive inhibitor GDPβS stops the binding of GTP to the regulatory site, and thus adenylate cyclase maintains its activity only until the prebound GTP is hydrolyzed. It has been recently demonstrated that the hydrolysis of GTP results in the formation of tightly bound GDP at the regulatory site and that reintroduction of GTP depends on a hormone-induced release of the prebound GDP (3). Addition of a hormone antagonist would therefore inhibit the replenishment of GTP at the regulatory site. From these considerations it follows that blocking of either the hormone receptor or the GTP site should yield a similar rate of decay for the activated adenylate cyclase, reflecting the time required for the hydrolysis of the bound GTP. It should be noted, however, that the rate of decay of adenylate cyclase activity may also be affected by the following processes. 1) Addition of a receptor blocking agent immediately stops the hormone-induced binding of GTP only if the dissociation of the hormone from the receptor is very rapid. Thus, in the case of hormones with high affinity for the receptor, the dissociation of the hormone, rather than the GTPase reaction, might be the rate-limiting step in the decay of adenylate cyclase activity. 2) Addition of GDPβS to the hormone plus GTP-activated adenylate cyclase may also cause a decay of enzyme activity due to a hormone-induced replacement of bound GTP by GDPβS. It is not likely, however, that this process would significantly affect the rate of decay of adenylate cyclase activity since GTP has high affinity to the regulatory site and its dissociation should be much slower than the GTPase reaction (cf. Refs. 15 and 16). The above considerations suggest that the rate constants determined with GDPβS or a receptor blocking agent may be either smaller or larger than the rate constant of the GTPase reaction, respectively. In practice, both GDPβS and propranolol gave similar koff values for the turkey erythrocyte adenylate cyclase. It thus appears that in this system interfering processes do not have significant effect on the decay of adenylate cyclase activity. In parotid membranes, however, the decay obtained with propranolol was somewhat slower than that obtained with GDPβS, and this might be due to a slow dissociation of epinephrine from the β-adrenergic receptor of the parotid system.

It has been recently shown that cholera toxin catalyzes an ADP ribosylation of the guanyl nucleotide binding component of adenylate cyclase (17-19). This modification inhibits the GTPase activity and thus results in the activation of adenylate cyclase (2, 17). With the present assay it should be possible to test whether other modulations of adenylate cyclase activity in various systems are also caused by a modification of the turn-off reaction.
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