Forskolin-activated Adenylate Cyclase

INHIBITION BY GUANYL-5'-YL IMIDODIPHOSPHATE*

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Forskolin-activated adenylate cyclase of purified rat adipocyte membranes in the absence of exogenous guanine nucleotides. Guanyl-5'-yl imidodiphosphate (Gpp(NH)p) inhibited the forskolin-activated cyclase immediately upon addition of the nucleotide at concentrations too low to activate adenylate cyclase (10⁻⁸ to 10⁻⁵ M). Inhibition seen with a very high concentration of Gpp(NH)p (10⁻⁴ M) lasted for 3–4 min and was followed by an increase in the synthetic rate which remained constant for at least 15 min. The length of the transient inhibition did not vary with forskolin concentrations above 0.05 μM but low Gpp(NH)p (10⁻⁸ M) exhibited a lengthened (6–7 min) inhibitory phase.

The transient inhibitory effects of Gpp(NH)p were eliminated by 10⁻² M isoproterenol, high (40 mM) Me, or preincubation with Gpp(NH)p in the absence of forskolin. While forskolin stimulated fat cell cyclase in the presence of Mn²⁺, this ion blocked the inhibitory effects of Gpp(NH)p. The well documented inhibitory effects of GTP on the fat cell adenylate cyclase system were also observed in the presence of forskolin. However, the inhibition by GTP is not transitory. These findings indicate that Gpp(NH)p regulation of forskolin-stimulated cyclase has at least two components: 1) an inhibitory component which acts through an undetermined mechanism and which acts immediately to decrease cyclase activity; and 2) an activating component which modulates the inhibited cyclase activity through the guanine nucleotide regulatory protein.

Regulation of adenylate cyclase by GTP involves the interaction of at least two integral membrane proteins. One is the catalytic subunit which is active after it complexes with a second component, the G-protein.¹ The catalytic subunit-G-protein complex is stabilized by GTP occupancy of a regulatory site on the stimulatory G-protein (1). Activation of adenylate cyclase by hydrolysis-resistant GTP analogues occurs in membranes from a variety of tissues (2, 3), including rat adipocytes (4), but only after a lag (hysteretic activation). Hormones and Mg²⁺ ion act to decrease the lag period (4, 5).

Forskolin is a diterpene which has been shown to activate rat adipocyte adenylate cyclase both in intact cells and purified plasma membrane preparations (6). In intact cells, forskolin dramatically increases the sensitivity of the cyclic AMP response to isoproterenol. This potentiation of cyclase activity is not observed in purified membrane preparations stimulated by isoproterenol in the presence of guanosine-5'-O-(3-thiotriphosphate or GTP (6). While a normal G-protein is not required for forskolin activation of adenylate cyclase in the cyc⁻ mutant of S49 mouse lymphoma (7), guanine nucleotides modify forskolin-activated cyclase from the cyc⁻ mutant (8) as well as from platelet (9) and rat cerebral cortical membranes (10). Neither the exact locus of forskolin action nor the site of guanine nucleotide modulation has been elucidated.

The present studies demonstrate that forskolin-activated fat cell adenylate cyclase is inhibited by Gpp(NH)p immediately upon addition of the nucleotide. The inhibition is transient but the length of the inhibitory phase is dependent on the concentration of Gpp(NH)p.

MATERIALS AND METHODS

Adipocyte Isolation—Adipocytes were isolated by a modification of the method of Rodbell (11) from parametrial, omental, and epididymal adipose tissue of 175–250 g male or female Sprague-Dawley rats (Charles River, CD strain) fed ad libitum on laboratory chow. Adipose tissue (5 g/10 ml of medium) was incubated for 1 h at 37 °C in Krebs-Ringer phosphate buffer containing 120 mM NaCl, 1.4 mM CaCl₂, 5.2 mM KCl, 1.4 mM MgSO₄, 3% bovine albumin (Fraction V; Armour), 10 mM Na₂HPO₄ (pH 7.4) supplemented with collagenase (Type 1, Millipore; 1 mg/ml). The digest was filtered through nylon mesh and pooled in 50-ml plastic conical centrifuge tubes (Sarstedt). Adipocytes were floated to the surface by a 15-s centrifugation in an International Clinical centrifuge at maximal speed. The infranatant was withdrawn and discarded. The adipocytes were washed free of collagenase by suspension in at least 2 volumes of buffer, centrifugation, and removal of the infranatant. Following two washes, the adipocytes were washed once with 2 volumes of homogenizing medium (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4)) and the infranatant was removed to yield a concentrated adipocyte suspension. (These procedures were carried out at 22 °C.)

Plasma Membrane Preparation—Adipocyte plasma membranes were prepared by a modification of the method of McKeel and Jarrett (12). One volume of free adipocytes was suspended in 2 volumes of homogenizing medium (at 22 °C) in a 50-ml glass tissue grinder (Arthur H. Thomas, type C) and immediately homogenized with eight up and down strokes of a motor-driven Fisher Dym-Mix stirrer, 1700–1900 rpm) Teflon pestle. The homogenates were centrifuged at 10,000 rpm in a Sorvall SS-34 rotor (30,000 × g maximum) for 20 min. The congealed fat cake and infranatant were removed and the pellet was resuspended in homogenizing buffer and centrifuged at 30,000 × g for 20 min. The supernatant was discarded, and the pellets were resuspended in

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² The abbreviations and trivial names used are: G-protein, guanine nucleotide binding regulatory component of adenylate cyclase; forskolin, 7-β-acetoxy-8,13-epoxy-1,α,δ,β,δ-a-trihydroxylabd-14-en-11-one; Gpp(NH)p, guanylatedyl-5'-y imidodiphosphate.
homogenizing buffer, layered over 32% (w/w) sucrose in 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), and centrifuged for 1 h in a Beckman SW28 rotor at 27,000 rpm. The plasma membranes (a white band at the sucrose interface) were collected, diluted with 8 volumes of homogenization buffer, and centrifuged in a Sorvall SS-34 rotor at 16,000 rpm. The pellet was suspended in homogenization buffer to a membrane protein concentration of approximately 1 mg/ml. The membranes were quick frozen in dry ice/ethanol and stored at −80 °C. The properties of the adenylate cyclase reported here are stable in these membranes for several months.

**Protein Determination**—Membrane protein determinations were made by the method of Lowry et al. (13) using bovine serum albumin fraction V as standard.

**Adenylate Cyclase Assay**—Unless otherwise cited, adenylate cyclase was assayed in a mixture containing 0.2 mM α-[32P]ATP (20–50 cpm/pmol), 0.1 mM [3H]cyclic AMP (0.1 cpm/pmol, as standard), 4 mM MgCl2, 0.2% bovine serum albumin fraction V, 10 mM creatine phosphate, 10 units/ml of creatine phosphokinase, 30 mM Tris-HCl, pH 7.5, in a final volume of 100 μl of reaction mix. The assay was initiated by adding 2–20 μg of membrane protein to the reaction mixture and incubation at 30 °C. Under these conditions steady state adenylate cyclase activity was linear with protein concentration and time over 30 min. The reaction was terminated by the addition of 1 ml of 1% sodium dodecyl sulfate. Cyclic AMP was purified by sequential chromatography on Dowex and alumina by the method of Salomon et al. (14).

**Materials**—(±)-Isoproterenol, ATP (sodium salt, grade 1, by phosphorylation of adenosine), creatine phosphate, creatine phosphokinase, and GTP were obtained through Sigma. α-[32P]ATP and [3H]AMP were purchased from New England Nuclear. Gpp(NH)p was obtained from Boehringer Mannheim. Forskolin was purchased from Calbiochem-Behring. Stock solutions of forskolin (10 mM) were maintained at −20 °C in 95% ethanol. The stock was diluted so that reaction mixtures contained less than 1% ethanol. These ethanol concentrations had no effect on adenylate cyclase activity. All other materials were of reagent grade.

**RESULTS**

Forskolin stimulated adenylate cyclase activity even in the absence of exogenous guanine nucleotide (Fig. 1). However, in the presence of high concentrations of forskolin (10 or 100 μM) a clear inhibitory effect of Gpp(NH)p on forskolin activation of adenylate cyclase was observed (Fig. 1). This paradoxical inhibition of adenylate cyclase by the guanine nucleotide analog was optimal at a Gpp(NH)p concentration of 0.01 μM but partially reversed by increasing the nucleotide concentration (Fig. 1).

The stimulation of adenylate cyclase by forskolin required a concentration of greater than 0.05 μM, was immediate in onset, and linear with time for 19 min (Fig. 2). Gpp(NH)p stimulation was hysteretic in nature with a sharp increase in activity occurring approximately 6 min after its addition (Fig. 2). Forskolin at a concentration of 0.05 μM had little effect on basal activity (not shown). However, the addition of 0.05 μM
forskolin in the presence of a saturating (100 μM) concentration of Gpp(NH)p shortened the lag but had no significant effect on the reaction rate (Fig. 2A). Higher forskolin concentrations did not change the length of the lag period which was approximately one-half that with Gpp(NH)p alone (Fig. 2, A and B). Because the stimulation of adenylate cyclase by forskolin alone was linear throughout the time course, the lag seen in the presence of Gpp(NH)p and forskolin actually represents a transient inhibition by Gpp(NH)p of forskolin-activated cyclase (Fig. 2). The inhibition of forskolin (0.1 μM or higher)-stimulated adenylate cyclase activity by Gpp(NH)p was immediate in onset but transitory in nature (Fig. 2). This inhibitory effect of high concentrations of Gpp(NH)p was confined to an initial 3-4 min period and was followed by a return of cyclase activity to a value (termed the secondary rate) which remained constant for at least 15 min (Fig. 2).

Adenylate cyclase stimulated by a low forskolin concentration (0.1 μM) was only slightly inhibited by Gpp(NH)p and the secondary synthetic rate was greater than that due to forskolin alone (Fig. 2 and Table I). Adenylate cyclase activity stimulated by higher concentrations of forskolin was inhibited by Gpp(NH)p to a greater degree, but the secondary rate was similar to that observed with forskolin alone (Fig. 2 and Table I).

Using saturating concentrations of Gpp(NH)p (100 μM), the initial inhibitory period induced by the nucleotide was approximately 3 min for all forskolin concentrations tested (Fig. 2). Fig. 1 demonstrated that the maximal inhibition in a 30-min assay was seen with 0.01 μM Gpp(NH)p. The time course using the low Gpp(NH)p concentration (Fig. 3) exhibited an initial inhibitory phase lasting approximately 6 min. The reversal of Gpp(NH)p inhibition by higher Gpp(NH)p concentrations was due, at least in part, to a shortening of the inhibitory phase.

Fig. 4 demonstrates the dependence of the initial inhibitory effects of Gpp(NH)p on the exposure time to Gpp(NH)p. Addition of 10 μM forskolin to membranes previously exposed to 100 μM Gpp(NH)p for 7 or 12 min resulted in an immediate increase in adenylate cyclase activity which was constant with time (Fig. 4). Addition of forskolin to membranes pre-exposed to Gpp(NH)p for 3 min or not pre-exposed also resulted in immediate increases in cyclase activity, but importantly, steady state activation was reached only after lags of approximately 1½ and 3 min, respectively (Fig. 4). The steady state rates of cyclic AMP synthesis were identical for all times of forskolin action.

**TABLE I**

<table>
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<th>Modulation of forskolin-stimulated adenylate cyclase activity by Gpp(NH)p</th>
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| Forskolin concentration (μM) | Adenylate cyclase activity over the first 3 min |
|---|
| Without | +Gpp(NH)p (100 μM) | Inhibition by Gpp(NH)p | Ratio |
| pmol/min/mg | % |
|---|
| 0 | 56 | 56 | 0 | |
| 0.1 | 200 | 150 | 25 | 2.5 |
| 1.0 | 700 | 450 | 36 | 1.5 |
| 10.0 | 1300 | 750 | 42 | 1.2 |
| 20.0 | 1500 | 750 | 50 | 1.0 |
| 50.0 | 1500 | 750 | 50 | 1.0 |
| 100.0 | 1500 | 750 | 50 | 1.0 |

* Ratio of forskolin-stimulated adenylate cyclase activity measured over 4-18 min in the presence of Gpp(NH)p (100 μM) divided by that in its absence.

Addition of forskolin to membranes preincubated without Gpp(NH)p resulted in the immediate increase to steady state cyclase activity regardless of the length of preincubation (Fig. 4, inset). Likewise, the inhibitory phase induced by Gpp(NH)p in these membranes was a constant 3 min for all the preincubation times tested (Fig. 4, inset). These findings indicate that: 1) the lag in reaching steady state activation after forskolin addition is due to the inhibitory effects of Gpp(NH)p on forskolin-stimulated activity; 2) the length of the transient...
inhibition is a function of the time of exposure to Gpp(NH)p; and 3) the mechanism(s) by which Gpp(NH)p controls the length of the inhibitory phase do not require the presence of forskolin.

Exposure of membranes to 10 μM forskolin for 2 or 6 min prior to the addition of 100 μM Gpp(NH)p did not alter the length (3 min) of the inhibitory phase (Fig. 5). Taken with Fig. 4, these data indicate that the length of the inhibitory phase is determined entirely by the length of exposure to Gpp(NH)p alone.

Membranes incubated with Gpp(NH)p for times greater than 6 min did not exhibit a lag in the steady state activation of cyclase upon addition of forskolin (Fig. 4). As observed in other systems (15), fat cell membranes incubated for 30 min with Gpp(NH)p and then extensively washed exhibited a consistently high adenylate cyclase activity which was not further activated by Gpp(NH)p. Forskolin stimulation of these membranes was immediate in onset, linear with time, and not inhibited by further addition of Gpp(NH)p (data not shown). These findings suggest that the elimination of the inhibitory effects of Gpp(NH)p observed in Fig. 4 are due to Gpp(NH)p acting on the membrane and are not a result of direct interaction of free Gpp(NH)p and forskolin.

The biphasic response of fat cell adenylate cyclase to GTP is well documented (16, 17). This response was evident in fat cell membranes incubated with forskolin in the presence of 10 μM isoproterenol (Fig. 6). The increase in cyclase activity only required 0.1 to 1 μM GTP and was followed by a sharp inhibition of activity with higher GTP concentrations (100-1000 μM). In the presence of 10 μM forskolin alone, the

FIG. 5. Time course for inhibition of forskolin-stimulated adenylate cyclase by Gpp(NH)p. Reactions were initiated by addition of adipocyte plasma membranes to adenylate cyclase reaction mix containing 10 μM forskolin and cyclic AMP accumulation was determined at the times indicated ( ). To identical tubes, 100 μM Gpp(NH)p was added 2 min ( ) and 6 min ( ) after initiation of the reaction.

FIG. 6. Effects of GTP and isoproterenol on forskolin-activated adenylate cyclase. Adipocyte plasma membranes were assayed for 20 min in the presence of 10 μM forskolin and the indicated GTP concentrations alone ( ) or 0.01 μM ( ) or 10 μM ( ) isoproterenol. Each point is the average of three determinations. The bars represent standard deviations when larger than the symbol.

activation of cyclase by low GTP concentrations was not seen, but the inhibitory effects of GTP were evident (Fig. 6). These GTP dose response curves qualitatively mimic those observed in control membranes (17). The inhibition of both control and forskolin activated fat cell cyclase by high GTP concentrations is constant with time (data not shown), as opposed to the transitory inhibition produced by Gpp(NH)p in the presence of forskolin (Fig. 2).

The inhibitory response of forskolin-stimulated adenylate cyclase to Gpp(NH)p was dependent on the nature and concentration of divalent cations. Increasing the Mg2+ concentration from 0.5 to 20 mM elevated the forskolin stimulation of adenylate cyclase activity in the absence or presence of Gpp(NH)p (Fig. 7A). Increasing the Mg2+ concentration also reduced the degree to which the forskolin-stimulated activity was inhibited by Gpp(NH)p. At 20 mM MgCl2, the Gpp(NH)p inhibition was virtually eliminated. Forskolin stimulated fat cell adenylate cyclase even if Mg2+ was replaced by Mn2+ (Fig. 7B). Under these conditions, the inhibition by Gpp(NH)p was greatly reduced. In addition, the inhibition of adenylate cyclase activity by Gpp(NH)p observed with 1 mM Mg2+ (Fig. 7A) was virtually eliminated by the inclusion of 4 mM MnCl2 (Fig. 7B).

Stimulation by forskolin alone was linear with time at all Mg2+ concentrations tested (Fig. 8). Increasing the Mg2+ concentration resulted in a shortening of the transient inhibitory phase (Fig. 8), from approximately 3½ min (4 mM MgCl2) to 1½ min (14 mM MgCl2) (Fig. 8, inset). With a very high MgCl2 concentration (40 mM) Gpp(NH)p is no longer capable of inhibiting the forskolin-activated cyclase (Fig. 8). In agreement with others (4, 18),

2 T. H. Hudson and J. N. Fain, unpublished data.
Forskolin-stimulated Adenylate Cyclase

log [Gpp(NH)p] (M)

FIG. 7. Effects of Mg²⁺ and Mn²⁺ on Gpp(NH)p inhibition of forskolin-stimulated adenylate cyclase. A, membranes were incubated for 20 min with 10 μM forskolin plus the following concentrations of MgCl₂: 0.5 mM (■—■), 1.0 mM (○—○), 2 mM (■—■), 8 mM (□—□), or 20 mM (▲—▲), and the indicated concentrations of Gpp(NH)p. Basal (no forskolin, no Gpp(NH)p) adenylate cyclase activities were 0.02 and 0.18 nmol/min/mg in the presence of 1 and 20 mM MgCl₂, respectively; with 100 μM Gpp(NH)p activities were 0.03 and 0.37 nmol/min/mg in the presence of 1 and 20 mM MgCl₂, respectively. B, adenylate cyclase activities were determined in the absence of added Me with 10 μM forskolin plus 4 mM MnCl₂ alone (■—■) or with 1 mM MgCl₂ (□—□) plus the indicated concentrations of Gpp(NH)p. Basal cyclase activity in the presence of 4 mM MnCl₂ was 0.04 nmol/min/mg protein and with 100 μM Gpp(NH)p was 0.04 nmol/min/mg. Each point represents the mean of three determinations.

Increasing the concentration of Mg²⁺ reduced the hysteretic lag in Gpp(NH)p (100 μM) activation of fat cell cyclase from 15 min (1 mM MgCl₂) to less than 1 min (40 mM MgCl₂). The addition of forskolin (10 μM) to these systems had the effect of decreasing the lag period by approximately one-half (data not shown).

The initial inhibitory phase induced by Gpp(NH)p on cyclase activated by all concentrations of forskolin was eliminated by isoproterenol (Figs. 9 and 10). The steady state cyclase activities produced by isoproterenol and Gpp(NH)p were dependent on the forskolin concentration (Figs. 9 and 10). Cyclic AMP accumulation was determined at the times indicated.

FIG. 9. Elimination of Gpp(NH)p inhibition of forskolin-activated adenylate cyclase by isoproterenol. Reactions were initiated by addition of adipocyte plasma membranes to adenylate cyclase reaction mix containing forskolin at a concentration of 1 μM (A), 10 μM (B), 100 μM (C) without (■—■) or with 100 μM Gpp(NH)p (□—□) or 100 μM Gpp(NH)p plus 10 μM isoproterenol (▲—▲). Cyclic AMP accumulation was determined at the times indicated.

FIG. 10. Kinetics of adenylate cyclase activation. Reactions were initiated by addition of adipocyte plasma membranes to adenylate cyclase reaction mixture containing: no additions (■—■), 10 μM isoproterenol (□—□), 100 μM Gpp(NH)p (▲—▲), or 100 μM Gpp(NH)p plus 10 μM isoproterenol (▲—▲). Cyclic AMP accumulation was determined at the times indicated.

FIG. 8. Elimination of inhibitory effects of Gpp(NH)p on forskolin activation of adenylate cyclase by high Mg²⁺. Adenylate cyclase reactions were initiated by addition of adipocyte plasma membranes to reaction mix containing either 10 μM forskolin alone (filled symbols) or with 100 μM Gpp(NH)p (open symbols) in the presence of MgCl₂ concentration of 4 mM (○, △); 14 mM (□, ▼); or 40 mM (▲). The inset shows, on an expanded scale, the time courses of forskolin plus Gpp(NH)p at MgCl₂ concentrations of: 4 mM (open circles); 14 mM (open triangles); and 40 mM (open squares).
As the forskolin concentration was increased, the stimulatory effects of isoproterenol plus Gpp(NH)p reached a plateau (Fig. 9B) and, finally, with 100 μM forskolin, the forskolin activity was slightly but significantly inhibited by isoproterenol plus Gpp(NH)p (Fig. 9C).

The data in Fig. 10 indicate that Gpp(NH)p did not have any inhibitory effect on adenylate cyclase activity in the absence of forskolin. The stimulation due to Gpp(NH)p occurred after a lag period of 4-5 min. The figure also shows that the effects of all agents were fairly linear over a 20-min incubation except for isoproterenol + Gpp(NH)p which increased slightly during the last 10 min as compared to the first 10 min of incubation.

DISCUSSION

The inhibition of forskolin-stimulated fat cell adenylate cyclase by Gpp(NH)p is immediate in onset and transient. This inhibition shares the following characteristics with hysteric activation of adenylate cyclase by Gpp(NH)p. 1) Mg2+ has been shown to be an antihysteretic agent in a variety of adenylate cyclase systems (6) including fat cell membranes (5). High Mg2+ concentrations eliminate the lag in Gpp(NH)p activation of control fat cell adenylate cyclase and, in a parallel fashion, the transient inhibitory effects of Gpp(NH)p on forskolin-stimulated activity (Fig. 8). 2) Hormones are widely reported to be antihysteretic agents (17-20), acting to increase the rate of cyclase activation by guanine nucleotides. In fat cell membranes β-agonists both eliminate the lag in activation of cyclase by Gpp(NH)p (Fig. 10) and the inhibitory phase of Gpp(NH)p action on forskolin stimulated enzymes (Fig. 9). 3) Prior incubation with Gpp(NH)p decreased the period of transient Gpp(NH)p inhibition of forskolin-stimulated cyclase (Fig. 4).

The data presented here suggest that Gpp(NH)p activates an inhibitory process simultaneously with its binding to the activating G-protein. The resulting adenylate cyclase activity is the summation of the inhibitory and stimulatory processes. The inhibitory effect is immediate in onset after the addition of Gpp(NH)p but only lasts for 6 min with a concentration (0.01 μM) of Gpp(NH)p which does not stimulate adenylate cyclase. In contrast, the stimulation of cyclase by higher concentrations of nonhydrolyzable GTP analogues occurs only after a lag period required for isomerization of the stimulatory G-protein (2-4).

In none of our studies was there any significant inhibition of basal adenylate cyclase activity by Gpp(NH)p (Figs. 2 and 10). Rodbell (4) did note a slight inhibition of basal adipocyte adenylate cyclase activity at 1 min but not at 2 min or any time thereafter. However, Hildebrandt et al. (8) did observe inhibition by Gpp(NH)p of basal adenylate cyclase activity in membranes from ccc-M. S49 mouse lymphoma cells.

In nonavian systems the binding of Gpp(NH)p to the activating G-protein appears to be immediate and not dependent upon GDP release based on both kinetic (4, 21) and biochemical (22) studies. Gpp(NH)p binding initiates a slow isomerization of the G-protein from a state which is not capable of activating the catalytic subunit to a state which can activate adenylate cyclase. This isomerization process is probably responsible for the lag period observed during hysteretic activation of cyclase by Gpp(NH)p. Strittmatter and Neer (23) have resolved catalytic subunits for G-protein in preparations of bovine cerebral cortex. Gpp(NH)p addition to a mixture of both catalytic subunits and G-protein(s) increased cyclic AMP formation but only after a lag. However, pretreatment of the purified G-protein with guanine nucleotide and Mg2+ ion resulted in the rapid appearance of activity after addition of the catalytic subunit preparation. Similar results have been reported by Iyengar (18), using cholate extracts of rat liver as the source of G-protein and membranes from ccc-M. S49 for catalytic activity. Taken together these studies suggest that the hysteretic nature of Gpp(NH)p activation is the result of isomerization of the G-protein.

Fat adipocyte adenylate cyclase exhibits a biphasic response to GTP (17). This response is observed in forskolin-treated membranes (Fig. 6). It has been postulated that the inhibitory effects of GTP may be mediated through a specific inhibitory guanine nucleotide regulatory pathway (24-26). The inhibitory pathway has been described in a variety of adenylate cyclase systems including human platelets (25) and ccc-M. mutant mouse lymphoma (8). The inhibitory pathway appears to be operable in all of these systems in the presence of forskolin (Fig. 6 and Refs. 8 and 9). The inhibition of these systems by GTP is immediate and the resulting rates are constant with time (21). The inhibition of cyclase by GTP is never complete but in the range of 25-50% of control values (24).

Possibly the inhibition of forskolin-activated adenylate cyclase by Gpp(NH)p involves binding to an inhibitory G-protein. If enough Gpp(NH)p is present it also binds to the stimulatory G-protein which then isomerizes to a form which is capable of stimulating adenylate cyclase and offsetting the effects of Gpp(NH)p bound to the inhibitory protein. However, this explanation cannot explain the results shown in Fig. 3 where a concentration (0.01 μM) of Gpp(NH)p, which was unable to activate adenylate cyclase, only inhibited the enzyme for 6 min. The sharp break in the curve suggests that an all-or-none transition occurs. In contrast, with a saturating concentration of Gpp(NH)p, this occurs after 3 min (Fig. 4). These data suggest that if a separate protein is involved in inhibition, it also isomerizes after Gpp(NH)p binding to a state which is no longer inhibitory. If the transient inhibition by Gpp(NH)p is mediated through the inhibitory G-protein, then treatment with pertussis toxin might abolish this effect (26). Pertussis vaccine administration to hamsters markedly decreases the inhibition of adenylate cyclase by adenosine and prostaglandins while having little effect on activation of adenylate cyclase by catecholamines (27). Katada and Ui (28) have shown that pertussis toxin ADP ribosylates a membrane protein different from the substrate for cholera toxin and this is associated with a decrease in the effects of agents which inhibit adenylate cyclase. Recently, Fain and O’Donnell (29) found that in adipocyte membrane preparations derived from rats injected 3 days previously with pertussis toxin the transient inhibition of forskolin-activated adenylate cyclase activity by Gpp(NH)p was abolished.

Seaman and Day (30) recently reported an inhibitory effect of Gpp(NH)p on forskolin-activated adenylate cyclase activity of rat striatal membranes which was reversed by Mn2+. If membranes were first incubated with Gpp(NH)p for 5 min, washed free of nucleotide, and then incubated with forskolin, the only effect of Gpp(NH)p was an enhancement of forskolin action on adenylate cyclase. Seaman and Daly (30) concluded that Gpp(NH)p inhibition of forskolin-activated adenylate cyclase was characteristic of cells which contain inhibitory guanine nucleotide regulatory proteins. Our data support this conclusion and indicate that GTP hydrolysis is not the only mechanism by which this protein can inhibit adenylate cyclase activity.

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T H Hudson and J N Fain


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