Mechanism and Properties of Inhibition of Purified Rat Brain Adenylate Cyclase by G Protein \( \beta\gamma \)-Subunits

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ABSTRACT—The mode of the inhibition of purified rat brain adenylate cyclase by the \( \beta\gamma \)-subunits of G protein \( (\beta\gamma) \) was studied. These subunits inhibited the catalytic activity of the cyclase with the maximal inhibition of 85% and the half-maximal inhibition at about 0.7 nM \( \beta\gamma \). The complex of \( \beta\gamma \) and adenylate cyclase isolated by density gradient centrifugation contained 1.8–2.0 mol \( \beta\gamma \) per mol of the cyclase when \( \beta\gamma \) was assayed by immunoblotting and by its inhibitory activity on adenylate cyclase. However, the \( \beta\gamma \) concentration-inhibition curves suggest that one of the two \( \beta\gamma \) molecules bound may be essential for the inhibition. The role for the second \( \beta\gamma \) molecule is unknown. As a tentative estimate, 70% of the adenylate cyclase activity remained inhibited by \( \beta\gamma \) when the complex was isolated. The inhibition was not dependent on \( G_{\alpha} \) or calmodulin. Although purified adenylate cyclase contained a protein (0.06–0.08 mol/mol of adenylate cyclase) that reacted with anti-\( G_{\alpha} \) antibody, this protein was not liberated from the cyclase when it formed a complex with \( \beta\gamma \). In addition, guanine nucleotide analogs little affected the cyclase activity or the inhibition by \( \beta\gamma \). The inhibition by \( \beta\gamma \) was reversed by the dilution of the complex, and the following re-addition of \( \beta\gamma \) suppressed the enzyme activity to about 15% of the initial activity again. These findings provide strong evidence that \( \beta\gamma \) inhibits adenylate cyclase directly and reversibly through the formation of the complex.

Keywords: Adenylate cyclase, Enzyme inhibition, G protein, \( \beta\gamma \)-Subunits, Brain (rat)

Adenylate cyclase (ATP pyrophosphate lyase [cyclizing], EC 4.6.1.1) is a component of the signal transduction system in plasma membranes. The enzyme is under dual regulation by the stimulatory and inhibitory receptors and G proteins. The activation of a G protein through the receptor promotes the binding of GTP to the G protein and accelerates the dissociation of G protein subunits into the GTP-binding \( \alpha \)-subunit and \( \beta\gamma \). The \( \alpha \)-subunit is unique for each G protein. The GTP-activated \( \alpha \)-subunit of \( G_\alpha \) directly activates adenylate cyclase. The dissociated \( \alpha \) and \( \beta\gamma \) of \( G_\alpha \) have been considered to inhibit the cyclase activity directly or indirectly (1, 2).

The inhibition of adenylate cyclase activity by the isolated \( \beta\gamma \) has been shown in both plasma membranes (3, 4) and the solubilized and purified cyclase preparations (5–10). Although \( \beta\gamma \) was considered in early studies to inhibit the cyclase activity indirectly by deactivating \( \alpha \), we (5) and Katada et al. (6) proposed that the inhibition of brain adenylate cyclase by \( \beta\gamma \) was direct. In recent cloning studies of cDNAs for several types of adenylate cyclase, Gilman and colleagues showed that \( \beta\gamma \) moderately inhibited purified recombinant type I adenylate cyclase activated with \( \alpha_\delta \) (10). The other five types of cloned adenylate cyclase are not inhibited by \( \beta\gamma \) (11, 12).

In the present study, we present evidence that \( \beta\gamma \) binds to and reversibly inhibits highly purified brain adenylate cyclase independent of \( \alpha \) or calmodulin.

Abbreviations used are: G protein, the guanine nucleotide-binding regulatory protein; \( \beta\gamma \), the complex of the \( \beta \)- and \( \gamma \)-subunits of G protein; \( G_\alpha \) and \( G_i \), the stimulatory and inhibitory G proteins of adenylate cyclase system, respectively; \( G_\alpha \), a G protein abundantly present in the brain; \( \alpha_\delta \), \( \alpha_\beta \), \( \alpha_\gamma \), and \( \alpha \), the \( \alpha \)-subunits of \( G_\alpha \), \( G_i \), \( G_\gamma \), and transductin, respectively; MOPS, 3-N-morpholinopropanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Gpp(NH)p, 5'-guanylylimidodiphosphate; GDP, guanosine 5'-diphosphate; EGTA, ethyleneglycol bis(amoetoxyethyl)tetraacetate.
MATERIALS AND METHODS

Materials

Forskolin-conjugated Affi-gel 10 was prepared by the method of Pfeuffer et al. (13). Antibodies to α₁/α₁₂ (AS/7), α₁/α₁₃ (GO/1), α₆ (RM/1) and β (SW/1 and MS/1) were purchased from Du Pont NEN Research Products (Boston, MA, USA). Male Wistar rats were used (Seiwa Experimental Animals, Yoshitomi, Fukuoka).

Buffers

Buffer 1 contained 10 mM MOPS, pH 7.4, 1 mM MgCl₂, 1 mM EDTA, 1 mM benzamidine hydrochloride, 10 μM benzethonium chloride, 1 μg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 500 mM NaCl and 0.13% (wt./vol.) Tween 60. Buffer 2 is buffer 1 lacking aprotinin and containing 100 mM NaCl instead of 500 mM NaCl. Buffer 3 consisted of 10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 1 mM EDTA, 1 mM benzamidine hydrochloride, 10 μM benzethonium chloride, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5% (vol./vol.) glycerol and 0.13% (wt./vol.) Tween 60.

Purification of adenylate cyclase

Adenylate cyclase was purified by affinity chromatography on forskolin-agarose according to the method of Pfeuffer et al. (13). The procedure was modified as follows: The membrane homogenate (13-15 mg protein/ml) prepared from 90 g of fresh rat brains was mixed with 1/19 volume of 20% (wt./vol.) Lubrol PX, stirred for 60 min and centrifuged for 60 min at 105,000 x g. The supernatant obtained was diluted with 4 volumes of buffer 1 and applied to the forskolin-agarose column (25 ml) equilibrated with the same buffer. The column was washed as described by Pfeuffer et al. (13). The column was equilibrated with the same buffer. The column was washed as described by Pfeuffer et al. (13). The cyclase was eluted with a gradient (150 ml) of 0 to 100 μM forskolin in buffer 2. The column was eluted as described in an Amicon concentrator equipped with a PM-30 membrane (Amicon, Lexington, MA, USA). To reduce the NaCl concentration, the concentrated sample was diluted with 3 volumes of buffer 3 lacking Tween and applied to the DEAE-5PW column (0.8 x 7.5 cm) equilibrated with buffer 3. The column was eluted with a gradient (30 ml) of 0-500 mM NaCl.

The cyclase was purified by 4,000- to 5,000-fold from the Lubrol-solubilized supernatant. The yield was 25-30 μg protein from 180 g of rat brain. The recovery of the cyclase activity from the supernatant was 3%-4%. The activity of the purified enzyme was 5-6 μmol cyclic AMP/min/mg protein in the presence of 100 μM forskolin, which is comparable to the enzyme activity reported by Pfeuffer et al. (13).

Assay of adenylate cyclase

Adenylate cyclase was assayed for 15 min at 30°C in 100 μl of the medium containing 20 mM HEPES, pH 7.0, 20 mM KCl, 3.2 mM MgCl₂, 0.2 mM ATP, 0.2 mg/ml BSA, 0.2 mM dithiothreitol and 0.015% Lubrol PX unless otherwise mentioned. The reaction was terminated by immersing the tubes in boiling water for 90 sec. The amount of cyclic AMP produced was determined by the cyclic AMP-binding protein assay (14).

Inhibition of adenylate cyclase activity by Pr

Purified adenylate cyclase and Pr purified as previously described (5) were preincubated together for 10 min at 23°C in 30 μl of buffer containing 20 mM HEPES, pH 7.4, 0.7 mg/ml BSA, 0.7 mM dithiothreitol and 0.05% Lubrol. The assay medium (65 μl) containing no ATP was added to the preincubated cyclase and Pr, and the mixture was further preincubated for 40 min at 6°C. ATP (5 μl) was added to the mixture and then adenylate cyclase activity was assayed as above in the presence of 3.2 mM MgCl₂.

Glycerol-density gradient centrifugation

Purified adenylate cyclase and Pr in 0.1 ml were preincubated for 10 min at 23°C and then layered on a 8%-30% (wt./vol.) glycerol-density gradient (4.4 ml) in 25 mM Tris-HCl, pH 8.0, buffer containing 1 mM dithiothreitol and 0.05% Lubrol PX. The gradient was centrifuged for 14 hr at 43,000 rpm (222,000 x g) at 4°C in a Beckman SW50.1 rotor (Beckman Instruments, Palo Alto, CA, USA), and fractions were collected from the bottom of the tubes.

Quantification of Pr

In Fig. 3, Pr were assayed by the Pr-stimulated ADP-ribosylation of α₁/α₁₂ with pertussis toxin as previously reported (5, 15). An aliquot of the fraction containing Pr or the control buffer was added to the reaction mixture containing α₁/α₁₂, pertussis toxin and [³²P]NAD. After the reaction, protein was precipitated and washed with trichloroacetic acid. The radioactivity incorporated into the protein was measured in a scintillation counter. Purified Pr was used for a standard curve to calculate the Pr concentration in a sample. Upon SDS-PAGE of the precipitated protein, radioactivity was incorporated only into the α₁- and α₁₂-subunits.

The low concentration of Pr seen in Fig. 3 was quantified by the inhibition of adenylate cyclase activity. A 50-μl aliquot of the fraction containing Pr was added to 5 μl of the purified cyclase (final concentration of 0.77 nM during the assay), and the mixture was preincubated for 5 min at 23°C. Then 40 μl of the assay medium omitting ATP was added to the mixture, and the preincubation
was further continued for 40 min at 6°C. After the preincubation, the adenylate cyclase activity was assayed in the presence of ATP and 3.2 mM MgCl₂. Each assay tube contained 0.77 nM of the added purified adenylate cyclase and the cyclase from the fraction. The enzyme concentration of the latter was separately determined by the enzyme assay in the presence of 50 mM MgCl₂, which abolished the inhibition by Pr contained in the fraction (see the text). The % inhibition of adenylate cyclase activity by Pr was calculated by dividing the enzyme activity determined as above by the activity of the same concentration of adenylate cyclase in the absence of Pr. The latter enzyme activity was calculated from the activity of the purified cyclase (0.25 μmol cyclic AMP/min/nmol enzyme in the absence of Pr) determined under the same conditions. Finally, the Pr concentration during the assay was determined, using the Pr concentration-% inhibition curve (the inserted figure of Fig. 3).

**Immunoblotting**

Immunoblotting of G protein subunits was conducted as described by Towbin et al. (16). Samples were subjected to SDS-PAGE (12% gel) and were transferred to an Immobilon PVDF membrane (Nihon Millipore, Tokyo). Antibodies indicated in the figure legends and ¹²⁵I-labeled secondary antibody (F(ab')₂ fragment) were used. The measurement of radioactivity on the Immobilon PVDF membrane was conducted in a Fujix bioimaging analyzer BAS2000 (Fuji Photo Film, Tokyo) which gave the radioactivity in a PSL unit (Photo-Stimulated Luminescence value). The unit was proportional to the total disintegration of ¹²⁵I during the exposure.

In Figs. 4 and 7, the fractions containing adenylate cyclase and associated Pr from density gradient centrifugation were combined and concentrated in a Millipore Molcut concentrator (nominal cut-off molecular weight of 30,000, Nihon Millipore) before SDS-PAGE. The amount of the concentrated cyclase was estimated by the density of silver-stained cyclase on a polyacrylamide gel, using the purified cyclase as standard, because the concentration procedure resulted in a decrease (about 40%) in the cyclase activity probably due to adsorption of the

![Fig. 1. SDS-PAGE-pattern of purified adenylate cyclase. Adenylate cyclase was eluted from a forskolin-agarose column with a gradient of forskolin, and each 7-ml fraction was collected. The cyclase was assayed in the presence of 100 μM forskolin. Portions of the eluted fractions were subjected to SDS-PAGE on a 5%–12% gradient gel. Protein was detected by the silver-staining technique. The arrows in the figure indicate copurified proteins. Standard proteins are shown in the left lane.](image-url)
enzyme to the filtration membrane and the partial inactivation of the enzyme.

Protein determination

The method of Schaffner and Weissmann (17) was used for microassay of protein. Briefly, protein precipitated on a filter with trichloroacetic acid was dyed with amido black. The dye adsorbed to the protein was solubilized with 100 μl of ethanol solution, and the optical density of the solubilized dye was measured at 630 nm in a microcuvette. A linear standard curve was obtained, using up to 2 μg of BSA. With 1 μg of BSA, the optical density at 630 nm was 0.23 - 0.25 and the standard deviation was 3% of the mean.

Miscellaneous procedures

SDS-PAGE was carried out by the method of Laemmli (18). The densities of silver-stained protein bands on the polyacrylamide gel were measured in a Shimadzu flying-spot scanner CS9000 (Shimadzu, Kyoto).

**RESULTS**

**Properties of adenylate cyclase inhibition by βγ**

Adenylate cyclase was purified from rat brain by successive chromatography on forskolin-agarose and DEAE-5PW columns as described in the Materials and Methods. Figure 1 shows that major proteins of Mr 180,000 and 135,000 were coeluted with adenylate cyclase activity from the forskolin-agarose column. Both the proteins are considered to be adenylate cyclase as discussed later. Densitometric measurement of silver-stained bands in SDS-PAGE of the purified cyclase preparation showed that the proteins of Mr 180,000 and 135,000 accounted for 77% of the total protein in the final enzyme preparation, and these two major proteins were present in a ratio of 1 : 2.0. Based on this ratio, the weight-average molecular weight of 150,000 for adenylate cyclase was calculated. We used the molecular weight of 150,000 and the purity of 77% to calculate the molar concentration of the cyclase. Proteins of Mr 66,000, 38,000, 36,000 and 28,000 (shown by the arrows in Fig. 1) and other minor proteins of Mr 55,000 and 43,000 were also coeluted with the enzyme activity.

The purified βγ derived from G; and Go strongly inhibited the catalytic activity of the purified cyclase as shown in Fig. 2. Cyclic AMP accumulating during the assay increased linearly up to at least 20 min both in the presence and absence of βγ. The inhibition was not observed when βγ were denatured by heating. In the presence of 3 mM MgCl₂ in an excess of Mg-ATP, the βγ concentration for half-maximal inhibition (EC₅₀) was about 0.7 nM and the maximum inhibition was 85%. In the presence of 50 mM excess MgCl₂, the βγ-dependent inhibition decreased. Particularly, the inhibition was almost abolished when the βγ concentration was less than 1 nM. This property was used to determine the concentration of adenylate cyclase in the fraction containing βγ as described later.

**Association of βγ with adenylate cyclase**

From the βγ-induced inhibition described above, it is considered that βγ interacted with adenylate cyclase. This was examined by density gradient centrifugation of βγ and the cyclase.

Purified adenylate cyclase was preincubated with a sufficient amount of βγ to inhibit the cyclase activity maximally and then subjected to the centrifugation in a glycerol-gradient. During the centrifugation, free βγ, assayed by the βγ-stimulated ADP-ribosylation of α₁/α₀ with pertussis toxin as previously reported (5, 15), was separated from the cyclase and remained near the top of the gradient (Fig. 3). On the other hand, adenylate cyclase, assayed in the presence of 50 mM or 3 mM Mg²⁺,
Fig. 3 Association of adenylate cyclase and G protein βγ-subunits (βγ) assessed by density gradient centrifugation. Purified adenylate cyclase (3.4 pmol) was preincubated for 10 min at 23°C with 230 pmol of βγ. The preincubated cyclase and βγ were then subjected to centrifugation in a 8%–30% (wt./vol.) glycerol-density gradient as described in the Materials and Methods. Fractions were collected from the bottom of the tube. A 5-μl or 50-μl aliquot of the fraction was assayed for adenylate cyclase activity in a final volume of 100 μl in the presence of 50 mM (●) or 3 mM (○) excess MgCl₂, respectively. From the enzyme assay in the presence of 50 mM excess MgCl₂, the concentration of adenylate cyclase in each fraction (◼) was estimated. βγ were assayed by the stimulation of pertussis toxin-catalyzed ADP-ribosylation of α/α (▲) as previously reported (5, 15). To determine the βγ concentrations in the fractions 1–7, 50 μl of the fraction was added to the purified cyclase (final 0.77 nM during the assay), and the % inhibition of the cyclase activity by the βγ contained in the fraction added was measured in the presence of 3.2 mM MgCl₂ as described in the Materials and Methods. The βγ concentration during the assay was determined from the curve shown in the inserted figure, in which % inhibition of adenylate cyclase activity (I) by βγ (in nM) was measured with 0.77 (×), 1.00 (+) and 1.54 (●) nM of purified adenylate cyclase under the same conditions as described above. The βγ concentrations in each fraction (▲) estimated by this method and the molar ratio of βγ to the cyclase (◼) are shown.

As shown in Fig. 2, assay of adenylate cyclase in the presence of 50 mM MgCl₂ nearly abolished the inhibition by a low concentration of βγ (<1 nM). This abolition was observed between the examined cyclase concentrations of 0.025 and 0.2 nM, which suggested that the concentration of adenylate cyclase in the fraction containing βγ shown in Fig. 3 could be determined by the assay in the presence of 50 mM MgCl₂ under these conditions. Actually, when adenylate cyclase centrifuged in a density gradient in the absence of βγ as a control in Fig. 3 was diluted 20-fold (final concentration) in the assay, the cyclase concentrations in the peak fractions of the enzyme determined by the standard assay in the presence of 3.2 mM MgCl₂ were within the range of 0.025–0.2 nM. Therefore, we assayed adenylate cyclase in the fraction (Fig. 3) in the presence of 50 mM MgCl₂ by using a final 20-fold dilution, assuming that the cyclase concentration in this fraction was similar to that of the control cyclase centrifuged in the absence of βγ. The determined concentrations of adenylate cyclase from fractions 1–5 in Fig. 3 were 0.05–0.09 nM during the assay, which were within the range of adenylate cyclase concentration (0.025–0.2 nM) allowed for the estimation. In addition, the concentrations of βγ from fractions 1–5 determined as described below were 0.07–0.22 nM during the assay. Therefore, the quantification of adenylate cyclase was performed under proper conditions.

The enzyme activity in the presence of 3 mM excess Mg²⁺, as shown in Fig. 3, was lower than that assayed in the presence of 50 mM Mg²⁺, although the enzyme assay in the presence of 50 mM and 3 mM Mg²⁺ gave similar activities in the absence of βγ (see Fig. 2). This indicates that the enzyme activity with 3 mM Mg²⁺ was under inhibition by βγ contained in the adenylate cyclase fractions. So we estimated the concentration of βγ in the
fraction. However, correct quantification of $\beta_T$ in these fractions by the ADP-ribosylation assay was difficult due to the low $\beta_T$ concentration. Then, we tried to use the inhibitory activity of $\beta_T$ on adenylate cyclase to estimate the $\beta_T$ concentration (Fig. 3). Briefly, an aliquot of each adenylate cyclase fraction was added to the purified cyclase (0.77 nM), and the % inhibition of the cyclase activity by $\beta_T$ contained in the fraction added was obtained from the cyclase assay with 3.2 mM MgCl$_2$ as described in the Materials and Methods. Then, the $\beta_T$ concentration was determined from the $\beta_T$ concentration-% inhibition curve shown in the inserted figure of Fig. 3. The $\beta_T$ concentration could be determined independently of the cyclase concentration in the fraction, because the total cyclase concentration during the assay was 0.77–1.54 nM including 0.77 nM purified enzyme, and no difference of the curve was observed with 0.77, 1.00 or 1.54 nM cyclase (the inserted figure of Fig. 3).

In fractions 1–5, the $\beta_T$ concentration was proportional to the cyclase concentration determined by the enzyme assay with 50 mM MgCl$_2$ (Fig. 3). The average molar ratio of $\beta_T$ to the cyclase in fractions 1–5 was 2.0 mol $\beta_T$ per mol of the cyclase. These results indicate that $\beta_T$ in the cyclase fraction actually sedimented as a complex with adenylate cyclase. In the fraction 6 and later, the $\beta_T$ concentration increased markedly due to the sedimentation of free $\beta_T$. When adenylate cyclase was omitted from the centrifugation, the amount of $\beta_T$ in the bottom fractions 1–5 was under the limit of detection.

The amount of $\beta_T$ in the cyclase fraction was also quantified by immunoblotting with anti-\(\beta\)-subunit antibody (SW/1) (Fig. 4). Using purified $\beta_T$ as the standard,
we estimated the content of $\beta$-subunit in the cyclase fraction to be 1.8 mol $\beta$ per mol of adenylate cyclase.

From the experiments described above, it was found that 1.8–2.0 mol of $\beta_\gamma$ was associated with 1 mol of adenylate cyclase. So, we tried to distinguish by kinetic analysis whether only one or two $\beta_\gamma$ molecules were required for the inhibition of adenylate cyclase activity.

Figure 5A shows the plots of the reciprocal of adenylate cyclase activity against the $\beta_\gamma$-subunits ($\beta_\gamma$) concentration. Line B shows the plots when adenylate cyclase activity in the presence of an excess of $\beta_\gamma$ (15% of the cyclase activity in the absence of $\beta_\gamma$) was subtracted from each enzyme activity before the plotting. Panel B: Hill plot. "i" is the inhibition at each $\beta_\gamma$ concentration, and "i" is the maximal inhibition in the presence of an excess of $\beta_\gamma$ (85% of the cyclase activity in the absence of $\beta_\gamma$). Data shown are representative of three independent experiments.

The content of the immunoreactive protein of 43 kDa was estimated by densitometric measurement of silver-stained proteins in a Shimadzu flying spot scanner after SDS-PAGE of purified adenylate cyclase. The amount of silver-stained protein of 43 kDa (probably the immunoreactive protein) was 1.8% of that of silver-stained adenylate cyclase on the same gel, that is, 0.06 mol of the immunoreactive protein per mol of the cyclase.

We also attempted to estimate the content from the experiment in Fig. 7, in which the radioactivity on a protein band reacting with anti-$\alpha$ antibody was proportional to the amount of the purified cyclase (0.21–0.88 pmol) loaded on the gel. To calculate the amount of the 43-kDa immunoreactive protein from its radioactivity, we used the radioactivity on the immunoblots of a known amount of $\beta$-subunit in Fig. 4. The experiments in Figs. 7 and 4 were conducted simultaneously under the same conditions, and we assumed for the calculation that anti-$\alpha$ and anti-$\beta$ antibodies used in these experiments reacted with their antigen to the same extent and the second antibody recognized these antibodies with the same efficiency. Therefore, the content of the immunoreactive protein was 0.08 mol per mol of adenylate cyclase.
From the estimation as described above, it is evident that the content of \( \alpha_s \), if it exists, is too low to serve as the site for the Pr binding in the inhibition.

In the adenylate cyclase assay conducted here, we did not use GTP, a physiological activator of \( \alpha_s \), or an ATP-regenerating system that may phosphorylate the GDP associated with \( \alpha_s \) to GTP. To determine whether \( \alpha_s \)-activated cyclase activity was involved in the Pr-dependent inhibition observed here, we examined the effects of guanine nucleotide analogs on adenylate cyclase activity.

Adenylate cyclase was preincubated for 10 min at 23°C in the presence of 5 mM Mg\(^{2+}\) and 100 \( \mu \)M Gpp(NH)p, a hydrolysis-resistant analog of GTP and a strong activator of \( \alpha_s \), and then the cyclase was assayed for 15 min at 30°C (final concentrations of 3.2 mM Mg\(^{2+}\) and 33 \( \mu \)M Gpp(NH)p). Although our previous report (21) on the Gpp(NH)p-dependent activation of partially purified adenylate cyclase with \( \alpha_s \) showed that preincubation for 30 min with Gpp(NH)p doubled the enzyme activity, we had to limit the preincubation time to 10 min due to instability of the highly purified adenylate cyclase used in the present study. The amounts of cyclic AMP formed in the presence and absence of Gpp(NH)p were 32.9 ± 0.3 and 32.4 ± 1.0 pmol (mean ± S.E.), respectively. The activation by Gpp(NH)p was not observed. Moreover, the preincubation of the cyclase with 5 mM MgCl\(_2\) and 30 \( \mu \)M GDP\(\beta\)S, a hydrolysis-resistant analog of GDP, in order to replace the guanine nucleotide bound to \( \alpha_s \) did not cause any reduction of the cyclase activity in the absence of Pr. There was no difference between the Pr concentration (0.11–114 nM)-inhibition curves after preincubation with or without GDP\(\beta\)S (data not shown). These results show that adenylate cyclase activity and the effect of \( \beta\gamma \) observed here were independent of \( \alpha_s \).

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**Fig. 6.** Immunoblotting of purified adenylate cyclase with the antibodies against G protein subunits. Immunoblotting was conducted as described in the Materials and Methods. Panel A: Lanes 1–3, 0.87 pmol of the purified adenylate cyclase ("C"); lanes 4–6, 1.1 pmol of the mixture of \( \alpha_o \) and \( \alpha_i \) ("\( \alpha_o / \alpha_i \)"). Antibodies used were AS/7 recognizing the carboxyl-termini of \( \alpha_o \), \( \alpha_i \), and \( \alpha_2 \) (lanes 1 and 4), GO/1 recognizing the carboxyl-termini of \( \alpha_o \) and \( \alpha_3 \) (lanes 2 and 5) and RM/1 recognizing the carboxyl-terminus of \( \alpha_i \) (lanes 3 and 6). Panel B: Lanes 1 and 4, 1.9 and 2.5 pmol of \( \beta\gamma \)-subunits ("\( \beta \)"), respectively; lanes 2 and 3, 0.87 pmol of purified adenylate cyclase ("C"). Antibodies used were SW/1 recognizing the carboxyl-terminus common to \( \beta_1 \), \( \beta_3 \) and \( \beta_5 \) (lanes 1 and 2) and MS/1 recognizing the amino-terminus of \( \beta_2 \) (lanes 3 and 4).
In addition to αs, Ca²⁺/calmodulin was also not involved in the inhibition by βγ. The presence of 1 mM EGTA, to eliminate the effects of Ca²⁺, in the assay medium showed no effect on the inhibition by βγ (data not shown).

Adenylate cyclase activity of the complex with βγ and the reversibility of the inhibition by βγ

We measured adenylate cyclase activity in the complex fraction obtained in Fig. 3 to determine if the cyclase activity in the complex fraction was inhibited to a similar extent as in the standard assay system (85% inhibition). The complex fraction was added to an equal volume of assay medium so that the dilution of the complex fraction was minimum (0.47 nM cyclase during the assay in a final volume of 100 μl) and also the detergent and glycerol brought in from the complex fraction did not exceed the concentration allowed for the assay. Then, the specific activity (pmol cAMP/min/pmol of the enzyme) of the cyclase in the complex fraction measured was about 60% of that of the control cyclase centrifuged in a density gradient in the absence of βγ. Because this result was quite different from that observed in the standard assay system, we considered that βγ might be dissociated from the cyclase due to the dilution of the complex with the assay medium containing ATP and Mg²⁺ and the elevation of temperature in the assay. Actually, dilution of the complex fraction by 10-fold with the buffer used for density-gradient centrifugation and the following addition of the diluted complex fraction to the assay medium (final concentration, 0.047 nM cyclase) increased the measured specific activity of the cyclase from about 60% to 86% of the control cyclase activity. We also tentatively tried to estimate the cyclase activity in the complex fraction that was not diluted. The % specific activities of the fraction assayed with various extents of dilution were plotted.

Fig. 7. Lack of the effect of βγ-subunits (βγ) on the liberation from adenylate cyclase of the protein immunoreactive to anti-α, antibody. Protein was immunoblotted with an antibody (RM/1) recognizing the carboxyl-terminus of αs. Purified adenylate cyclase was centrifuged in the absence or presence of βγ as in Fig. 3. Lanes 1, 2 and 5, untreated adenylate cyclase (0.41, 0.88 and 0.21 pmol, respectively); lane 3, adenylate cyclase centrifuged with βγ (corresponding to the fractions 2–5 in Fig. 3) ("E1") (0.28 pmol enzyme); lane 4, the cyclase centrifuged without βγ ("E2") (0.19 pmol enzyme). The radioactivities (12-hr exposure) of the immunoreactive bands of untreated adenylate cyclase (0.41, 0.88 and 0.21 pmol) were 1268 (lane 1), 3053 (lane 2) and 682 (lane 5) PSL unit. The radioactivities of the immunoreactive bands of "E1" and "E2" were 1033 (lane 3) and 747 (lane 4) PSL unit, respectively, and the contents of the immunoreactive protein were 3700 ("E1") and 3900 ("E2") PSL unit/pmol enzyme, respectively. The plot of radioactivities (PSL unit) against the amounts of the immunoreactive protein in untreated adenylate cyclase was linear. (PSL unit: see the legend of Fig. 4 and the Materials and Methods)
against the cyclase concentration during the assay. Then, the approximately linear line obtained was extrapolated to the cyclase concentration (0.94 nM) in the undiluted complex fraction. As a result, the % specific activity at 0.94 nM cyclase on the plot was estimated to be about 30% of the control (70% inhibition). The estimated % inhibition, which was close to the maximal inhibition in the standard assay system, was tentatively considered to indicate that the adenylate cyclase in the complex stayed nearly fully inhibited by pr under the assay conditions employed here.

If the dissociation of pr from the cyclase suggested above is the case, the cyclase activity enhanced by dilution must be inhibited again by the re-addition of pr. In Fig. 8, adenylate cyclase activity of the complex fraction (diluted to 0.08 nM cyclase) assayed under standard conditions was indeed inhibited again by the re-added pr. Both enzyme activities in the complex fraction and the control adenylate cyclase were inhibited to the maximum (about 15% of the initial activity of the control cyclase) with similar concentrations of pr. These results show that the inhibition by pr is reversible. The effects of dilution and pr re-addition described above may be explained if the inhibition is assumed to be a reversible bimolecular interaction between adenylate cyclase and pr as suggested above, although the formation or dissociation of the complex in a buffer system containing detergent may not be a simple process.

DISCUSSION

In the present study, we confirmed the previous observations (5, 6) that the enzyme activity of brain adenylate cyclase was effectively inhibited by pr of G proteins. The inhibition shown here was reversible and dependent on the pr concentration. This suggests that the inhibition by pr described here may serve as the physiological mechanism for adenylate cyclase regulation in the brain.

Because both pr- and pr-subunits have subtypes, various forms of pr exist. Illíezeg-Lluhi et al. (22) recently reported that pr generated by the combination of p1 or p2 with r1, r2 or r3 showed similar inhibitory effects on the activity of type I adenylate cyclase except pr from transducin which inhibited the enzyme activity relatively weakly. It is not certain that only particular forms of pr inhibit adenylate cyclase activity.

In the present study, pr associated reversibly with adenylate cyclase. Because 1.8–2.0 mol of pr was found to be associated with one mole of adenylate cyclase, we tried to distinguish whether the inhibition required only one or two pr molecules. The results suggested that one of the two pr molecules bound to the cyclase was essential for the inhibition. The property and the function of the other pr molecule suggested is unknown, but the possibility remains that the binding of the second pr molecule serves to stabilize the inhibition complex. However, it should be noted that the ratio mentioned above is based on the molecular weights determined by SDS-PAGE and the protein concentrations assayed by using bovine serum albumin as the standard.

Our previous (9) and present studies have shown that calmodulin and the a-subunit were not the binding site for pr in the inhibition observed here. The content of the protein immunoreactive to anti-a antibody (0.06–0.08 mol/mol of the cyclase) was too low to serve as the site of the pr binding. In addition, the immunoreactive protein was not liberated from the cyclase even in the presence of pr. Thus it is very unlikely that the inhibition requires a or calmodulin.

The adenylate cyclase purified here contained major proteins of Mr 180,000 and 135,000 as clearly visible bands on a polyacrylamide gel. Both the proteins appear
to be adenylate cyclase on the basis of the reported molecular weights of mammalian adenylate cyclase (105 to 180 kDa) (7, 13, 23–27). Particularly, even Ca\(^{2+}\)/calmodulin-sensitive adenylate cyclase purified from the brain by calmodulin-affinity chromatography has a molecular weight of 135,000–140,000 (24–26). In addition, Orlando et al. (26) found a Ca\(^{2+}\)/calmodulin-sensitive cyclase of Mr 175,000 from the brain whose apparent molecular weight changed to 135,000 in the course of purification. Other forskolin-binding proteins (glucose carriers) have been reported, but they have much smaller molecular weights (Mr 40,000–60,000) than adenylate cyclase (28). Therefore, the two major bands on the gel may be isozymes or they might be generated by a modification such as proteolysis or by a difference in sugar chains covalently attached to the enzyme. The complex of adenylate cyclase and \(\beta_2\)-subunit contained both forms of the cyclase (Mr 180,000 and 135,000). However, it is not yet certain whether or not \(\beta_2\) inhibits the two forms of the enzyme to the same extent.

We have no direct evidence that the adenylate cyclase purified here is the type I cyclase that is localized in neural tissues (29). However, the sensitivity of the purified enzyme to Ca\(^{2+}\)/calmodulin (9) or to \(\beta_2\)_subunit indicates that the purified cyclase is the type I enzyme or a similar one. Type II adenylate cyclase, which is also found in the brain, is activated by \(\beta_2\) and is not sensitive to Ca\(^{2+}\)/calmodulin (11, 12).

The nature of minor proteins that copurified with the adenylate cyclase is not known. The protein of Mr 38,000 might be the same protein reported by Mollner et al. (30) who found that the proteins of Mr 38,000 associated with adenylate cyclase cross-reacted with the antibody against synaptophysin. The copurified protein of Mr 36,000 was similar in molecular weight to the G protein \(\beta\)-subunit. However, this minor protein did not cross-react with the antibody against the carboxyl- or amino-terminal peptide of the \(\beta_1\)-subunit, whose carboxyl terminal amino acid sequence is also shared by \(\beta_2\) and \(\beta_3\)-subunits (31). Therefore, this protein is not \(\beta_1\) and is unlikely to be \(\beta_2\) or \(\beta_3\).

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