# Inhibition of catalytic unit of adenylate cyclase and activation of GTPase of $N_i$ protein by $\beta\gamma$-subunits of GTP-binding proteins

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Received 1 March 1986; revised version received 1 May 1986

A protein factor which inhibited adenylate cyclase was purified to apparent homogeneity from rat brain and identified as the $\beta\gamma$-subunits of the GTP-binding regulatory proteins of adenylate cyclase. (i) The $\beta\gamma$-subunits (protein factor) inhibited the partially purified catalytic unit of adenylate cyclase in the presence of an activator, forskolin or the stimulative regulatory protein ($N_s$), to 60 and 40% of the control, respectively; inhibition of the catalytic unit in the presence of forskolin required no guanine nucleotides. (ii) The subunits enhanced the GTPase activity of the purified $\alpha$-subunit of the inhibitory regulatory protein ($N_i\alpha$) 3.8-fold. (iii) The subunits stimulated ADP-ribosylation of $N_i\alpha$ catalyzed by islet-activating protein (pertussis toxin). ADP-ribosylation had no effect on the GTPase activity of $N_i\alpha$ in the presence of the $\beta\gamma$-subunits. The results suggest that direct inhibition of the catalytic unit by the $\beta\gamma$-subunits liberated from $N_i$ is essential for the receptor-mediated inhibition of adenylate cyclase.

*Adenylate cyclase* *Enzyme inhibition* *$N_i$ protein* *GTPase* *ADP-ribosylation* *Islet-activating protein* *(Rat brain)*

## 1. INTRODUCTION

Adenylate cyclase activity is regulated by the stimulative and inhibitory GTP-binding regulatory proteins termed $N_s$ and $N_i$, respectively, which act as the communicator between the catalytic unit of adenylate cyclase and the receptors in cell membranes [1,2]. Both $N_s$ and $N_i$ consist of 3 subunits namely $\alpha$, $\beta$ and $\gamma$ [2,3]. The $\alpha$-subunits (GTP-binding subunits) from the two GTP-binding proteins are clearly different but the $\beta$-subunits are indistinguishable [4]. Binding of GTP analogues or $F^-$ to their $\alpha$-subunits appears to dissociate the proteins into $\alpha$- and $\beta\gamma$-subunits [5,6]. The $\alpha$-subunit of $N_i$ ($N_i\alpha$) has GTPase activity [7–10]. ADP-ribosylation of $N_i\alpha$ by islet-activating protein (IAP), pertussis toxin, was reported to attenuate the receptor-mediated inhibition of adenylate cyclase [11]. The catalytic unit of adenylate cyclase has been shown to be activated by the $\alpha$-subunit of $N_s$ to which GTP is bound [5]. On the other hand, the mechanism of inhibition of adenylate cyclase by $N_i$ has not been extensively studied at the molecular level. Katada et al. [12] have proposed that the $\beta\gamma$-subunits liberated from $N_i$ suppress the GTP-dependent activation of $N_s$ and thus indirectly reduce the cyclase activity. However, whether $N_s$ is actually involved in the inhibition by $N_i$ is currently a point in dispute [13].

In [14], we reported that the partially purified GTP-binding regulatory proteins inhibited the forskolin-activated catalytic unit of adenylate cyclase even in the absence of guanine nucleotides. We supposed that the inhibitory effect was exerted by $N_i$ but failed to show the inhibitory activity in purified $N_i\alpha$ [7]. Recently we found that the inhibitory activity was associated with a protein factor of $M_r$ 35000 [15] which we previously reported as stimulating ADP-ribosylation of purified $N_i\alpha$ by IAP and augmenting the GTPase activity of

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00145793/86/$3.50 

$N_i\alpha$ [7]. Here, we have purified this inhibitory protein factor and identified it as the $\beta\gamma$-subunits of the GTP-binding regulatory proteins.

## 2. MATERIALS AND METHODS

[$\gamma$-$^{32}$P]GTP and [$^{32}$P]NAD were purchased from New England Nuclear. IAP was a generous gift from Dr Michio Ui (Hokkaido University, Sapporo). Heptylamine-Sepharose was prepared as described in [16].

### 2.1. *Buffers*

Buffer A contained 20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol (DTT), 20 $\mu$M $AlCl_3$, 6 mM $MgCl_2$, and 10 mM NaF, pH 8.0. Buffer B was the same as buffer A except that $AlCl_3$, $MgCl_2$ and NaF were omitted. Buffer C consisted of 50 mM Tris, 0.2 M sucrose, 15 mM $MgCl_2$, 7 mg/ml soybean phospholipid, 14 mM cholate, 1 mM DTT and 25% saturated ammonium sulfate, pH 7.7. Buffer D contained 20 mM Hepes, 0.2 M sucrose, 15 mM $MgCl_2$, 7 mg/ml soybean phospholipid, 0.1% (w/v) Lubrol PX and 1 mM DTT, pH 7.7.

### 2.2. *Partially purified catalytic unit*

The catalytic unit of adenylate cyclase was partially purified from rat brain according to [17]. The preparation was free of $N_s$ activity and GTPase activity of $N_i\alpha$ [14,17]. The specific activity in the presence of 5 mM $MnCl_2$ was 1.2–1.7 nmol cyclic AMP/min per mg protein at 30°C.

### 2.3. *Purification of $N_i\alpha$ and $N_s$*

$N_i\alpha$ and $N_s$ were purified essentially as in [7]. The second protein peak in the flow-through fractions from a heptylamine-Sepharose column (1.6 × 16 cm) was collected as the $N_i\alpha$ preparation. The specific activity of the GTPase was 4.1–4.6 nmol $P_i$ released/min per mg protein. Gel electrophoresis of the purified $N_i\alpha$ preparation is shown in fig.2B. The specific activity of the $N_s$ preparation obtained was 156 nmol cyclic AMP/min per mg protein.

### 2.4. *Purification of the $\beta\gamma$-subunits*

Synaptosome-rich membranes were prepared from about 40 g rat brains and membrane protein was solubilized with cholate as described [7]. The concentration of protein during solubilization was 8 mg/ml. The supernatant supplemented with 20 $\mu$M $AlCl_3$ and 10 mM NaF was diluted with buffer A containing 25 mM NaCl to reduce the cholate concentration to 21 mM. The supernatant (539 mg protein) was then divided into two portions, each being applied separately to a DEAE-Sephacel column (2.2 × 34 cm) which had been equilibrated with buffer A containing 21 mM cholate and 25 mM NaCl. The column was washed with the same buffer and adsorbed protein was eluted with 360 ml of a linear gradient of 0–250 mM NaCl in buffer A containing 21 mM cholate. The $\beta\gamma$-subunits were eluted with about 160 mM NaCl and mostly separated from the GTPase activity of $N_i\alpha$ which was eluted with about 110 mM NaCl. Fractions containing $\beta\gamma$ were recovered and concentrated to about 20 ml by ultrafiltration with an Amicon PM-10 membrane. The concentrate was applied to a Sephadex G-25 column (1.6 × 30 cm) in buffer A containing 0.5% (w/v) Lubrol PX. Protein eluted was divided into two fractions and each fraction applied separately to a TSK DEAE-5PW column (0.8 × 7.5 cm) in the same buffer. The $\beta\gamma$-subunits eluting near the second protein peak in the flow-through fractions were again applied to a Sephadex G-25 column which had been equilibrated with buffer A containing 9.3 mM cholate and 100 mM NaCl, and then the eluate loaded on a heptylamine-Sepharose column (1 × 21 cm) in the same buffer. The $\beta\gamma$-subunits were eluted with 50 ml of a linear gradient in buffer B starting with 7 mM cholate and 150 mM NaCl and ending with 30 mM cholate and 25 mM NaCl. The column was further washed with the ending buffer. Peak fractions of $\beta\gamma$ (about 10 ml) were concentrated to about 300 $\mu$l and subjected to TSK G3000SW gel chromatography (0.8 × 30 cm) in 30 mM Tris, 1 mM EDTA, 1 mM DTT, 0.3 M NaCl and 21 mM cholate, pH 7.5.

### 2.5. *Assay of activities*

Before the assay of activities, protein in column fractions was precipitated with ammonium sulfate as described [7]. Buffer C was used for dilution of column fractions. Protein precipitated was dissolved in buffer D.

GTPase activity was assayed at 37°C for 10 min [14]. For assay of the $\beta\gamma$ subunits, $N_i\alpha$ (1.5 pmol

$P_i$ released/min per tube) and $\beta\gamma$ in 10 $\mu$l buffer D were preincubated for 30 min at 23°C before GTPase assay.

Stimulation of ADP-ribosylation by the $\beta\gamma$-subunits was assayed as follows. $N_i\alpha$ (10 pmol $P_i$ released/min per tube) and $\beta\gamma$ in 8.4 $\mu$l buffer D were preincubated for 30 min at 23°C and then mixed with 16.6 $\mu$l assay solution to give final concentrations of 25 mM Tris, 2.5 mM $MgCl_2$, 1 mM ATP, 0.1 mM GTP, 10 mM thymidine, 5 mM nicotinamide, 10 $\mu$M [$^{32}$P]NAD (5–10 Ci/mmol), 2 mM DTT and 2.5 $\mu$g/ml IAP. The reaction conducted at 25°C for 30 min was stopped by the addition of 250 $\mu$l of 15% (w/v) trichloroacetic acid containing 1% (w/v) SDS. Precipitated protein was repeatedly washed with 10% (w/v) trichloroacetic acid and the radioactivity measured in a scintillation counter. Radioactivity was incorporated only into $N_i\alpha$ as shown in fig.2B.

To measure the inhibition of adenylate cyclase, the catalytic unit (12 pmol cyclic AMP/min in the presence of $MnCl_2$) and the $\beta\gamma$-subunits were preincubated for 60 min at 23°C in 25 $\mu$l of medium containing 80 mM sucrose, 2.8 mg/ml soybean phospholipid, 15 mM $MgCl_2$, 0.04% (w/v) Lubrol PX, 1 mM DTT, 1 mg/ml bovine serum albumin and 20 mM Hepes, pH 7.7. Adenylate cyclase activity was assayed at 30°C for 10 min as in [18] except that GTP was omitted and 10 $\mu$M forskolin was included in the assay medium.

Protein concentration was determined by using bovine serum albumin as a standard [19]. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to [20].

## 3. RESULTS AND DISCUSSION

A protein factor found in the partially purified GTP-binding regulatory proteins has been shown by us to have three different activities; stimulation of IAP-catalyzed ADP-ribosylation of $N_i\alpha$, activation of GTPase of $N_i\alpha$ and inhibition of the catalytic unit of adenylate cyclase [7,14,15]. The protein factor was purified and identified as the $\beta\gamma$-subunits of the GTP-binding regulatory proteins in the present study. The $\beta\gamma$-subunits (protein factor) were solubilized with cholate from a synaptosome-rich membrane fraction prepared from rat brain and its activity assayed by the stimulation of ADP-ribosylation of $N_i\alpha$, the increment of GTPase activity of $N_i\alpha$ and the inhibition of adenylate cyclase activity. Purification was conducted by successive chromatography steps using DEAE-Sephacel, TSK DEAE-5PW, heptylamine-Sepharose and TSK G3000SW as described in section 2. Fig.1 shows the final step of purification by G3000SW gel chromatography. The $\beta\gamma$-subunits were eluted from the column in association with the second protein peak. No $N_s$ or phosphodiesterase activities were detected in this fraction. During a series of chromatography steps, activities for the stimulation of ADP-ribosylation, GTPase activation and inhibition of adenylate cyclase were not separated and showed similar elution profiles. This indicates that these activities can all be ascribed to the same protein. The $\beta\gamma$-subunits were purified 212-fold from a cholate extract with a recovery of 7.5% on the basis of its activity for the stimulation of ADP-ribosylation.

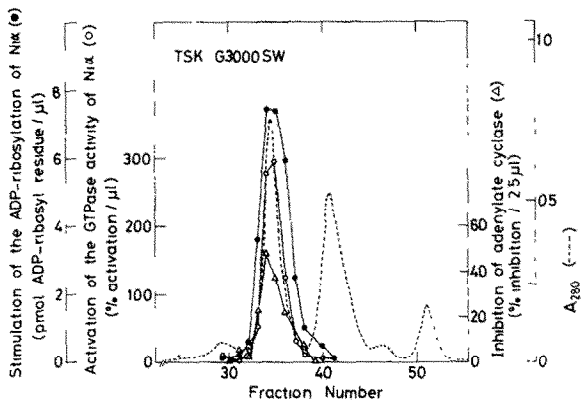


Fig.1. Purification of the $\beta\gamma$-subunits by G3000SW gel chromatography. The cholate extract (530 mg protein) from a synaptosome-rich membrane fraction was subjected to successive chromatography steps on DEAE-Sephacel, TSK DEAE-5PW and heptylamine-Sepharose. Fractions containing $\beta\gamma$ (0.51 mg protein) were then applied to a TSK G3000SW column (0.8 × 30 cm, 0.3 ml/fraction) and 0.19 mg protein of the purified $\beta\gamma$-subunits were obtained (see section 2). The $\beta\gamma$-subunits were assayed by the stimulation of IAP-catalyzed ADP-ribosylation of $N_i\alpha$ (●), activation of the GTPase of $N_i\alpha$ (○) and inhibition of the forskolin-activated catalytic unit of adenylate cyclase (△). (– – –) Absorbance at 280 nm. The purified $\beta\gamma$-subunits (0.1 $\mu$g protein) caused 3.25 pmol of net increase of ADP-ribosylation, 101% activation of GTPase activity and 15% inhibition of the forskolin-activated catalytic unit under standard assay conditions.

Fig.2A shows polyacrylamide gel electrophoresis of the protein purified as described above. Stained protein bands of $M_r$ 36000, 35500 and about 9000 were observed. Their estimated $M_r$ values were very close to the reported values for the $\beta$ ($M_r$ 35000) and $\gamma$ ($M_r$ ~10000) subunits of $N_i$ and $N_s$ [3,21]. Moreover, the protein purified here and the $\beta\gamma$ from $N_i$ have a similar stimulatory effect on ADP-ribosylation of $N_i\alpha$ by IAP [8,22]. Thus, we concluded that the purified protein was identical to the $\beta\gamma$-subunits. It is not known whether the $\beta\gamma$ purified here were derived from $N_i$ or $N_s$. If it is taken into consideration that $N_i$ occupies nearly 1.5% of the membrane protein from bovine brain [23] and $N_s$ is usually a minor membrane component, most of the $\beta\gamma$ are likely to have been dissociated from $N_i$ and some could have been derived from $N_s$. Both peptides of $M_r$ 36000 and 35500 seem to be the $\beta$. Two similar $\beta$-subunits in brain have been reported by others [23].

Brain has been shown to contain at least two IAP substrates of $M_r$ 41000 and 39000 [8,10,23].

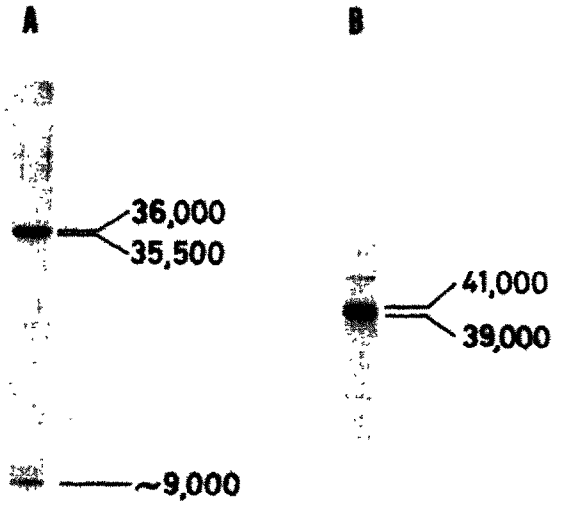


Fig.2. Polyacrylamide gel electrophoresis of the purified $\beta\gamma$-subunits and $N_i\alpha$. The purified $\beta\gamma$-subunits (6 $\mu$g) (A) and $N_i\alpha$ (3 $\mu$g) (B) were subjected to gel electrophoresis in the presence of SDS. Protein was stained with Coomassie blue. The concentrations of gels were 15% (A) and 12% (B), respectively. Both peptides of $M_r$ 41000 and 39000 in (B) were substrates for IAP.

In particular, the peptide of $M_r$ 39000, the $\alpha$-subunit of the GTP-binding protein called $N_o$ [23], is a major IAP substrate in brain but is not found in liver. The $N_i\alpha$ preparation used here contained both $\alpha$-subunits of $M_r$ 41000 and 39000 (fig.2B).

Fig.3 shows the inhibitory action of purified $\beta\gamma$ on the forskolin-activated catalytic unit which is free of $N_s$ and $N_i\alpha$. Maximum inhibition was achieved with 1 $\mu$g $\beta\gamma$. No guanine nucleotides were required for inhibition. Similar amounts of $N_i\alpha$ failed to inhibit the cyclase activity. Because no $N_s$ and guanine nucleotides were included in the reconstituted system, we conclude that the inhibition is a result of the direct interaction of the $\beta\gamma$-subunits and the catalytic unit. We obtained a similar curve for the $\beta\gamma$-dependent inhibition by using the catalytic unit which had been activated by $N_s$. Maximum suppression was observed in the presence of about 1 $\mu$g $\beta\gamma$. We suppose that the binding of GTP to $N_i$ liberates $\beta\gamma$ which subsequently inhibit the catalytic unit directly. Therefore, once $\beta\gamma$ are liberated, GTP will no longer be necessary for inhibition of the catalytic unit.

Table 1 shows the $\beta\gamma$-dependent inhibition of the catalytic unit which was activated in the

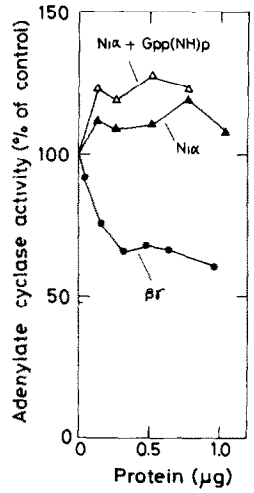


Fig.3. Inhibition of the forskolin-activated catalytic unit by the purified $\beta\gamma$-subunits. The $\beta\gamma$-subunits (●) or $N_i\alpha$ in the presence (△) or absence (▲) of 60 $\mu$M Gpp(NH)p were preincubated with the catalytic unit of adenylate cyclase and then assayed for adenylate cyclase in the presence of 10 $\mu$M forskolin. Adenylate cyclase activity in the absence of $\beta\gamma$ and $N_i\alpha$ was 180 pmol cyclic AMP/10 min.

Table 1

Inhibition of $N_s$-activated catalytic unit by the purified $\beta\gamma$-subunits

| $N_s$ ($\mu$g) | $\beta\gamma$ ($\mu$g) | Adenylate cyclase activity (pmol AMP/10 min) | Inhibition $\Delta$(pmol cyclic AMP/10 min) | (%) |
|---|---|---|---|---|
| 0.11 | 0 | 188 | | |
| 0.11 | 1.06 | 76 | 112 | 59.6 |
| 0.43 | 0 | 225 | | |
| 0.43 | 1.06 | 128 | 97 | 43.1 |
| 1.28 | 0 | 289 | | |
| 1.28 | 1.06 | 181 | 108 | 37.4 |

Catalytic unit was preactivated for 3 h at 23°C in the presence of $N_s$ and 60 $\mu$M Gpp(NH)p before the addition of the purified $\beta\gamma$-subunits. Adenylate cyclase activity was assayed in the absence of forskolin. Adenylate cyclase activity in the absence of $N_s$ and $\beta\gamma$ was 21 pmol cyclic AMP/10 min. The activation of the catalytic unit nearly reached a plateau at 0.6 $\mu$g $N_s$ in this system

presence of Gpp(NH)p and different amounts of $N_s$. The net decrease in adenylate cyclase activity by $\beta\gamma$ seemed to be constant and independent of the amount of $N_s$, but the relative inhibition by $\beta\gamma$ was significantly reduced with increasing amounts of $N_s$. A possible explanation for the result obtained is that $\beta\gamma$ deactivated $N_s$ and thus reduced the cyclase activity [12]. However, this is less likely to occur in the presence of sufficient $MgCl_2$. Another possibility is the existence of two forms of the catalytic unit; one susceptible to direct inhibition by $\beta\gamma$ and the other not. If the catalytic unit sensitive to $\beta\gamma$ has higher affinity for $N_s$ than the other form of the catalytic unit, the constant net decrease in adenylate cyclase activity by $\beta\gamma$ seems to be explained without difficulty.

As shown in fig.4, a complex of $N_i\alpha$ and $\beta\gamma$ is a much more effective substrate for IAP than $N_i\alpha$ alone. At the plateau, 4 pmol ADP-ribosyl residue was incorporated into $N_i\alpha$, which means that about 50% of $N_i\alpha$ used for the reaction was ADP-ribosylated. Very low efficiency of the ADP-ribosylation may be partly due to insufficient formation of $\alpha\beta\gamma$ complex and denaturation of $N_i\alpha$ during the reaction. GTPase activity of $N_i\alpha$ was also increased 3.8-fold by $\beta\gamma$ (fig.4). The $\beta\gamma$-subunits probably bind to the $N_i\alpha$-GDP complex

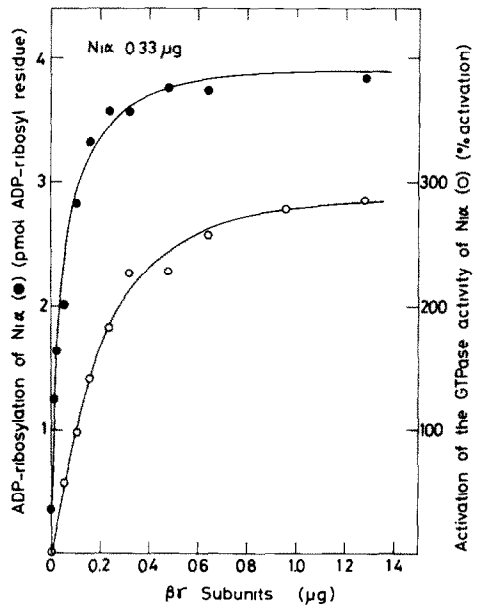


Fig.4. Stimulation of IAP-catalyzed ADP-ribosylation and the activation of GTPase activity of $N_i\alpha$ by the purified $\beta\gamma$-subunits. $N_i\alpha$ was preincubated with purified $\beta\gamma$ before ADP-ribosylation by IAP (●) and the assay of GTPase activity (○). ADP-ribosylation was conducted as described in section 2, except that 0.33 $\mu$g/tube (1.5 pmol $P_i$ released/min per tube) of $N_i\alpha$ was used and the reaction continued for 60 min. GTPase activity in the absence of $\beta\gamma$ was 13 pmol $P_i$ released/10 min.

and accelerate the exchange of GDP for GTP at the guanine nucleotide binding site on $N_i\alpha$, which results in an apparent increase of the turnover rate of GTPase activity.

ADP-ribosylation of $N_i\alpha$ did not affect its GTPase activity (fig.5), which confirmed our previous observation [7]. The result apparently conflicts with reports that IAP attenuated GTPase activity of $N_i$ enhanced by the stimulation of inhibitory receptors [24–26]. However, the result suggests that ADP-ribosylation by IAP only prevented $N_i$ from interacting with the receptors without affecting the binding and hydrolysis of GTP.

Here, we showed that the $\beta\gamma$-subunits of the GTP-binding regulatory proteins directly inhibited the catalytic unit of adenylate cyclase. $N_s$ and guanine nucleotides were not necessary for the inhibition. During the preparation of this manuscript, Smigel [27] reported similar inhibition

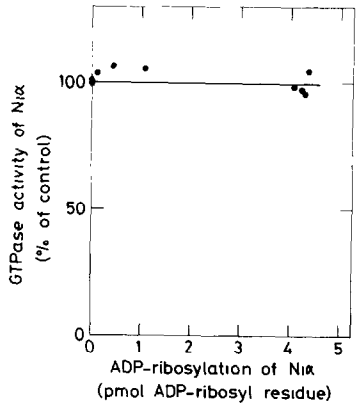


Fig.5. Lack of effect of ADP-ribosylation on the GTPase activity of $N_i\alpha$. $N_i\alpha$ (0.33 $\mu$g protein) and $\beta\gamma$ (0.48 $\mu$g protein) were preincubated and ADP-ribosylated by various concentrations (0–2.5 $\mu$g/ml) of IAP as in fig.4. $N_i$ was precipitated with ammonium sulfate as described in section 2 and resolubilized in 10 $\mu$l buffer D. After incubation at 23°C for 10 min, GTPase activity was assayed. The GTPase activity of $N_i\alpha$ treated in the absence of IAP was 25 pmol $P_i$ released/10 min.

of the purified catalytic unit by $\beta\gamma$, but he considered that the inhibition was due to deactivation of $N_s$ that was present in the catalytic unit preparation. The direct inhibition by $\beta\gamma$ seems to explain, at least partly, $N_i$-mediated inhibition of the cyclase in S49 $cyc^-$ cell membranes which lack functional $N_s$ [13,25]. Although $N_i\alpha$ but not $\beta\gamma$ was reported to inhibit adenylate cyclase in $cyc^-$ membranes [28], $N_i\alpha$ failed to inhibit the catalytic unit in the present study and in [27]. We believe that direct inhibition of the catalytic unit by $\beta\gamma$ is one of the mechanisms of the receptor-mediated inhibition of adenylate cyclase.

## ACKNOWLEDGEMENTS

We are very grateful to Dr Michio Ui for providing us with islet-activating protein. This work was partly supported by research grants (60770210 and 60226016) from the Ministry of Education, Science and Culture of Japan.

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