

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 α -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 α , β and γ [2,3]. The α -subunits (GTP-binding subunits) from the two GTP-binding proteins are clearly different but the β -subunits are indistinguishable [4]. Binding of GTP analogues or F^- to their α -subunits appears to dissociate the proteins into α - and $\beta\gamma$ -subunits [5,6]. The α -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 α -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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$N_{i\alpha}$ [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

$[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and $[\text{}^{32}\text{P}]\text{NAD}$ 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 μM 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 $\times$ 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 μM 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 $\times$ 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 $\times$ 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 $\times$ 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 $\times$ 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 μl and subjected to TSK G3000SW gel chromatography (0.8 $\times$ 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 μ 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 μ l buffer D were preincubated for 30 min at 23°C and then mixed with 16.6 μ 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 μ M [^{32}P]NAD (5–10 Ci/mmol), 2 mM DTT and 2.5 μ g/ml IAP. The reaction conducted at 25°C for 30 min was stopped by the addition of 250 μ 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 μ 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 μ 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_i 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.

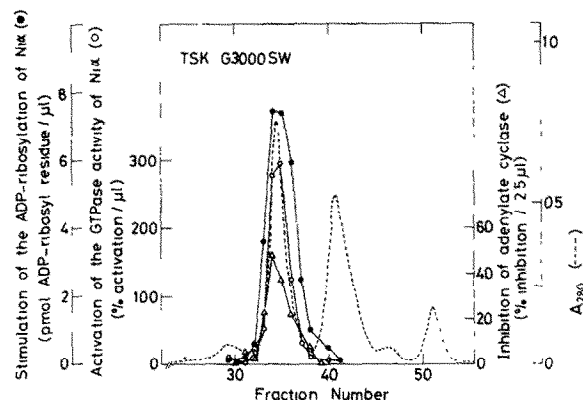


Fig.1. Purification of the $\beta\gamma$ -subunits by G3000SW gel chromatography. The cholate extract (530 mg protein) from a synaptosome-rich membrane fraction was subjected to successive chromatography steps on DEAE-Sephacel, TSK DEAE-5PW and heptylamine-Sepharose. Fractions containing $\beta\gamma$ (0.51 mg protein) were then applied to a TSK G3000SW column (0.8 $\times$ 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 $N_i\alpha$ ($\bullet$), activation of the GTPase of $N_i\alpha$ ($\circ$) and inhibition of the forskolin-activated catalytic unit of adenylate cyclase (Δ). (---) Absorbance at 280 nm. The purified $\beta\gamma$ -subunits (0.1 μ 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 β (M_r 35000) and γ (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 β . Two similar β -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].

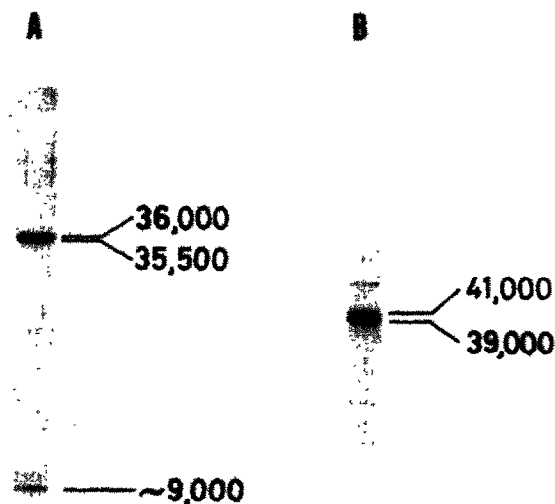


Fig.2. Polyacrylamide gel electrophoresis of the purified $\beta\gamma$ -subunits and $N_{i\alpha}$. The purified $\beta\gamma$ -subunits (6 μg) (A) and $N_{i\alpha}$ (3 μg) (B) were subjected to gel electrophoresis in the presence of SDS. Protein was stained with Coomassie blue. The concentrations of 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 α -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 α -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 μg $\beta\gamma$. 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 μg $\beta\gamma$. 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

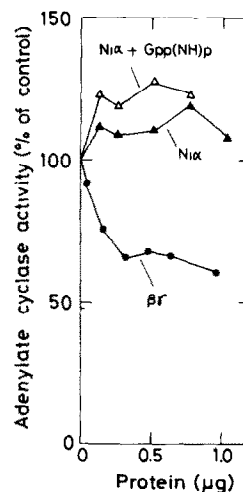


Fig.3. Inhibition of the forskolin-activated catalytic unit by the purified $\beta\gamma$ -subunits. The $\beta\gamma$ -subunits (●) or $N_{i\alpha}$ in the presence (Δ) or absence (▲) of 60 μM Gpp(NH)p were preincubated with the catalytic unit of adenylate cyclase and then assayed for adenylate cyclase in the presence of 10 μM 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 (μg)	$\beta\gamma$ (μg)	Adenylate cyclase activ- ity (pmol AMP/10 min)	Inhibition Δ (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 μM 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 μg 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

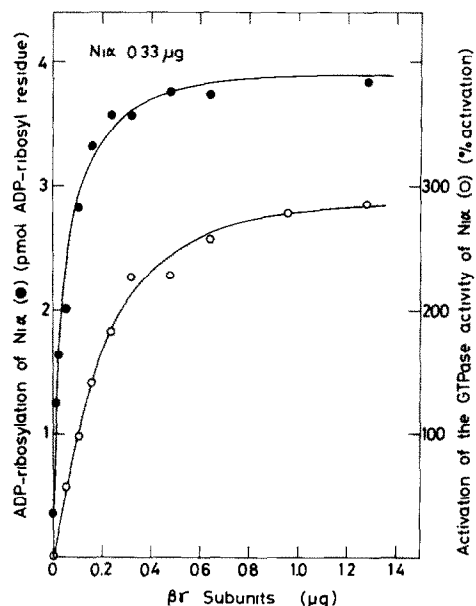


Fig.4. Stimulation of IAP-catalyzed ADP-ribosylation and the activation of GTPase activity of $N_i\alpha$ by the purified $\beta\gamma$ -subunits. $N_i\alpha$ was preincubated with purified $\beta\gamma$ before ADP-ribosylation by IAP (●) and the assay of GTPase activity (○). ADP-ribosylation was conducted as described in section 2, except that 0.33 μg /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

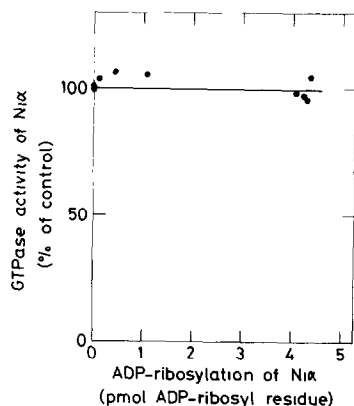


Fig.5. Lack of effect of ADP-ribosylation on the GTPase activity of N₁α. N₁α (0.33 μg protein) and βγ (0.48 μg protein) were preincubated and ADP-ribosylated by various concentrations (0–2.5 μg/ml) of IAP as in fig.4. N₁ was precipitated with ammonium sulfate as described in section 2 and resolubilized in 10 μl buffer D. After incubation at 23°C for 10 min, GTPase activity was assayed. The GTPase activity of N₁α treated in the absence of IAP was 25 pmol P_i released/10 min.

of the purified catalytic unit by βγ, 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 βγ 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_iα but not βγ was reported to inhibit adenylate cyclase in cyc⁻ membranes [28], N_iα failed to inhibit the catalytic unit in the present study and in [27]. We believe that direct inhibition of the catalytic unit by βγ 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.

REFERENCES

- [1] Rodbell, M. (1980) *Nature* 284, 17–22.
- [2] Gilman, A.G. (1984) *Cell* 36, 577–579.
- [3] Bokoch, G.M., Katada, T., Northup, J.K., Ui, M. and Gilman, A.G. (1984) *J. Biol. Chem.* 259, 3560–3567.

- [4] Manning, D.R. and Gilman, A.G. (1983) *J. Biol. Chem.* 258, 7059–7063.
- [5] Northup, J.K., Smigel, M.D., Sternweis, P.C. and Gilman, A.G. (1983) *J. Biol. Chem.* 258, 11369–11376.
- [6] Katada, T., Bokoch, G.M., Northup, J.K., Ui, M. and Gilman, A.G. (1984) *J. Biol. Chem.* 259, 3568–3577.
- [7] Enomoto, K. and Asakawa, T. (1984) *FEBS Lett.* 171, 233–239.
- [8] Neer, E.J., Lok, J.M. and Wolf, L.G. (1984) *J. Biol. Chem.* 259, 14222–14229.
- [9] Sunyer, T., Codina, J. and Birnbaumer, L. (1984) *J. Biol. Chem.* 259, 15447–15451.
- [10] Milligan, G. and Klee, W.A. (1985) *J. Biol. Chem.* 260, 2057–2063.
- [11] Katada, T. and Ui, M. (1982) *Proc. Natl. Acad. Sci. USA* 79, 3129–3133.
- [12] Katada, T., Northup, J.K., Bokoch, G.M., Ui, M. and Gilman, A.G. (1984) *J. Biol. Chem.* 259, 3578–3585.
- [13] Hildebrandt, J.D., Codina, J. and Birnbaumer, L. (1984) *J. Biol. Chem.* 259, 13178–13185.
- [14] Enomoto, K. and Asakawa, T. (1983) *Biochem. Int.* 6, 81–91.
- [15] Enomoto, K. and Asakawa, T. (1985) *Jap. J. Pharmacol.* 39, suppl. 110P.
- [16] Shaltiel, S. (1974) *Methods Enzymol.* 34, 126–140.
- [17] Strittmatter, S. and Neer, E.J. (1980) *Proc. Natl. Acad. Sci. USA* 77, 6344–6348.
- [18] Enomoto, K. and Asakawa, T. (1982) *Biomed. Res.* 3, 122–131.
- [19] Schaffner, W. and Weissmann, C. (1973) *Anal. Biochem.* 56, 502–514.
- [20] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [21] Northup, J.K., Sternweis, P.C. and Gilman, A.G. (1983) *J. Biol. Chem.* 258, 11361–11368.
- [22] Tsai, S.-C., Adamik, R., Kanaho, Y., Hewlett, E.L. and Moss, J. (1984) *J. Biol. Chem.* 259, 15320–15323.
- [23] Sternweis, P.C. and Robishaw, J.D. (1984) *J. Biol. Chem.* 259, 13806–13813.
- [24] Aktories, K., Schultz, G. and Jakobs, K.H. (1983) *FEBS Lett.* 156, 88–92.
- [25] Aktories, K., Schultz, G. and Jakobs, K.H. (1983) *FEBS Lett.* 158, 169–173.
- [26] Burns, D.L., Hewlett, E.L., Moss, J. and Vaughan, M. (1983) *J. Biol. Chem.* 258, 1435–1438.
- [27] Smigel, M.D. (1986) *J. Biol. Chem.* 261, 1976–1982.
- [28] Katada, T., Bokoch, G.M., Smigel, M.D., Ui, M. and Gilman, A.G. (1984) *J. Biol. Chem.* 259, 3586–3595.