

Guanine nucleotide dependent and independent reconstitution of G-proteins with adenylate cyclase: stimulation or attenuation of the enzyme by Gi α subunits

Dianne L. Newton and Werner A. Klee

Laboratory of Molecular Biology, National Institute of Mental Health, Bethesda, MD 20892, USA

Received 7 August 1990

The α subunits of five members of the Gi family of bovine brain proteins were reconstituted with adenylate cyclase in phospholipid vesicles. Our results support both the views that much of the inhibition of the enzyme by Gi is due to the action of its liberated $\beta\gamma$ subunits, and that the α subunits themselves interact with the enzyme. Inhibition of basal or Gs-stimulated adenylate cyclase activity is small or undetectable by α subunits of Go, Go* and Gi-2B. On the other hand, adenylate cyclase activity is stimulated by the α subunits of Gi-1 and Gi 2A. The G proteins act in the absence of added GTP when reconstituted with phospholipids of low but not high fluidity.

Adenylate cyclase; G protein; Reconstitution

1. INTRODUCTION

Stimulation of adenylate cyclase is caused by hormone receptor activation of G_s, and inhibition is mediated by one or more species of G_i. Upon binding GTP, the α subunit becomes activated, dissociates from the $\beta\gamma$ subunits, and in the case of G_s, directly stimulates the catalytic moiety of adenylate cyclase. A likely mechanism for inhibition is that the $\beta\gamma$ subunits, released upon activation of G_i, reverse G_s activation by regenerating the heterotrimeric protein [1–6]. An alternative mechanism postulates a direct interaction between the α subunits of G_i and the enzyme. Thus, the adenylate cyclase activity of S49 cyc[−] cells, a cell line lacking G_s, can be inhibited by guanine nucleotides, NaF or somatostatin, suggesting that the activated α subunit of a G_i may inhibit the enzyme [7–10]. Furthermore, the GTP γ S-activated α subunits of Gi-1 [11] and of transducin [12] inhibit G_s-activated adenylate cyclase.

Correspondence address: D.L. Newton, Laboratory of Molecular Biology, National Institute of Mental Health, Bethesda, MD 20892, USA

Abbreviations: G-protein, guanine nucleotide-binding protein; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate); CHAPSO, 3-(3-cholamidopropyl)dimethylammonio)-1-(2-hydroxy-1-propanesulfonate); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β -aminoethyl ether)-N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); GppNHp, guanosine 5'-(β , γ -imido)triphosphate

Here, we report on the abilities of the α subunits of five G_i proteins to interact with adenylate cyclase in a reconstituted system. None was able to inhibit adenylate cyclase activity by more than 15%. However, Gi-1 α and Gi-2A α , stimulated adenylate cyclase to the same activity level as did G_s. The G-proteins stimulate or inhibit cyclase activity in a GTP-independent manner in liposomes of C₁₆–C₁₈ lipids but require GTP in C₁₀–C₁₄ lipids.

2. MATERIALS AND METHODS

The G proteins, Go*, Gi-1, Go, Gi-2A and Gi-2B, were purified from bovine brain [13] and separated from one another by chromatography on Mono-Q columns ([14] and Newton and Klee, manuscript in preparation). The α subunits of the G-proteins, were prepared by chromatography on Blue Sepharose or octyl-Sepharose (Newton and Klee, manuscript in preparation). The holo G-proteins were quantitated by the method of Lowry et al. [15]; the α subunits by the dinitrofluorobenzene method [16]. The G_s was prepared as described [17] except that AMF was not included and octyl-Sepharose chromatography was substituted for the heptylamine-Sepharose chromatography. Wheat germ agglutinin was purchased from E-Y Laboratories. Calmodulin was prepared according to [18] and calmodulin-Sepharose according to [19]. The forskolin affinity column was prepared by the method of Pfeuffer et al. [20]. [³H]cAMP, [α -³²P]ATP and 2,4-dinitrofluoro[3,5-³H]benzene were from New England Nuclear. GppNHp and GTP γ S were obtained from Boehringer Mannheim. Forskolin, CHAPS, CHAPSO, and sodium cholate were from Calbiochem. Cholesterol and polyoxyethylene 9-lauryl ether (lubrol) from Sigma.

2.1. Preparation of adenylate cyclase

All operations were at 2–4°C. Four frozen rat brains (Pelfreeze) were homogenized in 80 ml buffer A (10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 5 mM MgCl₂, 1 μ M leupeptin, 10 μ M soybean trypsin inhibitor and 0.1 mM phenylmethylsulfonyl fluoride). The

homogenate, adjusted to 240 ml with buffer A, was centrifuged for 30 min at 40000 rpm. The pellet was resuspended in 52 ml buffer B (buffer A brought to 1 mM MgCl_2 and 0.09% lubrol) with a ground glass homogenizer and centrifuged for 30 min at 40000 rpm. The supernatant solution was stirred with 12 ml wheat germ agglutinin agarose (equilibrated with buffer B) for 40 min. The agarose was washed with 24 ml of buffer B followed by 36 ml of buffer C (10 mM Tris-HCl, pH 7.5, 1 μM leupeptin, 10 μM soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM CHAPS). The enzyme was eluted with 27 ml 0.5 M *N*-acetylglucosamine in buffer C. The eluate was applied either to calmodulin-Sepharose (Procedure A) or forskolin-Affigel (Procedure B).

(A) For chromatography on calmodulin-Sepharose, the eluate was adjusted to contain 2 mM CaCl_2 and 3 mM MgCl_2 before application at 0.5 ml/min to 2 ml calmodulin-Sepharose in buffer D (40 mM Tris-HCl, pH 7.5 containing 0.2 M NaCl, 3 mM MgCl_2 , 0.2 mM CaCl_2 , 0.1 mM dithiothreitol, 1 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ soybean trypsin inhibitor and 1 mM CHAPS). The column was washed at 2 ml/min with 12 ml each of buffer D, buffer D containing 0.4 M NaCl, and buffer D containing 0.05 M NaCl before elution with 10 ml buffer E (40 mM Tris-HCl, pH 7.5 containing 0.05 M NaCl, 1 mM MgCl_2 , 2 mM EGTA, 0.1 mM dithiothreitol, 1 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ soybean trypsin inhibitor and 1 mM CHAPS). The eluate was concentrated by passage over a wheat germ agglutinin column (0.5 ml), equilibrated with buffer F (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM MgCl_2 , 1 mM EGTA, 0.5 $\mu\text{g/ml}$ leupeptin, 0.1 mg/ml soybean trypsin inhibitor, 1 mM CHAPS, 0.1 mM phenylmethylsulfonyl fluoride), washed with 5 ml buffer F and eluted with 2 ml 0.5 M *N*-acetylglucosamine in buffer F. The activity ranged from 1.8 to 4.3 nmol/min per ml when assayed in the presence of 10 μM GppNHP, 100 μM forskolin and 5 mM MnCl_2 .

(B) For chromatography on forskolin-Affigel, the eluate (27 ml) was concentrated to 3 ml by ultrafiltration through an Amicon PM-30 membrane. The concentrate was applied to 3.5 ml forskolin-Affigel in buffer F. After 30 min, the gel was washed successively with 20 ml each of buffer F, buffer F made 1 mM NaCl and buffer F again. The column was eluted with 15 ml of 50 μM forskolin in buffer F. Forskolin was removed by adsorption of the eluate to 0.2 ml of wheat germ agglutinin agarose in buffer F. The resin was washed with 2 ml of buffer F before elution with 0.8 ml 0.5 M *N*-acetylglucosamine in buffer F. Activity ranged between 0.3 to 0.8 nmol/min per ml enzyme solution in the presence of 10 μM GppNHP, 100 μM forskolin and 5 mM MnCl_2 . After either procedure A or B, the enzyme was stored as 50–100 μl aliquots in liquid N_2 and was free of detectable amounts of G proteins.

Lipids were suspended at 10 mg/ml in 10 mM Tris-HCl, pH 7.5 immediately before use and were sonicated to clarity under N_2 . In experiments with cholesterol, 167 μl of cholesterol (10 mg/ml in $\text{CH}_3\text{Cl}/\text{MeOH}$, 4:1) was added to 0.5 ml of lipid (10 mg/ml in $\text{CH}_3\text{Cl}/\text{MeOH}$, 4:1). This mixture was dried in a rotary evaporator and lyophilized overnight before addition of 0.5 ml 10 mM Tris-HCl, pH 7.5 and sonication. Asolectin (95% purified soy phosphatides) was obtained from Associated Concentrates, Woodside, NY, and each of the synthetic phosphatidylcholines, with saturated fatty acids of chain length 10–18, were obtained from Calbiochem.

2.2. Reconstitution

Adenylate cyclase and G proteins were added to buffer G (40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl_2 , 2 mM EDTA and 0.1 mM DTT) containing 100 $\mu\text{g/ml}$ ovalbumin and 400 $\mu\text{g/ml}$ lipid. The type of lipid and amounts of proteins are indicated in the figure legends. The mixtures (0.6 ml) were dialyzed for 5 h at 2–4°C against 1 l of buffer G which was changed after 2 h. Aliquots (80 μl) were assayed in quintuplicate for adenylate cyclase activity after the addition of 20 μl of 450 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1.9 mg/ml ovalbumin, 1.0 mM [α - ^{32}P]ATP (2 μCi), 30 mM MgCl_2 , 40 nM [^3H]cAMP (16000 cpm) and 50 μM GppNHP. After 60 min at 30°C, assays were terminated by the addition of 1 ml of 5% trichloroacetic acid containing 1 mM cAMP and 1 mM ATP. [^{32}P]cAMP was assayed as described [21].

3. RESULTS AND DISCUSSION

When adenylate cyclase was reconstituted into asolectin in the absence of G-proteins, activity was unaffected by either $\text{GTP}\gamma\text{S}$ or AlF_4^- (Fig. 1). G_s stimulated activity, and AlF_4^- but not $\text{GTP}\gamma\text{S}$ stimulated further. A mixture of the purified holo-G-proteins was without effect when added alone, but inhibited G_s stimulation both in the presence and absence of guanine nucleotides. When pure phospholipids with fatty acid moieties of 10 to 14 carbons in length are used in place of asolectin, guanine nucleotides do stimulate G_s activity. An increase in chain length of the lipid to 16 or 18 results in the loss of GppNHP stimulation (Fig. 2). A guanine nucleotide augmentation of inhibition by G_o/G_i is also seen in the presence of dimyristoyl phosphatidylcholine (C_{14}) (Fig. 3). Although G_i and G_s alone can modulate adenylate cyclase in the presence of short chain phospholipids (C_{14}), much larger effects are observed when GppNHP is added. Thus, in lipids of low fluidity, such as C_{16} , C_{18} , or asolectin, G proteins exert their effects in the absence of added guanine nucleotides. Liposomes of higher fluidity allow the expected nucleotide requirement to be observed. The addition of cholesterol, which is believed to decrease the fluidity of the C_{10} – C_{14} lecithins and increase that of dipalmitoyl phosphatidylcholine (C_{16}) [22,23] was found to decrease GppNHP stimulated activity with the short chain lipids and not affect activity with C_{16} (Fig. 4). Thus, the guanine nucleoside triphosphate sensitive activity of G-proteins may require a fluid membrane environment, whereas the activity of GDP-complexed

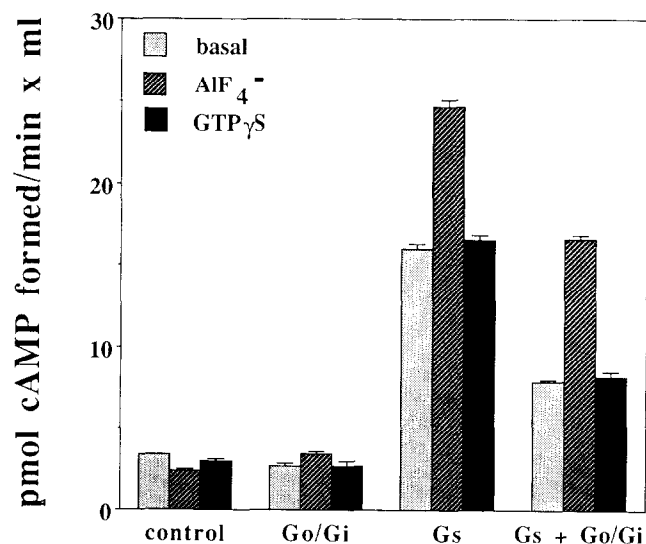


Fig. 1. Reconstitution of adenylate cyclase with G_s and/or G_o/G_i in asolectin vesicles. Adenylate cyclase (forskolin affinity purified) was reconstituted with G_s or a G_o/G_i mixture (0.9 nM) or both. When indicated, the assay was conducted in the presence of 10 mM NaF plus 10 μM AlCl_3 or 10 μM $\text{GTP}\gamma\text{S}$.

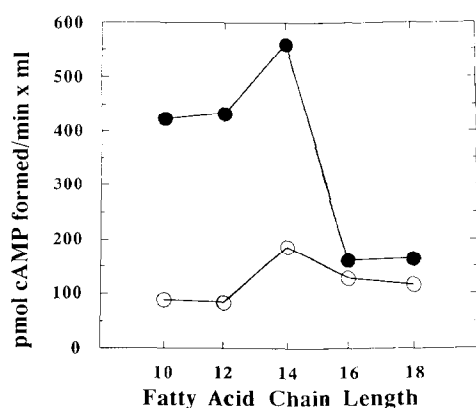


Fig. 2. The effect of the fatty acid chain length of phosphatidylcholines on the stimulation of adenylate cyclase by G_s and GppNHp. Calmodulin Sepharose purified adenylate cyclase was reconstituted with G_s and assayed in the presence (closed symbols) or absence (open symbols) of 10 μ M GppNHp.

G-proteins may be optimal with lipids of lower fluidity. Among the possible reasons for an effect of lipid viscosity on guanine nucleotide sensitive reconstitution are diffusional barriers raised against GDP dissociation. The GDP liganded state of the G-proteins may have properties similar to those of the GTP state if the environment is suitable.

When reconstituted in dimyristoylphosphatidylcholine, the α subunits of G_{o^*} and G_{i-2A} had no effect on G_s stimulated activity, whereas the α subunits of G_o and G_{i-2B} inhibited slightly (Fig. 5). This inhibition could well be due to a direct effect of these α subunits on the catalyst because the same low level of inhibition was observed when activity was assayed in the absence of G_s . In contrast, the α subunits of G_{i-1} and G_{i-2A} were found to stimulate adenylate cyclase activity to levels comparable to those achieved by G_s . It is unlikely that the stimulation of adenylate cyclase caused by the

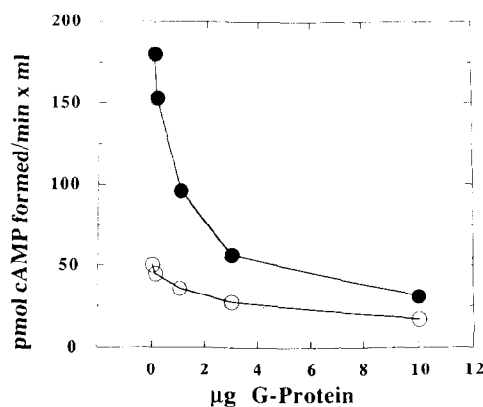


Fig. 3. The effect of G_o/G_i on G_s stimulated adenylate cyclase in dimyristoylphosphatidyl choline. Forskolin affinity purified cyclase was reconstituted with G_s and the indicated concentrations of G_o/G_i , and assayed in the presence (closed symbols) or absence (open symbols) of 10 μ M GppNHp.

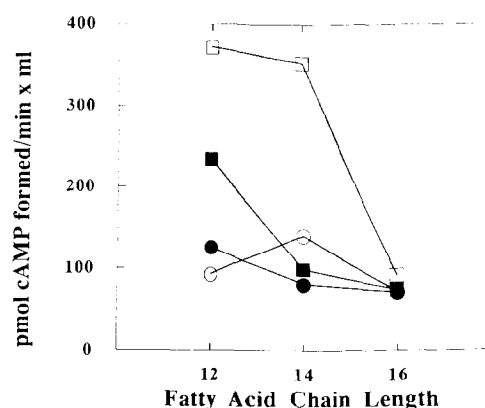


Fig. 4. The effect of cholesterol on the reconstitution of G_s with adenylate cyclase. Calmodulin-Sepharose purified adenylate cyclase was reconstituted with G_s in the presence of the indicated lipids with (closed symbols) or without (open symbols) cholesterol. Adenylate cyclase activity was measured in the presence ($\square$) or absence ($\circ$) of 10 μ M GppNHp.

α subunits of G_{i-1} and G_{i-2A} is due to contamination of these α subunits by G_s because: (1) G_s is well separated from all of the G_i proteins on octyl-Sepharose, the last step in their purification from brain membranes. (2) The G_o/G_i mixture is resolved into four proteins by chromatography on a Mono Q column. Each of these was always rechromatographed. The G-proteins which chromatograph on either side of G_{i-1} (G_{o^*} and G_o) and G_{i-2A} (G_o and G_{i-2B}) do not stimulate adenylate cyclase activity. (3) The α subunits of G_{i-1} and G_{i-2A} were prepared by different methods: octyl-Sepharose chromatography for the α subunit of G_{i-1} and Blue Sepharose chromatography for G_{i-2A} . Thus, any remaining G_s contaminant of G_{i-1} should have been efficiently removed. It is also unlikely that the stimulation is due to removal of the $\beta\gamma$ subunits

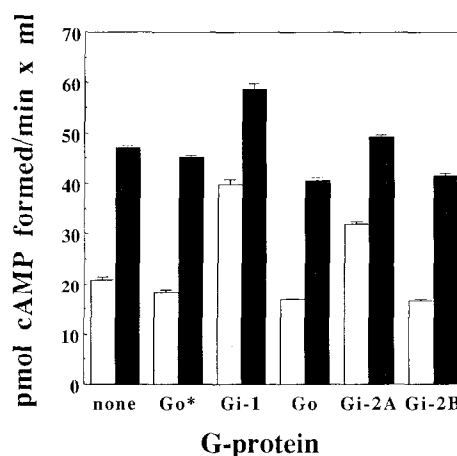


Fig. 5. The effects of α subunits of G_{o^*} , G_{i-1} , G_o , G_{i-2A} and G_{i-2B} on basal and G_s -stimulated adenylate cyclase activity. Calmodulin-Sepharose purified cyclase was reconstituted with the α subunits of G_{o^*} , G_{i-1} , G_o , G_{i-2A} or G_{i-2B} (180 nM) with (solid bars) or without (stippled bars) G_s in dimyristoylphosphatidylcholine. Adenylate cyclase activity was measured in the presence of 10 μ M GppNHp.

from the available pool because our experiments were performed in 10 μ M GppNHp and 5 mM Mg^{2+} , conditions known to cause dissociation of $\alpha\beta\gamma$ heterotrimers [24] and any such activity would be shared by all of the α subunits and not restricted to two of them.

Current views of the mechanism of inhibition of adenylate cyclase by G_i hold that much or all of such inhibition is due to the reassociation of $\beta\gamma$ subunits with the α subunits [1–6]. However, there is evidence that the α subunit of the inhibitory G-proteins may directly interact with the catalyst [7–12,14]. The data we have presented add to this evidence. When we reconstituted adenylate cyclase with the α subunits of the individual G_i proteins, we found that some of the α subunits inhibited activity by a small amount and that G_{i-1} α and G_{i-2A} α subunits stimulated adenylate cyclase activity. Our results with G_{i-1} α are in contrast to those of Katada et al. [11,14] which showed the protein to inhibit the enzyme in pertussis toxin treated membranes and in detergent solutions. Using recombinant, and therefore unmyristoylated α subunits, Linder et al. [25] found no effects on adenylate cyclase in detergent solutions. The fact that G_{i-1} α can have stimulatory or inhibitory activity depending on the assay system suggests that this, and perhaps other, G-proteins may behave as partial agonists. Thus, depending upon the extent to which the enzyme is activated by G_s in the assay system used, inhibition or stimulation may be seen. Although we have not been able to accurately measure the amounts of G_s used in our experiments, we estimate that they are at least two orders of magnitude lower than those of the G_i proteins used. Our experiments support the prevailing view that inhibition results primarily from $\beta\gamma$ subunits reassociating with α_s . Because G_i proteins are much more abundant than G_s in cells, direct interactions of the α subunits of G_i may also contribute to the regulation of adenylate cyclase activity.

Acknowledgement: We thank Richard A. Sreaty for excellent technical assistance.

REFERENCES

- [1] Northup, J.K., Sternweis, P.C. and Gilman, A.G. (1983) *J. Biol. Chem.* 258, 11361–11368.
- [2] Gilman, A.G. (1984) *Cell* 36, 577–579.
- [3] Katada, T., Bokoch, G.M., Northup, J.K., Ui, M. and Gilman, A.G. (1984) *J. Biol. Chem.* 259, 3568–3577.
- [4] Bockaert, J., Deterre, P., Pfister, C., Guillon, G. and Chabre, M. (1985) *EMBO J.* 4, 1413–1417.
- [5] Smigel, M.D. (1986) *J. Biol. Chem.* 261, 1976–1982.
- [6] Cerione, R.A., Gierschik, P., Staniszewski, C., Benovic, J.L., Codina, J., Somers, R., Birnbaumer, L., Spiegel, A.M., Lefkowitz, R.J. and Caron, M.G. (1987) *Biochemistry* 26, 1485–1491.
- [7] Hildebrandt, J.D., Hanoune, J. and Birnbaumer, L. (1982) *J. Biol. Chem.* 257, 14723–14725.
- [8] Jakobs, K.H. and Schultz, G. (1983) *Proc. Natl. Acad. Sci. USA* 80, 3899–3902.
- [9] Katada, T., Bokoch, G.M., Smigel, M.D., Ui, M. and Gilman, A.G. (1984) *J. Biol. Chem.* 259, 3586–3595.
- [10] Roof, D.J., Applebury, M.L. and Sternweis, P.C. (1985) *J. Biol. Chem.* 260, 16242–16249.
- [11] Katada, T., Oinuma, M. and Ui, M. (1986) *J. Biol. Chem.* 261, 5215–5221.
- [12] Cerione, R.A., Staniszewski, C., Gierschik, P., Codina, J., Somers, R.L., Birnbaumer, L., Spiegel, A.M., Caron, M.G. and Lefkowitz, R.J. (1986) *J. Biol. Chem.* 261, 9514–9520.
- [13] Milligan, G. and Klee, W.A. (1985) *J. Biol. Chem.* 260, 2057–2063.
- [14] Katada, T., Oinuma, M., Kusakabe, K. and Ui, M. (1987) *FEBS Lett.* 213, 353–358.
- [15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275.
- [16] Nishio, K. and Kawakami, M. (1982) *Anal. Biochem.* 126, 239–241.
- [17] Sternweis, P.C., Northup, J.K., Smigel, M.D. and Gilman, A.G. (1981) *J. Biol. Chem.* 256, 11517–11526.
- [18] Newton, D.L., Burke, Jr., T.R., Rice, K.C. and Klee, C.B. (1987) *Methods Enzymol.* 139, 405–417.
- [19] Klee, C.B. and Krinks, M.H. (1978) *Biochemistry* 17, 120–126.
- [20] Pfeuffer, E., Dreher, R.-M., Metzger, H. and Pfeuffer, T. (1985) *Proc. Natl. Acad. Sci. USA* 82, 3086–3090.
- [21] Salomon, Y., Londos, C. and Rodbell, M. (1974) *Anal. Biochem.* 58, 541–548.
- [22] Chapman, D. (1983) *Membr. Fluidity Biol.* 2, 5–42.
- [23] Lee, A.G. (1983) *Membr. Fluidity Biol.* 2, 43–88.
- [24] Codina, J., Hildebrandt, J.D., Birnbaumer, L. and Sekura, R.D. (1984) *J. Biol. Chem.* 259, 11408–11418.
- [25] Linder, M.E., Ewald, D.A., Miller, R.J. and Gilman, A.G. (1990) *J. Biol. Chem.* 265, 8243–8251.