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Effects of lovastatin on adenylyl cyclase activity and G proteins in GH_4C_1 cells

Antonio Chiloeches[a], Marina Lasa[a], Fuensanta Brihuega[a], Agustín Montes[b], María J. Toro[a,*]

[a]*Department of Biochemistry and Molecular Biology, University of Alcalá, Crta. Madrid-Barcelona Km. 33,600, 28871 Alcalá de Henares, Madrid, Spain*
[b]*Department of Physiology and Pharmacology, University of Alcalá, Crta. Madrid-Barcelona Km. 33,600, 28871 Alcalá de Henares, Madrid, Spain*

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Abstract **We studied the effect of lovastatin, a cholesterol lowering drug, on the basal state of G-proteins in GH_4C_1 cells. Our data show that the addition of lovastatin markedly decreased the amount of the α-subunits of the G_s and G_i-proteins in the plasma membrane. The decrease of αs was correlated with a decrease in adenylyl cyclase activity, and both effects were reverted by the presence of mevalonate. As the attachment of G protein subunits to the membrane is dependent on γ-subunit prenylation, we assume that the mechanism through which lovastatin exerts its effects on G-proteins is the lack of mevalonate for the synthesis of prenyl residues. In conclusion, our data indicate that some of the effects of lovastatin are mediated through changes in the basal state of G-protein in the membrane and consequently on adenylyl cyclase activity.**

Key words: G-protein; Adenylyl cyclase; Cholesterol; Lovastatin

1. Introduction

Adenylyl cyclase (AC) activity is under a dual regulation by G-proteins that can couple cell surface receptors to stimulate (G_s) or inhibit (G_i) ligands to the catalytic unit of AC [1]. Both G_s and G_i are heterotrimeric proteins. Each consists of a distinct α-subunit that can be ADP-ribosylated with CTX and PTX, respectively, and a highly conserved $\beta\gamma$-dimer [2].

G-proteins transduce information from a variety of membrane receptors and photopigments to a less populous group of effectors that include AC, cyclic GMP phosphodiesterase, phospholipase C, and several ion channels. Of the three G-protein subunits the β- and γ-subunits do not dissociate under physiological conditions and thus behave essentially as a monomer. $G\beta\gamma$ is required for efficient coupling of $G\alpha$-subunits to cell surface receptors, and functions as a membrane anchor, which stabilizes the basal GDP-bound $G\alpha$ for interaction with the appropriate cell surface receptor [3,4].

All the existing $G\gamma$ structures isolated contain the consensus CAAX domain (C = cysteine; A = aliphatic amino acid; X = any amino acid) as their carboxyl-terminus. Retinal γ_1 and brain γ_2 proteins have been shown to contain farnesyl [5,6] and geranylgeranyl [7,8] modifications in the C-terminal cysteine residues, respectively. On the other hand, the $G\alpha$ proteins of the G_i subfamily are myristoylated [9] and members of the G_s and G_q subfamilies of α units are palmitoylated [10,11]. In addition, members of the G_i subfamily are both palmitoylated and myristoylated at separate sites [10]. Unlike myristoylation, which is usually an irreversible cotranslational modification, palmitoylation is a dynamic posttranslational protein modification, and in each GTP cycle a palmitoyl residue is added and hydrolyzed from the αs subunit [12].

The prenyl residues necessaries for posttranslational modification of proteins are synthesized from mevalonate. Cultured cells in the presence of whole serum mainly derive their cholesterol from plasma lipoproteins, and their mevalonate by synthesis from acetyl CoA because they maintain low levels of hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase. In the absence of cholesterol in the medium, cells maintain high levels of HMG-CoA reductase to increase both mevalonate and cholesterol synthesis [13,14]. The discovery of potent inhibitors of HMG-CoA reductase has been of great therapeutic benefit to hypercholesterolemia patients. The prototype of these inhibitors, lovastatin, has been throughly studied and much is known about its cholesterol-lowering mechanism. Little attention has been paid, however, to the mechanism(s) by which lovastatin inhibits DNA synthesis and cell proliferation. In recent years, the role of hormones that act through the cAMP signalling pathways on cellular growth has become evident [15], and functional abnormalities of $G\alpha s$ have been characterized in several human secretory tumors [16]; so we decided to study how lovastatin affects the basal steady-state of G_s and G_i proteins and their regulated effector AC in endocrine (GH_4C_1) cells.

2. Materials and methods

2.1. Materials

^{32}P was from New England Nuclear (Boston, MA). Lovastatin was kindly provided by Merck Sharp and Dohme Res. Lab. (Rahway, NJ). Culture media, sera and antibiotic were from Gibco (Grand Island, NY), and culture flasks and plates were purchased from Nunc (Roskilde, Denmark). Antibodies to $G\alpha_s$ and $G\alpha_i$ were from Calbiochem (Palo Alto, CA). All other reagents were obtained from Sigma (St. Louis, MO), Calbiochem or Merck and were of the maximal purity available.

2.2. Cell culture and cellular treatments

Pituitary GH_4C_1 cells were grown in monolayers as previously described [17] in Dulbecco's modified Eagle's Medium supplemented with 10% foetal calf serum (FCS) in a water saturated atmosphere of 5% CO_2 and 95% air.

At the beginning of the experiments, the cells were rinsed with saline and incubated with fresh media containing 10% human-lipoprotein deficient serum (h-LPDS) for 24 h at 37°C. After this preincubation period, the cells were rinsed and incubated with the different media as described in the figure legends for 48 h.

Lovastatin was converted to the lactone sodium salt as described by Kita et al. [18], and diluted in water (5 mM). Mevalono-lactone was diluted in water (10 mM). All of these compounds were added to the medium supplemented with 10% h-LPDS.

At the end of the incubation, the cells were homogenized by frozen in liquid nitrogen and thawed at 32°C for three times. The homogenates were kept on ice until assay.

*Corresponding author. Fax: (34) (91) 885-4585.

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2.3. Isolation of lipoprotein-deficient serum

Human lipoprotein-deficient serum (h-LPDS) was prepared by ultracentrifugal flotation in potassium bromide [19] at 1.210 g/ml density [20]. After extensive dialysis against 50 mM Tris, 150 mM NaCl, 0.01% EDTA, pH 7.4, buffer, h-LPDS was sterilized by passage through a Millipore filter (Millex-GV; 0.22 μm) and kept frozen at −20°C.

2.4. Adenylyl cyclase assay

Incubations were performed at 32°C for 10 min in a final volume of 50 μl containing 0.1 mM [α-^{32}P]ATP (approx. 10×10^6 cpm/assay), 2.0 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM [^{3}H]cAMP (approx. 15,000 cpm/assay), 0.1% bovine serum albumin, a nucleoside triphosphate regenerating system (20 mM creatine phosphate, 26 U/ml creatine kinase, 25 U/ml myokinase, 25 mM Tris-HCl, pH 7.6), 10 μl homogenate, 20 μM GTP and, when present: 10 mM NaF, 250 nM VIP, 100 μM forskolin. After 10 min of incubation, the reaction was stopped by addition of 100 μl of stopping solution (10 mM ATP, 10 mM cAMP and 1% sodium dodecyl sulfate (SDS)). The [^{32}P]cAMP formed was measured by a modification [21] of the method of Salomon et al. [22]. Values are expressed as pmol of cAMP generated per min per mg cell protein.

2.5. ADP-ribosylation of $G\alpha_s$ and $G\alpha_i$ in cell homogenates

CTX from Sigma (5 mg/ml) and PTX from Calbiochem (0.6 mg/ml) were first activated for 30 min at 32°C in 10 mM Tris-HCl, pH 7.6, 50 mM dithiothreitol. The ADP-ribosylations were performed as described by Ribeiro-Neto et al. [23] using 5 μg activated CTX or 0.6 μg activated PTX, 5×10^6 cpm [^{32}P]NAD and 5–10 μg of protein homogenated. The final incubation volume was in both cases 60 μl. After incubation for 30 min at 32°C the reactions were stopped by adding 1 ml of 20% ice-cold trichloroacetic acid. After standing on ice for 10 min the mixture was centrifuged in a table top centrifuge at $1200 \times g$ for 30 min at 4°C. The resulting pellets were washed with ether, resuspended in 20 μl Laemmli's sample buffer [24] at room temperature and loaded onto 12.5% SDS-polyacrylamide gels; electrophoresis was performed according to Laemmli. Autoradiography was performed on the dried SDS gels by exposing Dupont (Cronex 4 NIF 100) films.

2.6. Immunoblotting

The cell pellets were homogenized in buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mg/ml soybean trypsin inhibitor, 0.1 mM phenylmethanesulfonyl fluoride, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml benzamidin) by freezing in liquid nitrogen and thawed at 32°C for three times. Nuclei and unbroken cells were pelleted by centrifugation at $500 \times g$ for 2 min, and the resulting supernatant was then spun at $50{,}000 \times g$ for 15 min. Membrane pellets were resuspended in homogenizing buffer at a concentration of 1–2 mg/ml of protein.

10 μg of membrane protein was fractionated by SDS-PAGE and transferred to nitrocellulose as described by Towbin et al. [25]. The filters were incubated either with anti-$G\alpha_s$ or anti-$G\alpha_i$ antibody, and the immunoreactive bands were detected by use of a horseradish peroxidase-coupled secondary antibody and the enhanced chemioluminiscence (ECL) system from Amersham Corp.

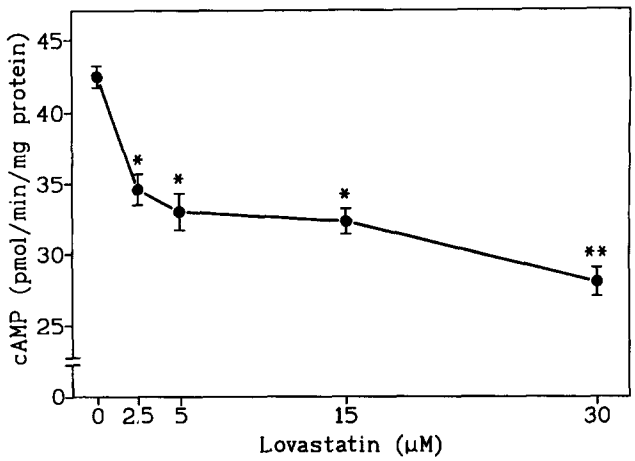

Fig. 1. Decreased AC activity in cells incubated with media supplemented with h-LPDS plus lovastatin. Cells were cholesterol-depleted by preincubation in medium supplemented with 10% h-LPDS for 24 h. Then, the media were removed and replaced with fresh media with 10% h-LPDS containing increasing concentrations of lovastatin for the following 48 h. Data shown are the means ± S.E.M. of four independent experiments performed in duplicate. *0.05 > P > 0.01; **0.01 > P > 0.001.

2.7. Measurements of cell protein

Proteins were determined essentially as described by Bradford [26], using bovine serum albumin as standard.

3. Results

3.1. Effect of lovastatin on AC activity

Lovastatin, a competitive inhibitor of HMG-CoA reductase, was studied for its effects on basal AC activity in GH_4C_1 cells. As shown in Fig. 1, addition of lovastatin to the cultures for 48 h markedly decreased the activity of AC. The inhibition was dose-dependent, being maxime at a concentration of 30 μM. Treatment of GH_4C_1 cells for 24 or 48 h with this inhibitor did not alter their viability; the protein content of each plate compared with untreated cells was not modified at 24 h and slightly decreased at 48 h (data not shown). It is important to remark that the drug was added at the end of the proliferating phase. These results suggest that the observed effects on AC caused by lovastatin are due to the decrease in mevalonate production which is a consequence of HMG-CoA reductase inhibition.

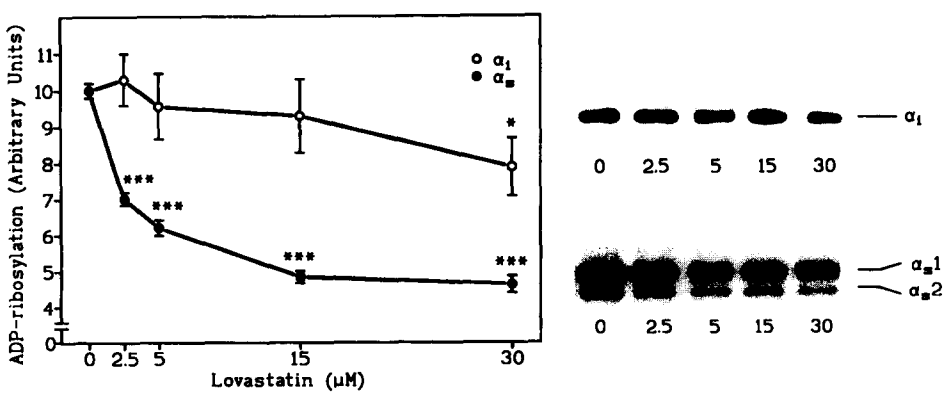

Fig. 2. Effect of lovastatin on ADP-ribosylation of $G\alpha_s$ and $G\alpha_i$. Cells were treated as indicated in Fig. 1. CTX and PTX-catalyzed ADP-ribosylation of $G_s\alpha$ and $G_i\alpha$ respectively was assayed as described in section 2. Data shown are the means ± S.E.M. of four independent experiments performed in duplicate. *0.05 > P > 0.01; ***P < 0.001. Representative autoradiogram bands showing ^{32}P-labeled α-subunits appears at the right of the plot.

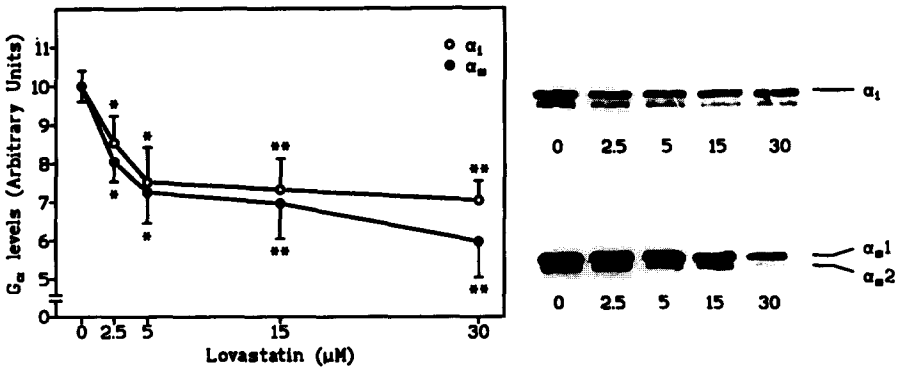

Fig 3. Treatment with lovastatin decreased $G\alpha_s$ and $G\alpha_i$ protein levels in plasma membranes. Membrane from cells treated as indicated in Fig. 1 were prepared, solubilized and polyacrylamide gel electrophoresis, immunoblotting, and autoradiography carried out as described in section 2. Data shown are the means ± S.E.M. of four independent experiments performed in duplicate. *$0.05 > P > 0.01$; **$0.01 > P > 0.001$. Representative immunoblot bands of both α-subunits appears at the right of the plot.

3.2. *Effect of lovastatin on G-protein α subunits*

It is generally accepted that AC activity is highly dependent on the activated state of the α-subunits of G_s and G_i proteins. One way to test the steady-state of these proteins is to ADP-ribosylate their α subunits with CTX or PTX respectively. As shown in Fig. 2, lovastatin decreased the amount of ADP-ribosylation in the CTX substrate ($G\alpha_s$) in a dose related manner, that correlated with the decrease in AC activity. By contrast, the effect of lovastatin on the ADP-ribosylation of PTX substrates, which include $G\alpha_i$, was very mild, the decrease at the highest dosis being only 15%.

The amount of both G protein α subunits in the membrane of cells treated with lovastatin was determined by immunoblotting. As shown in Fig. 3, the presence of lovastatin produced a decrease in the amount of both proteins in the plasma membrane, being about 40% for $G\alpha_s$ and 30% for $G\alpha_i$.

3.3. *Mevalonate suppresses the effect of lovastatin on AC and G-proteins*

The importance of mevalonate on AC activity on GH_4C_1 cells was tested directly by examining the ability of exogenous mevalonate to overcome the effect of lovastatin on basal AC activity. Mevalonate was added toghether with lovastatin for the 48 h incubation. As shown in Fig. 4, exogenous mevalonate counteracted the effect of lovastatin on AC activity. Similarly to what occurs with AC activity mevalonate added to the media, counteracted the effect of lovastatin on ADP-ribosylation of both $G\alpha_s$ and $G\alpha_i$ (Fig. 5), as well as on the amount of both proteins measured by immunoblotting (Fig. 6).

4. Discussion

The results of this study show that the HMG-CoA reductase inhibitor lovastatin decreases basal AC activity in GH_4C_1 cells. The effect was concentration dependent and was prevented by the addition of mevalonate. This clearly indicates that mevalonate availability is important for AC activity. Our data also indicate that suppression of mevalonate synthesis decreases the amount of $G\alpha_s$ and $G\alpha_i$ in the cell membrane, as measured by both immunoblotting and ADP-ribosylation. This decrease was also dose dependent, parallel to the inhibition produced in AC activity and also reverted by the presence of mevalonate.

Although none of the G-protein subunits contain regions that might obviously associate with a lipid bilayer, the heterotrimer is bound to the plasma membrane due in part to the fact that the γ-subunits are prenylated [5–8]. The carboxyl-terminal posttranslational modifications of G-protein γ-subunits are important determinants of the subcellular location of the G-subunits. The mutation of the modified cysteine into serine in γ_2 redistributes the β [27] and α [28] subunits from the membrane to the cytosol in transfected cells. The decrease in the amount of α_s and α_i bound to the membrane in cells treated with lovastatin should be due, at least in part, to the lack of prenyl residues in the γ subunits.

In addittion of their effects on cholesterol and mevalonate synthesis, lovastatin blocks the cell cycling in G1 and G2/M phases of cells grown in vitro [29–30] as well as suppresses growth of tumors in vivo [31]. We have found that when lova-

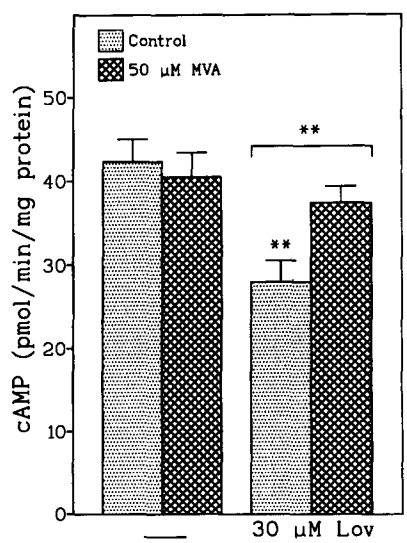

Fig. 4. Effect of mevalonate on AC activity in lovastatin-treated cells. Cells were preincubated with medium supplemented with 10% h-LPDS for 24 h. The media were removed and replaced with fresh media with 10% h-LPDS alone or 10% h-LPDS plus 30 μM lovastatin in absence or presence of 50 μM mevalonate (MVA) for the following 48 h. Each bar represent the means ± S.E.M. of duplicate determinations from four experiments. *$0.01 > P > 0.001$.

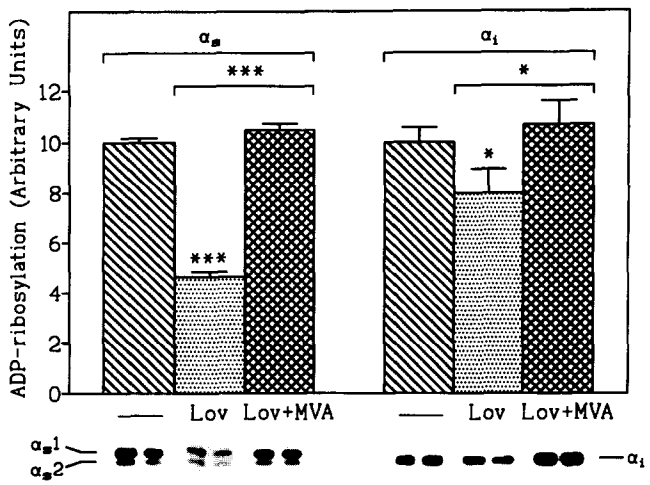

Fig. 5. The addition of mevalonate revert the effect of lovastatin on ADP-ribosylation of $G\alpha_s$ and $G\alpha_i$. CTX or PTX-catalyzed ADP-ribosylation of $G\alpha_s$ and $G\alpha_i$, respectively were assayed in cells treated as described in Fig 4. Lov = 30 μM lovastatin; MVA = 50 μM mevalonate. Each bar represents the means ± S.E.M. of four determinations carried out in duplicate. Representative autoradiogram bands showing ^{32}P-labeled $G_s\alpha$ and $G_i\alpha$ proteins appears below the appropriate columns for each group. *$0.05 > P > 0.01$; ***$P < 0.001$.

statin was added to cultures of GH_4C_1 in media supplemented with h-LPDS in the exponential phase, the rate of growth was markedly decreased (data not shown). The actual mechanism by which lovastatin blocks mitogenesis is not known, but inhibition of isoprenylation of proteins, such as inhibition of farnesylation of the oncogene product p21ras, has been proposed [32]. An activated mutant form of $G\alpha_s$ has been identified in pituitary tumors and postulated to be an oncogen [33]. Recently, it has been shown that the effects of GTPase-deficient

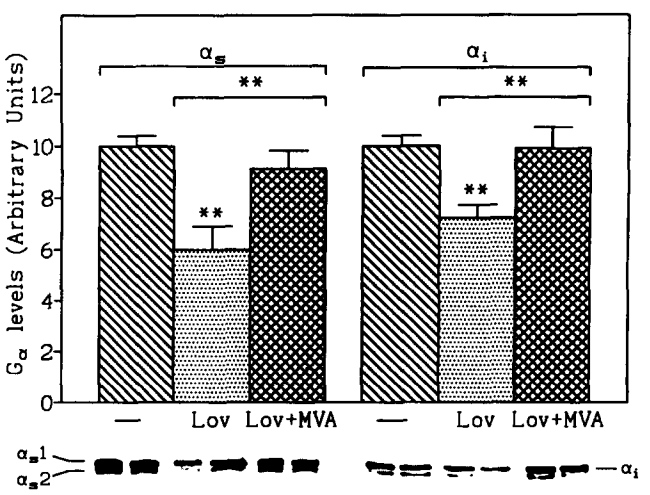

Fig. 6. Recovery of $G\alpha_s$ and $G\alpha_i$ protein levels in membranes of lovastatin-treated cells by addition of mevalonate. Membranes from cells incubated as indicated in Fig. 4 were prepared, electrophoresed and transferred to nitrocellulose membranes as described in section 2. The histogram and the immunoblots shown are of membranes prepared from cells incubated as indicated in Fig. 4. Lov = 30 μM lovastatin; MVA = 50 μM mevalonate. Values shown are the means ± S.E.M. of four independent determinations performed in duplicate. Panels below each column are representative immunoblots using the indicated antibodies. **$0.01 > P > 0.001$.

forms of $G\alpha_s$ found in human secretory tumors constitutively stimulate immediate early genes, thus supporting their oncogenic potential [34]; these effects are clearly mediated by the cAMP/PKA pathway and are therefore distinct from those exerted by the Ras oncogene. Although we do not know if the cAMP pathway exerts any influence on the rate of growth of the pituitary somato-mamotroph cell lines, like GH_4C_1 cells, our data show that lovastatin can decrease the AC activity by decreasing the amount of Gαs in the membrane and so decreases any proliferating effects mediated through the cAMP pathway by $G\alpha_s$ oncogenic proteins.

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References

[1] Gilman, A.G. (1984) Cell 36, 577–579.
[2] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[3] Freissmuth, M. and Gilman, A.G. (1989) J. Biol. Chem. 264, 21907–21914.
[4] Graber, S.G., Figler, R.A. and Garrison, J.C. (1992) J. Biol. Chem. 267, 1271–1278.
[5] Fukada, Y., Takao, T., Ohguro, H., Yoshizawa, T., Akino, T. and Shimonishi, Y. (1990) Nature 346, 658–660.
[6] Lai, R.K., Perez-Sala, D., Cañada, F.J. and Rando, R.R. (1990) Proc. Natl. Acad. Sci. USA 87, 7673–7677.
[7] Mumby, S.M., Casey, P.J., Gilman, A.G., Gutowski, S. and Sternweis, P.C. (1990) Proc. Natl. Acad. Sci. USA 87, 5873–5877.
[8] Yamane, H.K., Farnsworth, C.C., Sie, H., Howald, W., Fung, B.K.-K., Clarke, S., Gelg, M.H. and Glomset, J.A. (1990) Proc. Natl. Acad. Sci. USA 87, 5868–5872.
[9] Mumby, S.M., Heukeroth, R.O., Gordon, J.I. and Gilman, A.G. (1990) Proc. Natl. Acad. Sci. USA 87, 728–732.
[10] Linder, M.E., Middleton, P., Hepler, J.R., Taussig, R., Gilman, A.G. and Mumby, S.M. (1993) Proc. Natl. Acad. Sci. USA 90, 3675–3679.
[11] Degtyarev, M.Y., Spiegel, A.M. and Jones, L.Z. (1993) Biochemistry 32, 8057–8061.
[12] Wedegaernet, P.B. and Bourne, H.R. (1994) Cell 77, 1063–1070.
[13] Brown, M.S. and Goldstein, J.L. (1980) J. Lipid Res. 21, 505–517.
[14] Goldstein, J.L. and Brown, M.S. (1990) Nature 343, 425–430.
[15] Dumont, J.E., Jauniaux, J.-C. and Roger, P.P. (1989) Trends Biochem. Sci. 14, 67–71.
[16] Spiegel, A.M., Shenker, A. and Weinstein, L.S. (1992) Endocr. Rev. 13, 536–565.
[17] Martin, T.J.F. and Tashjian Jr., A.H. (1977) in: Biochemical Actions of Hormones (Litvack, G. ed.) Academic Press, New York, vol. 4, pp. 269–312.
[18] Kita, T., Brown, M.S. and Goldstein, J.L. (1980) J. Clin. Invest. 66, 1094–1100.
[19] Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345–1353.
[20] Rothblat, G.H., Bamberger, M. and Phillips, M.C. Methods Enzymol. 129, 628–644.
[21] Bockaert, J., Hunzickes-Dunn, M. and Birnbaumer, L. (1976) J. Biol. Chem. 251, 2653–2663.
[22] Salomon, Y., Landos, C. and Rodbell, M. (1979) Anal. Biochem. 58, 541–598.
[23] Ribeiro-Neto, F., Mattera, R., Grenet, D., Sekura, R.D., Birnbaumer, L. and Field, J.B. (1987) Mol. Endocrinol. 1, 472–481.
[24] Laemmli, M. (1970) Nature 227, 680–685.
[25] Towbin, H., Staehelim, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[26] Bradford, M.M. (1976) Anal. Biochem. 102, 344–352.

[27] Simonds, W.F., Butrynski, J.E., Gautam, N., Unson, C.G. and Spiegel, A.M. (1991) J. Biol. Chem. 266, 5363–5366.
[28] Muntz, K.H., Sternweis, P.C., Gilman, A.G. and Mumby, S.M. (1992) Mol. Biol. Cell 3, 49–61.
[29] Habenitch, A.J.R., Glomset, J.A. and Ross, R. (1980) J. Biol. Chem. 255, 5134–5140.
[30] Fairbanks, K.P., Witte, L.D. and Goodman, D.S. (1984) J. Biol. Chem. 259, 1546–1551.
[31] Maltese, W.A., Defendini, R., Green, R.A., Sheridan, K.M. and Donley, D.K. (1985) J. Clin. Invest. 76, 1748–1754.
[32] Gibbs, J. (1991) Cell 65 1–4.
[33] Landis, C.A., Masters, S.B., Spada, A., Pace, A. M., Bourne, H.R. and Vallar, L. (1989) Nature 340, 692–696.
[34] Gaiddon, C., Boutillier, A.-L., Monnier, D., Mercken, L. and Loeffler, J.-P. (1994) J. Biol. Chem. 269, 22663–22671.