

# Effect of mevalonate availability on the association of G-protein $\alpha$ -subunits with the plasma membrane in GH<sub>4</sub>C<sub>1</sub> cells

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**Abstract** We show that the levels and activity of the  $\alpha$ -subunits of G<sub>s</sub> and G<sub>i</sub> proteins in plasma membrane of GH<sub>4</sub>C<sub>1</sub> cells are regulated by the availability of mevalonate (MVA), and not by changes in cholesterol cell content. Changes in the levels of MVA, induced by modulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, determine the amount of both membrane-bound G $\alpha$ -subunits, which correlated with the activity of their effector adenylyl cyclase. Lipoprotein deficient serum (LPDS) decreases cholesterol content and increases both HMG-CoA reductase activity and G $\alpha$ -subunits in the membrane. Cholesterol and 25-hydroxycholesterol (25-HC) each repress HMG-CoA reductase and diminish G $\alpha$ -subunit levels. However, while cholesterol cell content is also decreased by 25-HC, exogenous cholesterol increases it. In addition, the decrease of both G $\alpha$ -subunits is reversed by the presence of MVA. This regulation appears to be mediated by nonsterol products generated from MVA. We assume that the first is the prenylation of the  $\gamma$ -subunits, since the attachment of G $\alpha$ -subunits to the membrane is dependent on this modification. However, as neither of our treatments completely abolished protein prenylation, we conclude that another MVA derivative is required in addition to prenyl residues to the presence and activity of  $\alpha$ -subunits in the membrane.

**Key words:** G-protein; Adenylyl cyclase; Mevalonate; Cholesterol; 25-Hydroxycholesterol

## 1. Introduction

Guanine nucleotide-binding regulatory proteins (G-proteins) are heterotrimers composed of subunits referred to as  $\alpha$ ,  $\beta$  and  $\gamma$ . These proteins are associated with the inner face of the plasma membrane, where they act as transducers between membrane-bound receptors and effectors, i.e. enzymes and ion channels. Probably the system studied in most depth is that of adenylyl cyclase (AC). This system is positively and negatively regulated by G<sub>s</sub> and G<sub>i</sub> proteins respectively [1].

A growing body of evidence indicates that several posttranslational modifications are required for the location of G-proteins in the inner surface of the membrane and for their activity. Covalent lipid modifications are found in both the  $\alpha$ - and  $\gamma$ -subunits of G-proteins. These modifications establish the interaction of the protein with the membrane. The  $\alpha$ -subunits of G<sub>s</sub> and G<sub>q</sub> subfamilies are palmitoylated [2,3] and members of the G<sub>i</sub> subfamily are both palmitoylated and myristoylated [2]. All of the isolated G-protein  $\gamma$ -subunits have been reported to be isoprenylated. Protein isoprenylation

is a posttranslational modification in which a farnesyl or geranylgeranyl isoprenoid is attached to a carboxyl-terminal cysteine residue of the consensus CAAX domain (C, cysteine; A, aliphatic amino acid; X, any amino acid) [4]. Retinal  $\gamma_1$  and brain  $\gamma_2$  proteins have been shown to contain farnesyl [5,6] and geranylgeranyl [7,8] modifications in the C-terminal cysteine residues, respectively. The carboxyl-terminal posttranslational modifications of G-protein  $\gamma$ -subunits are important determinants of the subcellular location of the G-subunits.  $\beta$ - and  $\gamma$ -subunits do not dissociate under physiological conditions and thus behave essentially as a monomer required for efficient coupling of G $\alpha$ -subunits to cell surface receptors, and function as membrane anchors for the  $\alpha$ -subunits [9,10]. Consistent with that, the substitution of the modified cysteine by serine in  $\gamma_2$  redistributes the subcellular location of both  $\beta$ - [11] and  $\alpha$ - [12] subunits. There is also evidence that suggests the involvement of the prenyl group in protein-protein interactions [13,14]. The biological significance of this modification is not fully understood, but in the best known case, that of the *ras* oncogene product (p21<sup>ras</sup>), the farnesyl moiety is required for the transforming activity of the protein and for its membrane anchorage [15–17].

It is well known that mevalonate (MVA) availability is required for growth in mammalian cells, besides being the key metabolite in the biosynthesis of cholesterol and a variety of non-sterol isoprenoid compound derivatives [18], among which are the prenyl residues for posttranslational modification. The formation of MVA, catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, is the key regulatory step of this pathway. In cultured mammalian cells, the activity of this enzyme is controlled by a feedback mechanism mediated by cholesterol cell content. In the absence of exogenous cholesterol the cells synthesize their own MVA and cholesterol, maintaining high levels of HMG-CoA reductase, while in the presence of exogenously added cholesterol or of its oxygenated derivative 25-hydroxycholesterol (25-HC), the activity of the enzyme is reduced, thereby turning off the MVA and cholesterol cellular synthesis [19].

Although there is an extensive literature concerning the relationships between MVA-derived non-sterol compounds, cell proliferation and G-proteins, mainly using inhibitors of HMG-CoA reductase (statins), which completely suppress prenyl synthesis, little attention has been focused on the relationship between MVA and cholesterol metabolism and G-proteins in physiological and basal conditions.

We have shown in a previous report [20] that lovastatin, a competitive inhibitor of HMG-CoA reductase, decreases the amount of G-protein  $\alpha$ -subunits associated with the plasma membrane in GH<sub>4</sub>C<sub>1</sub> cells, a clonal rat pituitary cell strain.

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The effect of lovastatin was reversed by exogenously added MVA, suggesting that the cause of this decrease is the lack of prenylation of the  $\gamma$ -subunit. In the present study, experiments were carried out to determine whether changes in cholesterol cell content and/or MVA availability, induced by the availability of the former, could regulate the amount of G-proteins in the cell membrane in a more physiological way. In order to get a mechanistic approach of the effect of cholesterol on this parameter, we have used cholesterol and 25-HC instead of lovastatin for the study of the changes in membrane localization of the GTP-binding proteins  $\alpha_s$  and  $\alpha_i$  in GH<sub>4</sub>C<sub>1</sub> cells, as well as in their regulated effector adenylyl cyclase.

In the present study we demonstrate that the localization and activity of G $\alpha_s$  and G $\alpha_i$  in the plasma membrane were significantly decreased by inhibiting HMG-CoA reductase and not by changes in free cholesterol content. Since the synthesis of MVA is regulated by cell cholesterol content, this strongly suggests that cholesterol, not directly, but through MVA synthesis, controls the presence and the activity of G $\alpha_s$  and G $\alpha_i$  in the plasma membrane. Therefore, in a non-stimulated situation, the rate of MVA synthesis is limiting for some signal transduction pathways via G-proteins.

## 2. Materials and methods

### 2.1. Materials

Culture media, sera and antibiotic were from Gibco (Grand Island, NY), and culture flasks and plates were purchased from Nunc (Roskilde, Denmark). Antibodies against G $\alpha_s$  and G $\alpha_i$  were from Calbiochem (Palo Alto, CA). [ $\alpha$ -<sup>32</sup>P]ATP, [<sup>3</sup>H]cAMP and [<sup>14</sup>C]HMG-CoA were from New England Nuclear (Boston, MA). All other reagents were obtained from Sigma (St. Louis, MO), Calbiochem or Bio-Rad and were of the maximal purity available.

### 2.2. Isolation of lipoprotein-deficient serum

Lipoprotein-deficient serum (LPDS) was prepared from fetal bovine serum (FBS) by ultracentrifugal flotation in potassium bromide [21] at 1.210 g/ml density [22]. After extensive dialysis against 50 mM Tris, 150 mM NaCl, 0.01% EDTA, pH 7.4. LPDS was sterilized by passage through a Millipore filter (Millex-GV 0.22  $\mu$ m) and kept frozen at –20°C.

### 2.3. Cell culture and cellular treatments

Pituitary GH<sub>4</sub>C<sub>1</sub> cells were grown in monolayers as previously described [23] in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in a water saturated atmosphere of 5% CO<sub>2</sub> and 95% air.

At the beginning of the experiments, the cells were rinsed with normal saline and incubated with fresh medium containing 10% FBS or 10% LPDS for 24 h at 37°C. After this preincubation, the cells were rinsed and incubated with the different media for 48 h as described in the figure legends.

Cholesterol (5 mg/ml) and 25-hydroxycholesterol (5 mM) were dissolved in ethanol. Before adding, ethanol was evaporated under N<sub>2</sub> flow. Mevalonolactone was diluted in water (10 mM). All of these compounds were added to the medium supplemented with 10% LPDS.

### 2.4. Immunoblotting of G $\alpha_s$ and G $\alpha_i$

Cell pellets were homogenized in buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, containing 5 mg/ml soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml benzamidine) by three times freezing in liquid nitrogen and thawing at 32°C. Nuclei and unbroken cells were pelleted by centrifugation at 500×g for 2 min, and the resulting supernatant was then centrifuged at 50000×g for 15 min. Membrane pellets were resuspended in homogenizing buffer at a concentration of 1–2 mg/ml of protein. 10  $\mu$ g of membrane protein was fractionated by SDS-PAGE and transferred to nitrocellulose membrane as described by Towbin et al. [24]. The nitrocellulose membranes were incubated with either anti-

G $\alpha_s$  or anti-G $\alpha_i$  antibody, and the immunoreactive bands were detected by the use of a horseradish peroxidase-coupled secondary antibody and the enhanced chemiluminescence (ECL) system (Amersham).

### 2.5. Measurement of cholesterol

The concentration of cellular free cholesterol was determined as previously described [25]. Lipids were extracted from the cells with chloroform-methanol (2:1), chloroform was evaporated under N<sub>2</sub> flow and the enzymatic reagent added. Values are expressed as  $\mu$ g of free cholesterol per mg cell protein.

### 2.6. HMG-CoA reductase activity

HMG-CoA reductase activity of freshly isolated cell lysates was measured with [<sup>14</sup>C]HMG-CoA as the substrate during a 2-h reaction essentially as described elsewhere [26]. Activity was calculated as pmol of mevalonolactone formed per min per mg cell protein.

### 2.7. Adenylyl cyclase assay

Assays were performed by incubation at 32°C for 10 min in a final volume of 50  $\mu$ l of a mixture containing 25 mM Tris-HCl, pH 7.6, 0.1 mM [ $\alpha$ -<sup>32</sup>P]ATP (approx.  $5 \times 10^6$  cpm/assay), 2.0 mM MgCl<sub>2</sub>, 1.0 mM EDTA, 1.0 mM [<sup>3</sup>H]cAMP (approx. 15000 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), 10  $\mu$ l homogenate and 20  $\mu$ M GTP. After 10 min of incubation, the reaction was stopped by addition of 100  $\mu$ l of stopping solution (10 mM ATP, 10 mM cAMP and 1% sodium dodecyl sulfate (SDS)). The [<sup>32</sup>P]cAMP formed was measured by a modification [27] of the method of Salomon et al. [28]. Values are expressed as pmol of cAMP generated per min per mg cell protein.

### 2.8. Measurement of cell protein

Proteins were determined as described by Bradford [29], using bovine serum albumin as standard.

## 3. Results

### 3.1. Effect of LPDS, cholesterol and 25-hydroxycholesterol on G-protein $\alpha$ -subunits

First of all, we tried to see the effect of LPDS on G $\alpha_s$  and G $\alpha_i$  protein membrane levels in GH<sub>4</sub>C<sub>1</sub> cells. As shown in Fig. 1, immunoblotting demonstrated that incubation of the

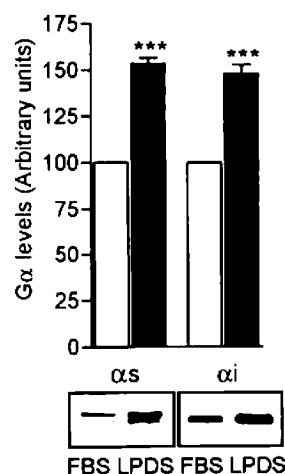


Fig. 1. Effect of LPDS on the levels of membrane-bound G $\alpha$ -subunits. Cells were preincubated with whole serum (10% FBS) or cholesterol-depleted by incubation in medium supplemented with 10% LPDS for 72 h. Membrane from cells was prepared and solubilized, and polyacrylamide gel electrophoresis, immunoblotting, and autoradiography were carried out as described in Section 2. Data shown are the means  $\pm$  S.E. of four independent experiments performed in duplicate. \*\*\* $P$  < 0.001. Representative immunoblot bands of both  $\alpha_s$ - and  $\alpha_i$ -subunits appear below each group of columns.

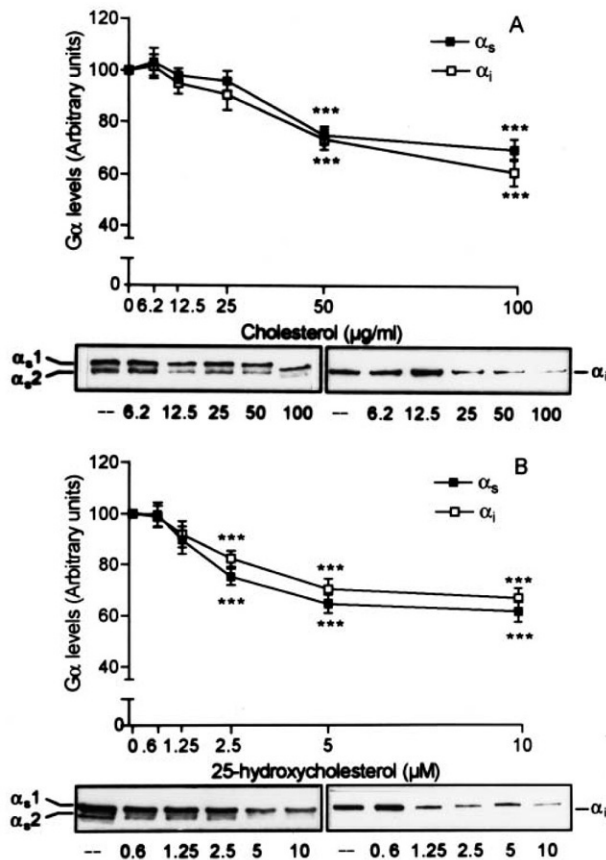


Fig. 2. Treatment with cholesterol (A) and 25-HC (B) decreases Gα<sub>s</sub> and Gα<sub>i</sub> protein levels in plasma membranes. Cells were cholesterol-depleted by preincubation in medium supplemented with 10% LPDS for 24 h. Then, the media were removed and replaced with fresh media with 10% LPDS containing increasing concentrations of cholesterol (A) or 25-HC (B) for the following 48 h. Immunoblotting of membrane proteins was assayed as described in Section 2. Data shown are the means ± S.E. of four independent experiments performed in duplicate. \*\*\**P* < 0.001. Representative immunoblot bands of both α<sub>s</sub>- and α<sub>i</sub>-subunits appear below the plot.

cells with LPDS for 72 h markedly increased the amount of both α<sub>s</sub>- and α<sub>i</sub>-subunits in plasma membrane, this increase being about 150% of that of controls incubated in normal FBS.

LPDS is a well known acceptor of cholesterol from the cells that decreased the cholesterol cell content (Table 1). Consequently cells responded with an increase in HMG-CoA reductase activity (Table 1).

In order to distinguish whether the effect of LPDS on α<sub>s</sub>- and α<sub>i</sub>-subunits could be due to the decrease in cholesterol cell content or to the increase in MVA synthesis, we studied the

effect of exogenously added cholesterol, and also the effect of oxygenated derivative 25-hydroxycholesterol (25-HC) on the cellular levels of cholesterol and HMG-CoA reductase (Table 1). While exogenously added cholesterol increased the cholesterol content and decreased the HMG-CoA activity, 25-HC decreased both cholesterol and enzyme activity.

When the amount of both Gα-subunits (α<sub>s</sub> and α<sub>i</sub>) in the plasma membrane were determined in cells incubated with increasing concentrations of either exogenous cholesterol (Fig. 2A) or 25-HC (Fig. 2B) for 48 h, a significant decrease in both subunits were observed in a dose-related manner with both treatments, returning to the values of control cells incubated with whole serum.

In all cases, treatment of cells either with LPDS, 25-HC or cholesterol did not alter the synthesis of total proteins as determined using [<sup>3</sup>H]leucine (data not shown).

### 3.2. Effect of LPDS, cholesterol and 25-hydroxycholesterol on AC activity

Since AC activity is highly dependent on the levels and association state of α<sub>s</sub> and α<sub>i</sub> in plasma membrane, we examined how the changes in α<sub>s</sub> and α<sub>i</sub> proteins promoted by cellular cholesterol manipulation affect the activity of their effector AC. LPDS significantly increased AC activity (Fig. 3A), while cholesterol and 25-HC decreased AC activity in a dose-related manner (Fig. 3B). These results correlate with the decrease in the amount of α<sub>s</sub> and α<sub>i</sub> subunits in plasma membrane.

### 3.3. MVA but not cholesterol suppresses the effect of 25-hydroxycholesterol on G-proteins and AC activity

The results observed on α<sub>s</sub> and α<sub>i</sub>, promoted by LPDS, are not related to changes in cholesterol cell content, but rather to the rate of MVA synthesis, which is a consequence of HMG-CoA reductase activity. To prove that the lack of MVA synthesis, and not cholesterol, was the cause of the decrease in α<sub>s</sub> and α<sub>i</sub> in the membrane, we directly examined the ability of exogenous MVA and cholesterol to overcome the inhibitory effect of 25-HC. When cells were incubated for 48 h with 25-HC in the presence of either MVA or cholesterol, only exogenous MVA was able to counteract the effect of 25-HC on α<sub>s</sub> and α<sub>i</sub> levels in plasma membrane (Fig. 4), while cholesterol did not have any effect. Similarly, MVA addition to the media reversed the inhibitory effect of 25-HC on AC while cholesterol did not suppress the effect of the inhibitory metabolite (Fig. 5).

## 4. Discussion

In the present study on GH<sub>4</sub>C<sub>1</sub> cells, we demonstrate that

Table 1  
Effect of LPDS, cholesterol and 25-hydroxycholesterol on cholesterol cell content and HMG-CoA reductase activity

	Cholesterol content (μg/mg protein)	HMG-CoA reductase (pmol/min/mg protein)
FBS	20.08 ± 2.8	34 ± 2.2 (100)
LPDS	11.92 ± 1.2***	280 ± 17 (730)***
LPDS+cholesterol (50 μg/ml)	50.52 ± 5.3+++	31 ± 3.5 (91)
LPDS+25-HC (2.5 μM)	8.67 ± 2.3 <sup>+</sup>	28 ± 3 (82)

Cells were preincubated with medium supplemented with 10% FBS or 10% LPDS for 24 h. The media were removed and replaced with fresh media with 10% FBS, or 10% LPDS in the absence or presence of the following additions: cholesterol or 25-hydroxycholesterol (25-HC). Enzyme activities were performed as described in Section 2. Values shown are the means ± S.E. of three independent determinations carried out in duplicate. Numbers in parentheses are percentages of control. \*\*\**P* < 0.001 compared to FBS; <sup>+</sup>0.05 > *P* > 0.01; +++*P* < 0.001 compared to LPDS.

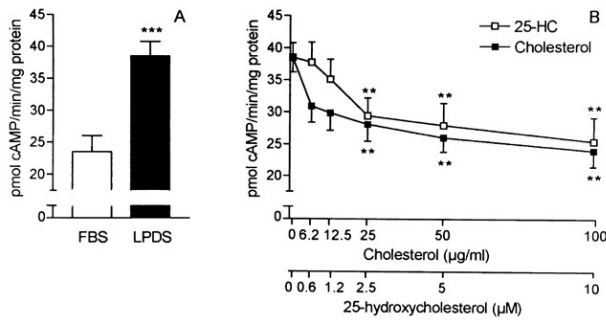


Fig. 3. Effect of LPDS (A), cholesterol and 25-HC (B) on AC activity. Cells were preincubated with medium supplemented with 10% FBS or 10% LPDS for 24 h. The media were removed and replaced with fresh media with 10% FBS or LPDS alone (A), or 10% LPDS containing increasing concentration of cholesterol or 25-HC. AC activity was carried out as described in Section 2. Data shown are the means  $\pm$  S.E. of four independent experiments performed in duplicate. \*\* $0.01 > P > 0.001$ ; \*\*\* $P < 0.001$ .

the location in plasma membrane and the activity of the  $G_{\alpha_s}$  and  $G_{\alpha_i}$  are regulated by a MVA-dependent mechanism, i.e. by the amount of MVA itself or by a non-steroid derivative. This type of regulation would be of physiological significance since  $G_s$  and  $G_i$  are associated with the activity of AC and to the generation of cAMP, as well as with other signal transduction pathways. Moreover, the rate of MVA synthesis is regulated by the amount of free cholesterol [18], and therefore, changes in cholesterol cell content would regulate the activity of  $G_s$  and  $G_i$  proteins indirectly.

Our data clearly indicate that incubation of GH<sub>4</sub>C<sub>1</sub> cells with LPDS decreases the cholesterol cell content by 50% and increases the amount of  $\alpha_s$ - and  $\alpha_i$ -subunits in the cell membrane and, consequently, their basal AC activity. Although  $G_{\alpha_i}$  is also increased in cells incubated with LPDS we only observed stimulation on AC activity. One pos-

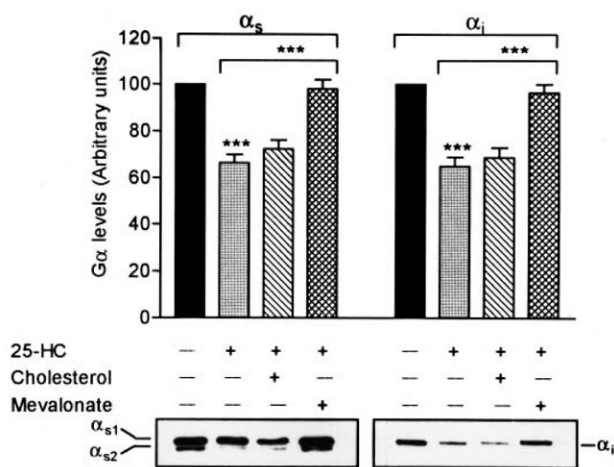


Fig. 4. MVA but not cholesterol recovers the  $G_{\alpha_s}$  and  $G_{\alpha_i}$  protein levels in membranes of 25-HC-treated cells. Cells were preincubated with medium supplemented with 10% LPDS for 24 h. The media were removed and replaced with fresh media with 10% LPDS alone or 10% LPDS plus 2.5  $\mu$ M 25-HC in the absence or presence of 50  $\mu$ g/ml of cholesterol or 50  $\mu$ M MVA for the following 48 h. Immunoblotting of membrane proteins was carried out as described in Section 2. Each bar represents the means  $\pm$  S.E. of duplicate determinations from four experiments. \*\*\* $P < 0.001$ . Panels below each group of columns are representative immunoblots using the indicated antibodies.

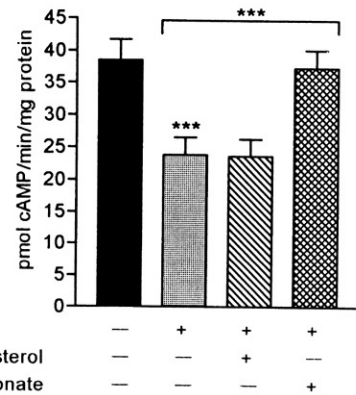


Fig. 5. The addition of MVA reverses the effect of 25-HC on AC activity. AC activity was assayed in cells treated as described in Fig. 4. 25-HC = 2.5  $\mu$ M 25-hydroxycholesterol; cholesterol, 50  $\mu$ g/ml; MVA = 250  $\mu$ M mevalonate. Each bar represents the means  $\pm$  S.E. of four determinations carried out in duplicate. \*\*\* $P < 0.001$ .

sible explanation is that not all mammalian AC are subject to inhibitory regulation by  $\alpha_i$ .  $G_{\alpha_i}$  inhibits types I and V AC [30,31], but these isoforms are not present in GH<sub>4</sub>C<sub>1</sub> cells (L. Birnbaumer, personal communication). By contrast, a high concentration of  $\alpha_i$  could be stimulating other isoforms of AC. In support of this hypothesis, Federman et al. [32] noted that activation of  $G_{\alpha_i}$  resulted in conditional activation (and not inhibition) of type II AC.

These changes can be attributed to the increase in the activity of HMG-CoA reductase and the consequent increase in MVA levels, because they were suppressed by the addition of two inhibitors of MVA synthesis: cholesterol itself and 25-HC. Since the cholesterol cell content was increased by exogenous cholesterol, and decreased by 25-HC, it seems to indicate that the effect of LPDS on the amount of  $G_{\alpha}$ -subunits in plasma membrane is independent of the cholesterol cell content. On the other hand, since the addition of MVA, but not cholesterol, was able to suppress the inhibitory effects of 25-HC over LPDS, it can be suggested that MVA itself, or a non-sterol MVA derivative, is responsible for the steady-state of  $G_s$  and  $G_i$  in the cell membrane.

One mechanism by which MVA is limiting for the localization of  $\alpha_s$ - and  $\alpha_i$ -subunits in plasma membrane is due to the fact that the association of  $G_{\alpha}$ -subunits with the membrane is dependent on prenylation and/or further carboxy-terminal processing of  $\gamma$ -subunits, which are indispensable for the high affinity of  $\beta\gamma$  for  $\alpha$ -subunits [33]. We assume that the changes in the amount of  $\alpha$ -subunits in the membrane, induced by the presence or absence of sterols, could be caused by the amount of prenyl residues derived from endogenous MVA. However, although it has been shown that a lack of prenylation of  $\gamma$ -subunits either by mutation [11] or by inhibition of prenyl synthesis in cells [12], produces non-prenylated proteins that remain cytosolic, it has not been shown that an increase in MVA causes a parallel increase in the amount of prenylated proteins and/or their location in plasma membrane. By contrast, Rilling et al. [34] have demonstrated that high MVA levels increase the lipid synthesis, without increasing the prenylation of one specific protein. On the other hand, Lutz et al. [35] have demonstrated, in rabbit reticulocyte lysates, that the synthesis of geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), the

precursors of the prenyl residue in  $\gamma$ -subunits and p21<sup>ras</sup> protein respectively, is product regulated. The synthesis of GGPP and FPP is several-fold higher in the presence of a prenyl acceptor (protein) than in its absence, without changes in the pools of prenyl PPs. If this regulation of the prenyl residues occurs in whole cells, it makes it unlikely that the increase in prenyl residues from endogenous or exogenous MVA could be the regulatory factor that increases the amount of  $\alpha$ -subunits in the cell membrane. This is also suggested by the fact that we observe a decrease in  $\alpha$ -subunits in the membrane in the presence of cholesterol and/or 25-HC, a situation in which it has been shown that protein prenylation is not inhibited and the synthesis of total proteins is not modified [36]. Together, all these facts seem to indicate that the effect of LPDS on the amount of  $\alpha$ -subunits in the cell membrane should be caused by some other MVA derivative in addition to the increase in prenyl residues, but the mechanism of this synergistic regulation remains to be elucidated.

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