Blockade of mevalonate production by lovastatin attenuates bombesin and vasopressin potentiation of nutrient-induced insulin secretion in HIT-T15 cells

Probable involvement of small GTP-binding proteins

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Small G-proteins (SMGs) require isoprenylation for their association with membranes. We have examined protein isoprenylation, subcellular distribution of SMGs, cytosolic Ca^{2+} changes and insulin secretion in HIT-T15 cells after treatment with lovastatin, which inhibits the production of isoprenoids by blocking mevalonate production by 3-hydroxy-3-methylglutaryl-CoA reductase. Numerous proteins in the 20–70 kDa range were found to be isoprenylated. Most of these proteins co-migrated with SMGs (21–27 kDa). Lovastatin treatment (25 μM, 24 h) decreased protein isoprenylation and affected the distribution of several SMGs, causing a large accumulation in the cytosol and a detectable decrease in membranes. Lovastatin selectively attenuated the potentiating action of bombesin and vasopressin, which activate phospholipase C in these cells, on insulin secretion stimulated by nutrients (glucose + leucine + glutamine). This lovastatin effect was overcome by mevalonate. Insulin secretion stimulated by nutrients alone or insulin release in the presence of the potentiating agents forskolin or phorbol myristate acetate remained unaffected. As the modulation of insulin secretion by isoprenaline and somatostatin were not altered by lovastatin, the drug does not non-selectively affect the binding of ligands to their receptors. Lovastatin did not interfere with the activation of phospholipase C by bombesin and vasopressin, since the rise in cytosolic Ca^{2+} induced by these agents was not changed. Limonene, proposed to block specifically prenyl-protein transferases of SMGs, did not alter protein isoprenylation patterns, but inhibited the stimulated insulin secretion. In conclusion, lovastatin selectively attenuated the potentiation of nutrient-induced insulin secretion by bombesin and vasopressin without affecting their activation of phospholipase C. The concomitant changes in SMG isoprenylation and their subcellular distribution after lovastatin treatment suggest that SMGs could play an important role in the bombesin and vasopressin action on insulin secretion.

INTRODUCTION

The generation of isoprenoids from acetyl-CoA is an important metabolic pathway whose products include cholesterol, dolichols and ubiquinones [1]. Recently, evidence has accumulated that some proteins are post-translationally modified by isoprenylation [1–5]. The isoprenylated proteins which have been identified include the yeast mating factor α, the nuclear lamin B, the γ-subunits of transducin and of other heterotrimeric G-proteins in brain, cyclic GMP phosphodiesterase, rhodopsin kinase, and, especially, the small-molecular-mass (20–28 kDa) GTP-binding proteins (SMGs) [1–7]. The latter include the *ras* family and *ras*-related SMGs, such as *rac*, *rap*, *ral* and *rab* [1–5]. These proteins (except *rab*) have a CAAX sequence at their C-terminus (C stands for cysteine, A is an aliphatic amino acid and X is any amino acid). It was found that two polyisoprenoids, geranylgeranyl and farnesyl (depending on whether leucine is the amino acid X or not), are linked to the cysteine via thioether bonds by specific prenyl-protein transferases [3–5]. *rab* proteins, with a CC or CXC motif at the C-terminus, are isoprenylated by distinct geranylgeranyl-protein transferase(s) [4,5]. The isoprenylated proteins become more hydrophobic, which facilitates their association with membranes [1–5,8].

Mevalonate (MVA) is the essential precursor of all isoprenoids. It is generated from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in the reaction catalysed by HMG-CoA reductase. This enzyme is under the feedback control of cholesterol and unknown non-steroid(s) [1] and can be competitively inhibited by compactin and lovastatin [9–11]. Using the HMG-CoA reductase blockers and the mutation of the C-terminal CAAX sequence of proteins, it was discovered that protein isoprenylation plays a crucial role in cell growth [5,12], cell transformation by *ras* [8], the GTP-binding activity of transducin [13], the NK cell killing ability [14], the antigen-mediated signal production in basophils [15,16] and the activity of NADPH oxidase in HL-60 cells [17].

SMGs have been found to participate in the secretion in yeast [4] and in the vesicle transport from the endoplasmic reticulum through the Golgi complex to the plasma membrane in eukaryotic cells [18]. In the present study, the possible role of SMGs in insulin secretion was examined by interfering with their isoprenylation after lovastatin treatment of HIT-T15 cells. It was found that lovastatin caused a large accumulation of several SMGs in the cytosol and some decrease of these proteins in membranes. Lovastatin selectively attenuated the potentiation by bombesin and vasopressin of nutrient-induced insulin secretion, and this inhibition could be reversed by addition of MVA. The effects of nutrients, forskolin, the phorbol ester phorbol 12-myristate 13-acetate (PMA), isoprenaline and somatostatin on insulin se-

Abbreviations used: SMGs, small-molecular-mass GTP-binding proteins; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; AVP, [arginine]vasopressin; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; KRBH, Krebs–Ringer bicarbonate–Hepes buffer; MVA, mevalonate; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; GTP[S], guanosine 5′-[γ-thio]triphosphate.

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cretion remained unaltered in lovastatin-treated cells. In contrast with its inhibitory effect on bombesin- and vasopressin-potentiated insulin secretion, lovastatin did not modify the rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) elicited by the two agonists, suggesting that the coupling from receptor to phospholipase C (PLC) is unperturbed in the treated cells. It is proposed that part of the potentiation by PLC-activating receptor agonists is exerted beyond the stimulation of the enzyme and that SMGs may be involved in this process.

MATERIALS AND METHODS

Materials

The sources of the materials used have been described elsewhere [19,20], except the following: forskolin was from Calbiochem, San Diego, CA, U.S.A.; bombesin from Bachem, Bubendorf, Switzerland; streptolysin-O from Wellcome Diagnostics, Dartford, U.K.; mevalonate and *d*-limonene from Fluka Chemie A.G., Buchs, Switzerland. [2-^{14}C]Mevalonolactone was from New England Nuclear, Dreiech, Germany. Lovastatin was generously given by Dr. Alfred W. Alberts of Merck Sharp & Dohme Research Laboratory, Rahway, NJ, U.S.A. The lactone form of lovastatin was transformed to the sodium salt as previously described [11], since the former is toxic to HIT cells.

Cell culture

The insulin-secreting cell line, HIT-T15, was originally provided by Dr. A. E. Boyd III (Tufts University, Boston, MA, U.S.A.). The cells (passages 70–79) were cultured in RPMI 1640 medium supplemented with 100 units of penicillin/ml, 100 μg of streptomycin/ml and 10% fetal-calf serum, and treated in the same medium containing vehicle, lovastatin or MVA for the indicated periods. For insulin-secretion experiments, cells were seeded on multiwell plates (1.77 cm^2 × 24 wells) or microtitre plates (0.28 cm^2 × 96 wells) at a density of 3×10^5 cells/cm^2. For other experiments, cells were cultured in flasks, trypsin-treated and transferred to a spinner flask for 3 h [19].

Protein isoprenylation

HIT-T15 cells [$(0.5–1) \times 10^6$] cultured in 24 multiwell plates (1.77 cm^2 each) were incubated for 24 h in RPMI 1640 medium supplemented with 10% fetal-calf serum and 40 μM [^{14}C]-mevalonolactone (2 μCi/ml). Vehicle, lovastatin (25 μM) or limonene (5 mM) was also included during the 24 h period. Then the cells were rinsed twice with the modified glucose-free Krebs–Ringer bicarbonate–Hepes buffer (KRBH) (containing, in mM: 136 NaCl, 4.8 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 5 $NaHCO_3$, 25 Hepes, at pH 7.4) supplemented with 0.1% BSA, and harvested in 200 μl of SDS buffer [10 mM Tris/HCl, pH 7.9, 7 mg of dithiothreitol/ml, 1 mM EDTA, 3% (w/v) SDS and 13% (w/v) glycerol]. After brief sonication (3 × 1 s) the samples were boiled for 2 min and loaded on a 12%-polyacrylamide gel. The isoprenylated proteins were detected by fluorography.

Detection of SMGs

The SMGs resolved by PAGE and blotted on nitrocellulose membranes were revealed by the [^{32}P]GTP-overlay technique exactly as described in detail previously [21].

Cholesterol measurement

About 10×10^6 cells were cultured in 150 cm^2 plates and treated for 24 h with vehicle, 25 μM lovastatin or 5 mM limonene. After washing three times with serum-free KRBH, the cells were extracted with 0.1 M NaOH for 2 h. Cholesterol was separated by t.l.c. on silica gel-60 plates and quantified at 380 nm by a scanner as described previously [22]. Protein content was determined as described by Lowry et al. [23].

Insulin secretion from intact cells

At 2 days after their seeding, the cells were washed twice with KRBH. Thereafter, they were preincubated for 30 min in the same glucose-free KRBH with indicated test agents at 37 °C. Subsequently, the medium was changed to one containing the various stimuli. Thereafter the cells were incubated for another 15 min. The supernatants were removed, centrifuged at 4 °C and kept at −20 °C until insulin assay. Attached cells were extracted with acid/ethanol for determination of insulin content [20].

Insulin secretion from permeabilized cells

For streptolysin-O permeabilization [24], cells seeded in culture plates were washed twice with Ca^{2+}-free KRBH and once with potassium glutamate buffer [25] at room temperature. Thereafter cells were rendered permeable in the potassium glutamate buffer containing 1.5 units of streptolysin-O/ml for 5 min at 37 °C. The permeabilizing solution was removed. The buffer containing 10.2 mM EGTA and different concentrations of Ca^{2+} and other test agents was then added. The cells were incubated for another 5 min. The supernatants were removed and the cells extracted as mentioned above for measurement of insulin secretion and content, respectively. Insulin was measured by radioimmunoassay with rat insulin as standard [20].

Cell permeability was examined by the Trypan Blue exclusion test. After streptolysin-O treatment, more than 95% of the cells were permeable to the dye.

$[Ca^{2+}]_i$ measurement

Cells were loaded with 1 μM fura-2 acetoxymethyl ester for 30 min at 37 °C in RPMI 1640 medium containing 10 mM Hepes and 1% fetal-calf serum. Cells were washed with glucose-free KRBH without BSA before their transfer into a cuvette (about 1×10^6 cells/ml). Fluorescence was measured in a fluorimeter with excitation and emission wavelengths at 340 and 505 nm respectively. Fluorescence from extracellular fura-2 was assessed by adding 100 μM Mn^{2+} at the beginning and the end of each trace [19,20]. $[Ca^{2+}]_i$ was calculated by using an equation as described previously [26].

Measurement of membrane potential

After two washes, about 3×10^6 cells were placed in a cuvette containing 2 ml of glucose- and BSA-free KRBH. The fluorescent probe bisoxonol [bis-(1,3-diethyl thiobarbiturate)trimethineoxonol] (100 nM) was added. Membrane potential was measured by monitoring the fluorescence at excitation and emission wavelengths of 540 and 580 nm respectively [19,20].

Statistics

The results are expressed as means ± S.E.M. and were analysed by two-tailed unpaired Student's *t* test.

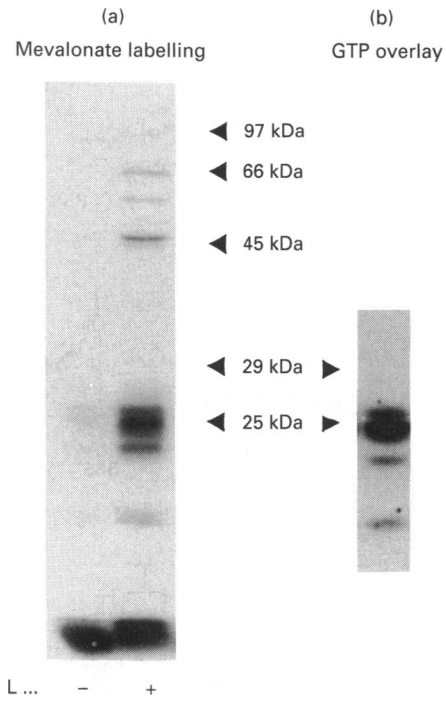

Figure 1 Effect of lovastatin on protein isoprenylation and comparison with small G-proteins in HIT cells

(**a**) HIT-T15 cells were incubated for 24 h with [^{14}C]MVA in the presence (+) or absence (−) of lovastatin (L; 25 μM). At the end of the incubation, the cells were washed twice with KRBH and immediately disrupted in SDS buffer as described in the Materials and methods section. The proteins were then resolved by PAGE (12% acrylamide) and the isoprenylated proteins were detected by fluorography. (**b**) HIT-T15 cells were washed twice with KRBH and disrupted by sonication in SDS buffer. The proteins were separated by PAGE (12% acrylamide), blotted on a nitrocellulose membrane and the SMGs were detected by incubation in the presence of [^{32}P]GTP as described previously [21].

RESULTS

Effect of lovastatin on cell growth, insulin and cholesterol content

It has been found that lovastatin inhibits proliferation in some cell systems [5,12]. After treatment of HIT cells for 24 h with 25 μM lovastatin, neither the number of cells nor their insulin content was altered. The values of the former were $(5.4 \pm 0.3) \times 10^5$ cells/well in controls ($n = 6$) and $(5.5 \pm 0.3) \times 10^5$ cells/well in lovastatin-treated cells ($n = 6$), and the latter values were 293 ± 11 ($n = 4$) and 266 ± 13 ($n = 4$) ng/10^6 cells respectively. Blockade of HMG-CoA reductase by lovastatin inhibits cholesterol production *de novo* and decreases the cell content of cholesterol. However, the high concentration of fetal-calf serum (10%) in the culture medium provides the cells with the external cholesterol contained in lipoproteins of the serum and prevents the decrease in cellular cholesterol during the lovastatin treatment. When cholesterol was measured, no difference was found in cholesterol content between control and lovastatin-treated cells: 20.7 ± 0.8 ($n = 4$) in control cells and 21.5 ± 0.2 μg/mg of protein ($n = 4$) in cells treated with 25 μM lovastatin for 24 h. Similarly, inclusion of 200 μM MVA, the product of HMG-CoA reductase, did not change cellular cholesterol level.

The cell morphology was, however, changed by lovastatin treatment. Control cells grew in clusters and overlapped. The boundaries between cells were ill-defined. After lovastatin treatment (25 μM, 24 h), the cells became more rounded and the cell borders were clearly visible (results not shown). This lovastatin effect could be prevented by addition of 200 μM MVA during the 24 h treatment period. After a higher dose (50 μM) of lovastatin, the cells did not resist washing and became easily detached from the substratum.

Lovastatin effects on protein isoprenylation

Isoprenylated proteins can be labelled with [^{14}C]MVA and made visible by autoradiography after separation on SDS/PAGE. An increase in the labelling of isoprenylated proteins was observed in 24 h lovastatin (25 μM)-treated cells (Figure 1). This indicates that the endogenous production of MVA via HMG-CoA reductase was blocked by lovastatin, since under control conditions endogenously produced MVA is expected to compete with the exogenously added [^{14}C]MVA for the labelling of the proteins. In the presence of lovastatin, cells mainly used the exogenously added [^{14}C]MVA as a precursor for isoprenylation, leading to the increased labelling of isoprenylated proteins. The labelled proteins distributed over a broad molecular-mass range between 20 and 70 kDa (Figure 1). Most of the isoprenylated proteins have not yet been identified. An isoprenylated band in the region of 70 kDa was detected, possibly corresponding to nuclear lamin B according to other studies [27,28]. Most of the isoprenylated proteins migrated, however, with an apparent molecular mass between 20 and 27 kDa (Figure 1).

Effects of lovastatin on the distribution and the electrophoretic mobility of small G-proteins

Several SMGs have been shown to be isoprenylated [1–5] and to require this post-translational modification for correct intracellular localization [1–5,8]. To probe a possible role of SMGs in insulin secretion, the effect of lovastatin on the distribution of these proteins between cytosol and membranes was assessed by the GTP-overlay technique [21]. As previously reported [21], a group of SMGs migrated in the range of 21–27 kDa, and thus co-migrated with the proteins labelled with [^{14}C]MVA (Figure 1). These SMGs were differentially distributed between cytosol and membranes, as seen in Figure 2. After 24 h treatment with 25 μM lovastatin, a dramatic increase in the amount of SMGs recovered in the cytosolic fraction was observed (Figure 2). A concomitant decrease in some of the proteins associated with the membranes was also detectable (Figure 2). In addition, a slight retardation in the electrophoretic mobility of the SMGs was noticed (Figure 2). These effects of lovastatin most probably result from the blockade of HMG-CoA reductase and the consequent impairment of the post-translational processing of the newly synthesized proteins.

Effects of lovastatin on insulin secretion from intact HIT cells

Figure 3 shows the effect of 24 h lovastatin treatment on secretagogue-stimulated and somatostatin-inhibited insulin secretion in HIT cells. Insulin secretion was increased 4-fold when cells were stimulated with a nutrient mixture (10 mM glucose, 5 mM leucine and 5 mM glutamine) during a 15 min incubation period. This effect was not significantly altered by lovastatin treatment (see legend to Figure 3 for absolute values). Bombesin and [arginine]vasopressin (AVP), which activate PLC in insulin-secreting cells [19,20,29], potentiated the mixed nutrient-induced secretion nearly 3-fold. In 25 μM lovastatin-treated cells, the potentiating effect of bombesin and AVP was attenuated by 58% and 62% respectively. The lovastatin effect was dose-

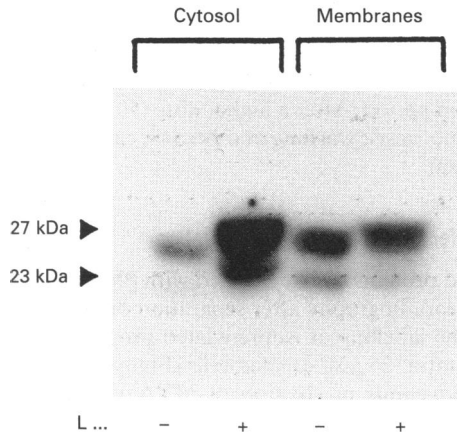

Figure 2 Effect of lovastatin on subcellular distribution of small G-proteins in HIT cells

HIT-T15 cells were incubated for 24 h in the presence (+) or absence (−) of 25 μM lovastatin (L). They were then disrupted by sonication in homogenization buffer (20 mM Tris/HCl, pH 7.5, and 5 mM EDTA) and centrifuged for 1 h at 100000 *g*. The supernatant obtained is referred to as 'cytosol' and the pellet as 'membranes'. The proteins from the two fractions were resolved by PAGE and then transferred to a nitrocellulose membrane. The SMGs were revealed by autoradiography after incubation of the nitrocellulose in the presence of [^{32}P]GTP as previously described [21].

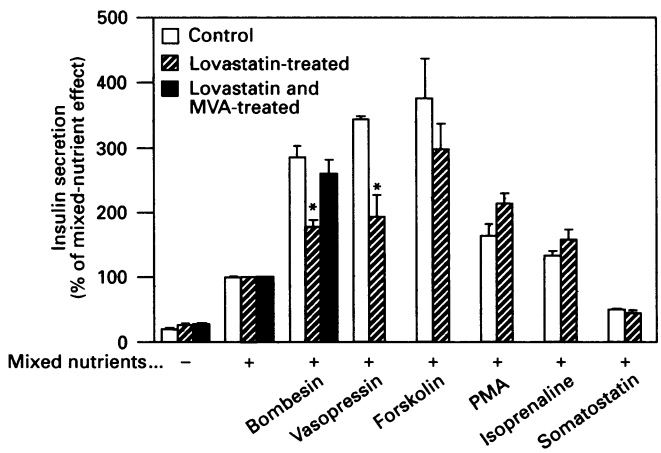

Figure 3 Effect of lovastatin on insulin secretion and reversal of lovastatin action on bombesin stimulation by MVA in HIT cells

Cells seeded in wells of culture plates were pretreated in culture medium with vehicle, 25 μM lovastatin alone or plus 200 μM MVA for 24 h. After washing, the cells were preincubated for 30 min in glucose-free KRBH containing vehicle, 25 μM lovastatin alone or plus 200 μM MVA. The medium was changed to one containing different test agents and the cells were incubated for 15 min. The mixed-nutrient-induced insulin secretion was 4.5 $\pm$ 0.5, 4.1 $\pm$ 0.4, 4.6 $\pm$ 0.5% of cell content in control (n = 23), lovastatin-treated alone (n = 23) and lovastatin plus MVA-treated cells (n = 12) respectively. Values are mean $\pm$ S.E.M. from three to five independent experiments in triplet or sextuplet: *$P < 0.01$ compared with control. The final concentrations were: bombesin, 0.1 μM; vasopressin, 1 μM; forskolin, 1 μM; PMA, 0.1 μM; isoprenaline, 1 μM; somatostatin, 0.1 μM. 'Mixed nutrients' were 10 mM glucose + 5 mM leucine + 5 mM glutamine.

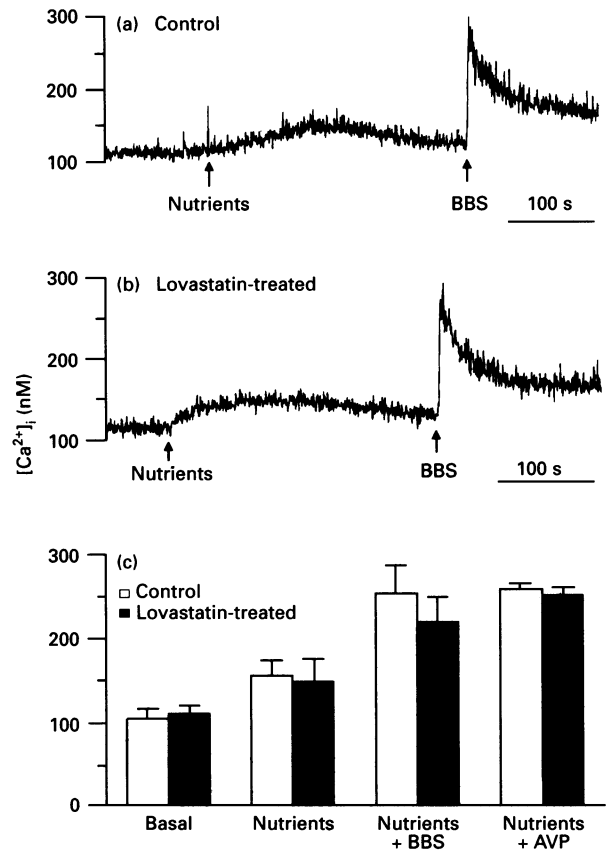

Figure 4 Effect of lovastatin on $[Ca^{2+}]_i$ in nutrient- and bombesin-stimulated HIT cells

Cells were pretreated for 20 h in culture medium with vehicle or 25 μM lovastatin. Thereafter, the cells were detached by mild trypsin treatment, transferred to a spinner flask and kept for 3 h at 37 °C. The spinner contained RPMI 1640 medium supplemented with 20 mM Hepes and 1% newborn-calf serum, with or without 25 μM lovastatin. The fura-2-loaded cells were preincubated for 30 min in glucose-free KRBH before stimulation. When present, 25 μM lovastatin was also included during the fura-2 loading and the preincubation period. The traces are representative of at least three experiments in each case. Values in the columns are means $\pm$ S.E.M. of four experiments. The final concentrations were: nutrients, 10 mM glucose + 1.5 mM leucine + 3 mM glutamine; bombesin (BBS), 0.1 μM; AVP, 1 μM.

dependent, with significant inhibition already seen at 6 μM and maximal action at 25 μM. In contrast, lovastatin did not significantly alter the potentiation caused by the cyclic-AMP-increasing agent forskolin (4-fold), to the protein kinase C activator PMA (1.7-fold), and to the β-adrenergic receptor agonist isoprenaline (1.4-fold). Furthermore, the inhibitory action (50%) of somatostatin on insulin secretion induced by the nutrient mixture was also unaffected by lovastatin. These results indicate that lovastatin does not interfere non-specifically with receptor activation and that it only selectively influences the action of receptor agonists which couple to PLC.

When 200 μM MVA was added together with 25 μM lovastatin during 24 h treatment of the cells, the attenuating effect of lovastatin on bombesin-potentiated insulin secretion was reversed by 88% (Figure 3), suggesting that the inhibitory effect of lovastatin is due to the blockade of HMG-CoA reductase.

Effects of lovastatin treatment on $[Ca^{2+}]_i$ and membrane potential

Certain γ-subunits of the heterotrimeric G-proteins have been reported to be isoprenylated [13,30,31]. If the γ-subunit of the G-protein(s) that couples bombesin and AVP receptors to PLC is isoprenylated in HIT cells, then lovastatin treatment could affect the generation of Ins(1,4,5)P_3 and, in turn, the rise in $[Ca^{2+}]_i$. To examine this possibility, $[Ca^{2+}]_i$ was measured in fura-2-loaded cells (Figure 4). Addition of a mixture of nutrients (10 mM glucose, 1.5 mM leucine and 3 mM glutamine) caused a $[Ca^{2+}]_i$ rise, with a peak of around 50 nM above basal. Subsequent

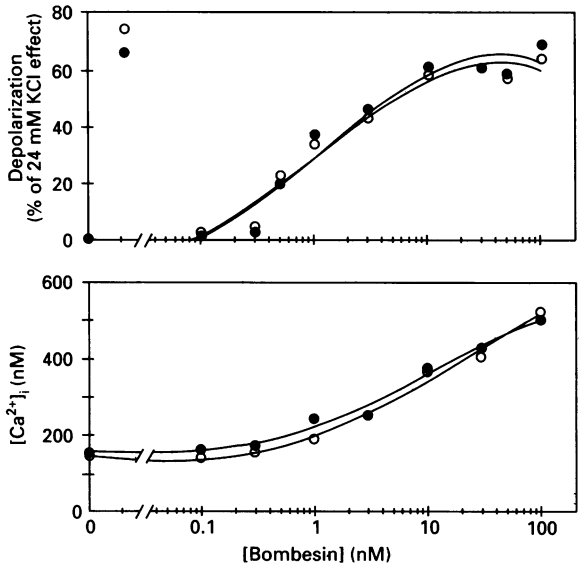

Figure 5 Effect of lovastatin on membrane potential and $[Ca^{2+}]_i$ in bombesin-stimulated HIT cells

Membrane potential and $[Ca^{2+}]_i$ were measured with the fluorescent probes bisoxonol and fura-2 respectively. Values are mean of two experiments.

Table 1 Effect of lovastatin on insulin release from streptolysin-O-permeabilized HIT cells

Cells on culture plates were treated with or without 25 μM lovastatin for 24 h. The cells were permeabilized with 1.5 unit of streptolysin-O/ml for 5 min. Thereafter, the cells were incubated with test agents for 5 min. Values are means ± S.E.M. from four independent experiments.

	Insulin secretion (% of cell content)		
Condition	Control	Lovastatin-treated	*P*
Ca^{2+} (0.1 μM)	1.02 ± 0.10	1.00 ± 0.10	> 0.05
Ca^{2+} (0.1 μM) + GTP[S] (100 μM)	2.32 ± 0.25	2.14 ± 0.29	> 0.05
Ca^{2+} (10 μM)	13.00 ± 1.19	10.09 ± 0.61	< 0.01

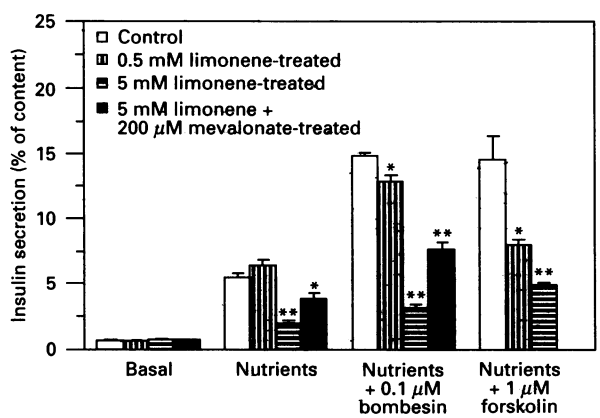

Figure 6 Effect of limonene on insulin secretion induced by various stimuli in HIT cells

Cells seeded in the wells of culture plates were treated in culture medium for 24 h with vehicle, limonene or mevalonate. Values are means ± S.E.M. from three experiments: $^*P < 0.05$ and $^{**}P < 0.01$ compared with control.

stimulation of cells with 0.1 μM bombesin evoked a biphasic $[Ca^{2+}]_i$ increase. The fast-onset $[Ca^{2+}]_i$ initial and the protracted $[Ca^{2+}]_i$ elevations were about 150 nM and 40 nM above basal respectively (Figure 4a). After 24 h treatment with 25 μM lovastatin, neither the basal nor the $[Ca^{2+}]_i$ rises induced by nutrients, bombesin and AVP were significantly affected (Figures 4b and 4c), although the kinetics of $[Ca^{2+}]_i$ in response to nutrients was somewhat modified (Figure 4b). In these experiments, supramaximal concentrations of bombesin were used. We therefore examined whether lovastatin altered the dose–response to bombesin on two different parameters, $[Ca^{2+}]_i$ increase and membrane potential.

Bombesin causes membrane depolarization in HIT cells [19]; its EC_{50} for both $[Ca^{2+}]_i$ increase and membrane depolarization was about 2–5 nM (Figure 5). Lovastatin treatment did not alter the dose–response to bombesin for either parameter measured at the peak effects. These results suggest that the coupling from receptors to PLC is unaltered in lovastatin-treated HIT cells.

Effects of lovastatin on Ca^{2+}-stimulated insulin release from permeabilized cells

To examine whether the exocytosis is affected by lovastatin treatment, insulin release was measured in streptolysin-O-permeabilized cells. As shown in Table 1, the poorly hydrolysable GTP analogue guanosine 5′-[γ-thio]triphosphate (GTP[S]) (100 μM), doubled insulin secretion at basal Ca^{2+} concentration (0.1 μM). Stimulatory concentrations of Ca^{2+} (10 μM) caused a 10-fold increase in the hormone release. After 24 h treatment with 25 μM lovastatin, the GTP[S] effect was unaltered, whereas the insulin release in response to high Ca^{2+} was weakly but significantly decreased.

Limonene effect on insulin secretion, $[Ca^{2+}]_i$ and protein isoprenylation

It has been reported that limonene and its metabolites selectively block the isoprenylation of SMGs, but not of nuclear lamin B, in NIH3T3 and mammary epithelial cells [28]. We compared the actions of limonene and lovastatin in HIT cells. As shown in Figure 6, treatment of cells with limonene inhibited the secretagogue-induced insulin secretion in a dose-dependent manner. However, in contrast with lovastatin, limonene exerted a general inhibitory effect, since nutrient-stimulated and forskolin-potentiated insulin secretion were also affected. In cells treated with 0.5 mM limonene, the mixed nutrient-induced insulin secretion was not affected, whereas bombesin and forskolin effects were inhibited by 14% and 47% respectively. At 2 mM of this agent, the secretion was respectively decreased by 48%, 67% and 60% in response to nutrients, bombesin and forskolin. After 5 mM limonene treatment, hormone secretion stimulated by these secretagogues was decreased by 74%, 82% and 70%. The limonene effect could only be partially overcome by 200 μM MVA. When the cells, which had been treated for 24 h with 2–5 mM limonene, were detached by mild trypsin treatment and kept in spinner culture for 3 h in order to measure the $[Ca^{2+}]_i$ in cell suspensions, we found that most of the cells died. The resting $[Ca^{2+}]_i$ levels in limonene-treated cells were very high (> 600 nM) and the cells did not respond to nutrient, bombesin or high KCl stimulation. Even addition of 1 μM ionomycin only caused a very minute $[Ca^{2+}]_i$ transient. The cholesterol content of the cells was not changed after 24 h treatment with 5 mM limonene (results not shown).

In [^{14}C]MVA-labelled cells, 5 mM limonene treatment for 24 h did not significantly alter the labelling of any of the proteins

observed (results not shown). Limonene treatment probably had major effects on protein content, as judged from the Coomassie Blue pattern observed after PAGE (results not shown). These findings demonstrate that in HIT cells limonene is not a specific inhibitor of SMG isoprenylation and that the drug is toxic to these cells.

DISCUSSION

Nutrients such as glucose and amino acids are the main physiological initiators of insulin release. The secretion induced by these agonists can be further potentiated by hormones and neurotransmitters which generate cyclic AMP by stimulating adenylate cyclase or cause phosphatidylinositol 4,5-bisphosphate breakdown via the activation of PLC [32]. The two products of the latter reaction, Ins(1,4,5)P_3 and diacylglycerol, respectively mobilize Ca^{2+} from intracellular stores and activate protein kinase C. In the present study, we clearly demonstrate that the potentiating effect of the two PLC agonists bombesin and AVP [19,20,29] on nutrient-induced insulin secretion is attenuated by the blockade of HMG-CoA reductase with lovastatin. Several lines of evidence indicate that lovastatin action is selective and not the result of a non-specific toxic effect on the cells. Firstly, neither the nutrient-stimulated insulin secretion nor the influence of other receptor ligands such as isoprenaline or somatostatin was affected by lovastatin treatment. Secondly, the enhanced secretion resulting from the direct activation of protein kinase C (by PMA) or of adenylate cyclase (by forskolin) were also not significantly altered. Thirdly, in streptolysin-O-permeabilized HIT cells, insulin release triggered by GTP[S] was not affected and that by micromolar Ca^{2+} concentrations was only weakly decreased after lovastatin treatment, indicating that the blockade of HMG-CoA reductase is not having a generalized effect on the secretory machinery. Fourthly, exogenously provided MVA, which by-passes the inhibition of HMG-CoA reductase, prevents the action of lovastatin on insulin release, pointing to the implication of MVA metabolites. The specific action of lovastatin on the signal transduction was also seen in other cells. Thus the drug inhibits the secretory and morphological responses of rat basophilic leukaemia cells to antigen, but not to ionomycin and PMA [16]. Lovastatin also selectively inhibits the signal pathways of the progressive growth factors and does not affect the competent ones [33].

Cholesterol, a very important membrane component, is one of the metabolic products of MVA. A decrease in the amount of cholesterol would greatly change the properties of the membranes (especially the plasma membrane) and might therefore have profound effects on cell growth and exocytosis. In our study, however, the cellular cholesterol content was not altered by lovastatin treatment. This is probably due to the fact that our cells in culture can obtain cholesterol from the lipoproteins present in the culture medium, and do not therefore depend on the endogenous steroid production. Similar observations have also been reported by others [14]. MVA metabolism is essential for the cell growth, and both of its products of steroid and non-steroid nature are needed [1]. The escape of HIT-cell growth from inhibition during 24 h lovastatin treatment might be due to the rather long half-life of the isoprenylated proteins involved. Indeed, the SMGs in membranes were only partially decreased after 24 h treatment with lovastatin. Failure of lovastatin (20 h treatment) to affect growth has also been reported for other cells [15]. The lovastatin effect on secretion and morphology could be reversed by MVA, the product of HMG-CoA reductase, pointing to the implication of its metabolites. However, steroid derivatives may not be involved, since cholesterol and dolichol did not restore the compactin-altered NK-cell-killing ability and HL-60 cell NADPH oxidase activity as well as lovastatin-induced secretory and morphological changes in basophils [14,15,17].

Isoprenoids constitute another important class of MVA metabolites. In fact, recently some of these compounds, in particular farnesyl and geranylgeranyl groups, have been shown to be covalently linked to several cellular proteins [1–5]. The post-translational addition of isoprenyl chains is crucial for the correct targeting of the proteins to the membranes [1–5,8]. Several proteins have been reported to be isoprenylated, including the SMGs of the *ras* and the *ras*-related family [1–5], the γ-subunit of heterotrimeric G-proteins [13,30,31] and lamin B [27]. Thus, *a priori*, a possible explanation for the effect of lovastatin on the potentiation of insulin secretion by bombesin and AVP was the inhibition of the isoprenylation of the γ-subunit of the heterotrimeric G-protein that links the receptors to PLC with a consequent uncoupling of the receptor from the phosphatidylinositol 4,5-bisphosphate breakdown. Indeed, lovastatin treatment has been reported to inhibit the antigen-induced Ins(1,4,5)P_3 generation and $^{45}Ca^{2+}$ influx and efflux in basophilic leukaemia cells [15]. This possibility was, however, ruled out, since the $[Ca^{2+}]_i$ rise induced by bombesin and AVP was not altered after the blockade of HMG-CoA reductase. Furthermore, the effects on insulin secretion of two other agonists, isoprenaline and somatostatin, whose receptors are coupled to heterotrimeric G-proteins (G_s and G_i respectively) [34,35], are unchanged in lovastatin-treated cells. These results indicate that, under our experimental conditions, the heterotrimeric G-proteins and, in particular, the one directly activating PLC remain functional, and suggest the involvement of a lovastatin-sensitive site beyond the generation of Ins(1,4,5)P_3 and diacylglycerol in the secretory response to bombesin and AVP.

SMGs, which control a number of important cellular functions and in particular the vesicular transport between organelles [1–5,8,18], constitute another important group of isoprenylated proteins. The farnesyl or the geranylgeranyl groups attached to the cysteines in the C-terminal region of these proteins have been demonstrated to be required for their attachment to membranes [1–5,8]. Thus in HIT cells the lovastatin treatment caused inhibition of protein isoprenylation and accumulation of newly synthesized SMGs in the cytosol. The accumulation of the SMGs in the cytosol was accompanied by a partial decrease in the amount of these proteins in the membrane fraction. Their incorrect subcellular location after lovastatin treatment, together with the fact that the non-isoprenylated SMGs are unable to interact with the regulatory protein GDP-dissociation inhibitor [4], may significantly perturb the functioning of several SMGs. Thus, although direct evidence that SMGs are involved in bombesin and AVP effects on insulin secretion is lacking, the specific action of lovastatin on the potentiation of nutrient-induced insulin release by PLC-activating ligands may be due to the incorrect post-translational processing of these proteins. Indeed, both protein kinase C and the intrinsic tyrosine kinase of some growth-factor receptors have been shown to affect the protein regulating the GTPase activity of *ras* [36,37], suggesting that the activation state of some SMGs may be controlled by bombesin and AVP receptors. Further studies are, however, required to test this hypothesis. It is of interest that compactin inhibited *N*-formylmethionyl-leucylphenylalanine-stimulated NADPH oxidase activity, which is regulated by an SMG [17,38].

In streptolysin-O-permeabilized HIT cells, the GTP[S]-induced secretion at resting Ca^{2+} concentration was not affected by lovastatin treatment. The failure of lovastatin to alter the

secretory and morphological responses to GTP[S] was also reported by others in permeabilized basophils [16]. The mechanism underlying the GTP[S] action on secretion is still not clarified. In insulin-secreting cells we have shown that poorly hydrolysable GTP analogues can trigger exocytosis via a PLC- and protein kinase C-independent pathway [39]. The involvement of a G-protein directly controlling exocytosis and named G_E has been postulated [40]. The identity of this protein remains, however, elusive. In any case, the G-protein controlling exocytosis in permeabilized cells is not affected by the blockade of HMG-CoA reductase during 24 h, suggesting that it does not require a post-translational isoprenylation. Alternatively the possibility remains that after 24 h of lovastatin treatment there is still enough of the isoprenylated proteins left to support the GTP[S] effect.

It has been reported that limonene specifically inhibits the isoprenylation of SMGs without effect on other higher-molecular-mass proteins such as nuclear lamin in some cells [28]. This limonene effect has been proposed to be due to its inhibition of the prenyl-protein transferase specific for SMGs [28]. In our study, treatment of HIT cells for 24 h with 5 mM of this agent, however, did not specifically suppress the isoprenylation of SMGs. In contrast, we observed that limonene has a general inhibitory effect on protein synthesis and insulin secretion induced by various stimuli in HIT cells. Limonene-treated cells tended to die after mild trypsin treatment and had an impaired $[Ca^{2+}]_i$ homoeostasis. The partial reversal of the inhibitory effect of limonene on insulin secretion by MVA could be due to the effect of limonene on HMG-CoA reductase activity via inhibition of synthesis of the enzyme [41]. Therefore, it is questionable whether limonene acts generally as a specific inhibitor of prenyl-protein transferases for SMGs.

In conclusion, the blockade of MVA production by the inhibition of HMG-CoA reductase with lovastatin decreased the isoprenylation of several SMGs and altered their subcellular distribution. This treatment selectively attenuated the potentiating effect of PLC-activating agonists on insulin secretion without affecting the coupling of the receptors to phosphatidylinositol 4,5-bisphosphate breakdown. Our results point to the involvement of an isoprenylated protein, possibly belonging to the SMG family, in the potentiation of nutrient-induced insulin secretion by bombesin and AVP.

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