



Inhibition by simvastatin, but not pravastatin, of glucose-induced cytosolic Ca^{2+} signalling and insulin secretion due to blockade of L-type Ca^{2+} channels in rat islet β -cells

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1 Hypercholesterolaemia often occurs in patients with type 2 diabetes, who therefore encounter administration of HMG-CoA reductase inhibitors. Alteration of pancreatic β -cell function leading to an impaired insulin secretory response to glucose plays a crucial role in the pathogenesis of type 2 diabetes. Therefore, it is important to examine the effects of HMG-CoA reductase inhibitors on β -cell function.

2 Cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) plays a central role in the regulation of β -cell function. The present study examined the effects of HMG-CoA reductase inhibitors on the glucose-induced $[\text{Ca}^{2+}]_i$ signalling and insulin secretion in rat islet β -cells.

3 Simvastatin, a lipophilic HMG-CoA reductase inhibitor, at $0.1\text{--}3\ \mu\text{g ml}^{-1}$ concentration-dependently inhibited the first phase increase and oscillation of $[\text{Ca}^{2+}]_i$ induced by $8.3\ \text{mM}$ glucose in single β -cells. The less lipophilic inhibitor, simvastatin-acid, inhibited the first phase $[\text{Ca}^{2+}]_i$ increase but was two orders of magnitude less potent. The hydrophilic inhibitor, pravastatin ($100\ \mu\text{g ml}^{-1}$), was without effect on $[\text{Ca}^{2+}]_i$.

4 Simvastatin ($0.3\ \mu\text{g ml}^{-1}$), more potently than simvastatin-acid ($30\ \mu\text{g ml}^{-1}$), inhibited glucose-induced insulin secretion from islets, whereas pravastatin ($100\ \mu\text{g ml}^{-1}$) had no effect.

5 Whole-cell patch clamp recordings demonstrated a reversible inhibition of the β -cell L-type Ca^{2+} channels by simvastatin, but not by pravastatin. Simvastatin also inhibited the $[\text{Ca}^{2+}]_i$ increases by L-arginine and KCl, agents that act *via* opening of L-type Ca^{2+} channels.

6 In conclusion, lipophilic HMG-CoA reductase inhibitors can inhibit glucose-induced $[\text{Ca}^{2+}]_i$ signalling and insulin secretion by blocking L-type Ca^{2+} channels in β -cells, and their inhibitory potencies parallel their lipophilicities. Precaution should be paid to these findings when HMG-CoA reductase inhibitors are used clinically, particularly in patients with type 2 diabetes.

Keywords: HMG-CoA reductase inhibitor; simvastatin; pravastatin; lipophilicity; islet; β -cell; cytosolic Ca^{2+} ; L-type Ca^{2+} channel; insulin secretion; diabetes

Abbreviations: $[\text{Ca}^{2+}]_i$, cytosolic calcium concentration, FBS, foetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IVGTT, intravenous glucose tolerance test; KRB, Krebs-Ringer bicarbonate buffer; TEA, tetraethylammonium

Introduction

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are specific, competitive inhibitors of the rate-limiting enzyme in cholesterol biosynthesis (Alberts, 1988). Extended clinical studies on the long-term (2–5.3 years) administration of HMG-CoA reductase inhibitors have demonstrated that these drugs were effective in lowering plasma cholesterol levels and reducing the incidence of cardiovascular disease (Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al.*, 1995; Byington *et al.*, 1995; Sacks *et al.*, 1996). Consequently, HMG-CoA reductase inhibitors have been widely used for the treatment of patients with hypercholesterolaemia (Hoeg, 1990). Hypercholesterolaemia often occurs in diabetic patients and atherosclerotic

cardiovascular disease is one of the major causes of death in diabetic patients. Therefore, diabetic patients are often administered HMG-CoA reductase inhibitors. Alteration of pancreatic β -cell function leading to an impaired insulin secretory response to glucose is thought to play a crucial role in the pathogenesis and progression of type 2 diabetes (non-insulin-dependent diabetes mellitus) (Perley & Kipnis, 1967; Kosaka *et al.*, 1977; Pfeifer *et al.*, 1981; O'Meara & Polonsky, 1994; Porte & Kahn, 1995). Therefore, it is of great importance to investigate the possible inhibitory effects of HMG-CoA reductase inhibitors on β -cell functions. It is well established that the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) plays a central role in the regulation of β -cell functions, including insulin secretion (Wollheim & Sharp, 1981; Gilon *et al.*, 1993; Ammala *et al.*, 1993; Yada *et al.*, 1994; Henquin, 1994). It has recently been shown that glucose-induced oscillations in $[\text{Ca}^{2+}]_i$ in a β -cell line, MIN6 cells, are linked to a long-lasting increase in mitochondrial Ca^{2+} (Nakazaki *et al.*, 1998), a factor which is considered to be involved in the maintenance of the β -cell metabolism (Nakazaki *et al.*, 1998; Kennedy *et al.*,

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Part of this work was presented in abstract forms at the 75th Annual Meeting of the American Endocrine Society, 1993, Abstracts 75: 1983, and the 10th International Symposium on Atherosclerosis, 1994, Abstracts: 242.

1996). Regulation of gene expression by $[Ca^{2+}]_i$ oscillations has recently been demonstrated (Dolmetsch *et al.*, 1998; Li *et al.*, 1998). Therefore, the present study examined the effects of HMG-CoA reductase inhibitors on the glucose-induced $[Ca^{2+}]_i$ signalling, including oscillations, and insulin secretion in islet β -cells.

There are two forms of HMG-CoA reductase inhibitors: a hydrophilic inhibitor, pravastatin, and lipophilic inhibitors such as simvastatin and lovastatin. Simvastatin is lipophilic because of its lactone ring. The open acid form of simvastatin (simvastatin-acid), an *in vivo* active metabolite, is partially hydrophilic (Serajuddin *et al.*, 1991). Pravastatin, occurring in the acid form, is hydrophilic (Serajuddin *et al.*, 1991). Pravastatin and both forms of simvastatin are equally active as HMG-CoA reductase inhibitors for lowering serum cholesterol levels. HMG-CoA reductase inhibitors have been reported to cause myotonia and acute rhabdomyolysis clinically and experimentally, though the frequency is very low (Tobert, 1988; 1995; Hurminghake, 1992; Nakahara *et al.*, 1992). The action of HMG-CoA reductase inhibitors to induce rapid cell damage in L6 myocytes has been shown to be closely related to the lipophilicity of these drugs (Nakahara *et al.*, 1994). In the present study, therefore, the comparative effects of simvastatin, simvastatin-acid, and pravastatin on glucose-stimulated β -cells were examined.

Methods

Preparation of islets and single β -cells and selection of β -cells

Islets and single β -cells were prepared as previously reported (Yada *et al.*, 1992; 1997). Briefly, islets of Langerhans were isolated from Wistar rats aged 8–12 weeks by collagenase digestion. Animals were anaesthetized with an intraperitoneal injection of pentobarbitone at 80 mg kg⁻¹. The abdomen was opened, and collagenase (3 mg ml⁻¹) dissolved in 5 mM Ca²⁺-containing Krebs-Ringer bicarbonate buffer (KRB) was injected into the common bile duct at the distal end after ligation of the duct proximal to the pancreas. The rats were killed by cervical dislocation. The pancreas was dissected out and incubated at 37°C for 17 min. Islets were hand-collected and either used for insulin release experiments or dispersed into single cells in Ca²⁺-free KRB. The single cells were plated on coverslips and maintained in short-term culture for up to 3 days in Eagle's minimal essential medium containing 5.6 mM glucose supplemented with 10% foetal bovine serum (FBS), 100 μ g ml⁻¹ streptomycin and 100 μ U ml⁻¹ penicillin at 37°C in a 95% air and 5% CO₂ atmosphere. The cells during this culture period responded to the test agents in a consistent manner. β -cells were selected according to their morphological and physiological criteria as reported previously (Yada *et al.*, 1995).

Solutions

KRB was composed of (in mM): NaCl 121.7, KCl 4.4, KH₂PO₄ 1.2, CaCl₂ 2.0, MgSO₄ 1.2, NaHCO₃ 5.0 and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES; at pH 7.4) 10 supplemented with 0.1% bovine serum albumin. The standard pipette solution for whole-cell patch clamp recording of Ca²⁺ channel current was composed of (in mM): aspartate 75, tetraethylammonium (TEA) 30, HEPES 11, EGTA 11, MgCl₂ 3, CaCl₂ 1, ATP-Na₂ 3 (Boehringer Mannheim, Indianapolis, IN) and GTP 0.1 (at pH 7.2) (Boehringer).

External solution used for superfusion to establish whole-cell clamp was composed of (in mM): Tris-HCl 100, TEA-Cl 30, HEPES 10 and CaCl₂ (at pH 7.3) 2.

Measurements of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by dual-wavelength fura-2 microfluorimetry combined with imaging as previously reported (Yada *et al.*, 1992; 1994). Briefly, cells on coverslips were incubated with 1 μ M fura-2 acetoxymethylester for 30 min at 37°C in KRB containing 2.8 mM glucose. Cells were then mounted in a chamber and superfused with KRB at a rate of 1 ml min⁻¹ at 37°C. Cells were excited at 340 and 380 nm alternately every 2.5 s, emission signals at 510 nm were detected with an intensified charge-coupled device (ICDD) camera, and ratio images were produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). Ratio values were converted to $[Ca^{2+}]_i$ according to calibration curves (Yada *et al.*, 1992).

Electrophysiological recordings

Voltage-dependent Ca²⁺ channel currents were recorded in single β -cells, under superfusion conditions at 1 ml min⁻¹ at 30°C, using the standard whole-cell configuration of the patch-clamp method (Hamill *et al.*, 1981). Pipettes were pulled from borosilicate glass (Sutter Instruments Co., Novata, CA, U.S.A.), coated with Sylgard 184 (Dow Corning, Midland, MI, U.S.A.) near their tips and fire polished. Their resistance was 2–7 M Ω when filled with the standard pipette solution. Membrane currents were measured using an Axopatch-200B amplifier and the software, pCLAMP 6 (Axon Instruments, Inc., Foster City, CA, U.S.A.). The potential was held at -70 mV and depolarizing voltage pulses to 0 mV with a duration of 100 ms were applied at a frequency of 0.2 Hz. The data were filtered at 5 kHz, digitized at 10 kHz, stored in a computer (IBM; Tokyo, Japan), and analysed with the Clampfit program. The reference potential was the zero-current potential of the pipette obtained immediately after correction for the junction potential and before establishment of the seal.

Measurement of insulin secretion

Measurements of insulin release were carried out as previously described (Yada *et al.*, 1994; 1997). Briefly, groups of five islets, isolated from Wistar rats 8–12 weeks of age, were first incubated for 30 min in KRB containing 2.8 mM glucose for stabilization. Islets were then incubated at 37°C for 30 min in 1 ml of KRB. Insulin concentration was determined by enzyme immunoassay using a kit (Morinaga, Yokohama, Japan).

Materials

Simvastatin, simvastatin-Na (simvastatin-acid) and pravastatin were synthesized and kindly provided by Sankyo Co. (Tokyo, Japan). Simvastatin was dissolved in 100% ethanol, while simvastatin-acid and pravastatin were in distilled water. For experiments, small aliquots of the stock solutions of HMG-CoA reductase inhibitors were added to KRB. The final concentration of ethanol was less than 0.1%, at which it had no effect on $[Ca^{2+}]_i$ in β -cells. Fura-2 and fura-2 acetoxymethylester were obtained from Dojin Chemical (Kumamoto, Japan). FBS was obtained from Life Technologies Inc. (New York, NY, U.S.A.). Mevalonic acid lactone was obtained from Nacalai-Tesque Chemical Co. (Kyoto, Japan). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Nacalai-Tesque.

Statistical analysis

The calculated values were expressed as the mean \pm s.e.mean (n =number of observations). The statistical analysis was carried out with the Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

Results

Temporal profile of the glucose-induced increase in $[Ca^{2+}]_i$ in single rat pancreatic β -cells

$[Ca^{2+}]_i$ in single β -cells at a basal glucose concentration of 2.8 mM ranged between 30 and 150 nM (76.8 ± 3.7 nM for 58 cells). An elevation of the glucose concentration from 2.8 mM to 8.3 or 16.7 mM increased $[Ca^{2+}]_i$ in β -cells in a biphasic manner: an initial peak at around 400–500 nM (first phase) (Figure 1) followed by a moderate elevation at around 100–200 nM which was occasionally superimposed with an oscillation of $[Ca^{2+}]_i$ (second phase), confirming previous reports (Yada *et al.*, 1992; 1994).

In the present study, the effects of HMG-CoA reductase inhibitors on the first phase $[Ca^{2+}]_i$ increase were studied unless otherwise indicated. To evaluate the effects of the inhibitors at the single cell level, glucose stimulation was carried out twice: first in the presence and second in the absence of inhibitors. In control experiments in which β -cells were stimulated twice with 8.3 mM glucose, 24 out of 29 cells responded to the first stimulation and 20 cells to the second stimulation with increases in $[Ca^{2+}]_i$. In 22 out of these 24 responding cells (92%), the response to the first stimulation was either larger than or comparable to the response to the second stimulation (Figure 1a) with regard to the amplitude of the first phase $[Ca^{2+}]_i$ increase. Therefore, if the experiments showed that the response to the first glucose stimulation obtained in the presence of HMG-CoA reductase inhibitors was consistently smaller than the response to the second stimulation obtained after washing out the inhibitors, the inhibition was evident at the single cell level. At the end of experiments, it was confirmed that the cells responded to 300 μ M tolbutamide with large increases in $[Ca^{2+}]_i$, the property characteristic of normal β -cells (Fujitani *et al.*, 1997).

Effects of simvastatin, simvastatin-acid and pravastatin on the glucose-induced first phase $[Ca^{2+}]_i$ increase in single β -cells

In the presence of 0.3 μ g ml⁻¹ simvastatin, the peak of the first phase $[Ca^{2+}]_i$ increase in response to 8.3 mM glucose was reduced (Figure 1b). Simvastatin at a higher concentration of 3 μ g ml⁻¹ strongly inhibited or even abolished the first phase $[Ca^{2+}]_i$ increase (Figure 1c). After washing out simvastatin, the first phase $[Ca^{2+}]_i$ increase in response to the second stimulation with 8.3 mM glucose was restored, indicating that the inhibition was reversible (Figure 1b and c).

The mean amplitude of the first phase $[Ca^{2+}]_i$ increase, averaged for the single β -cells examined, was also reduced by simvastatin at 0.1–3 μ g ml⁻¹ ($P < 0.05$ for 0.1 μ g ml⁻¹, $P < 0.02$ for 0.3 μ g ml⁻¹, $P < 0.001$ for 1 μ g ml⁻¹, and $P < 0.0001$ for 3 μ g ml⁻¹ simvastatin) (Figure 2). Thus, simvastatin inhibits the first phase $[Ca^{2+}]_i$ response to glucose in a concentration-dependent manner.

Simvastatin-acid at 1 and 10 μ g ml⁻¹, concentrations at which simvastatin had inhibitory effects, was without effect on

the first phase $[Ca^{2+}]_i$ increase in response to 8.3 mM glucose. However, simvastatin-acid at 30 μ g ml⁻¹ mildly (Figure 3a) and at 100 μ g ml⁻¹ strongly (Figure 3b) inhibited the first phase $[Ca^{2+}]_i$ increase in a reversible manner. The mean

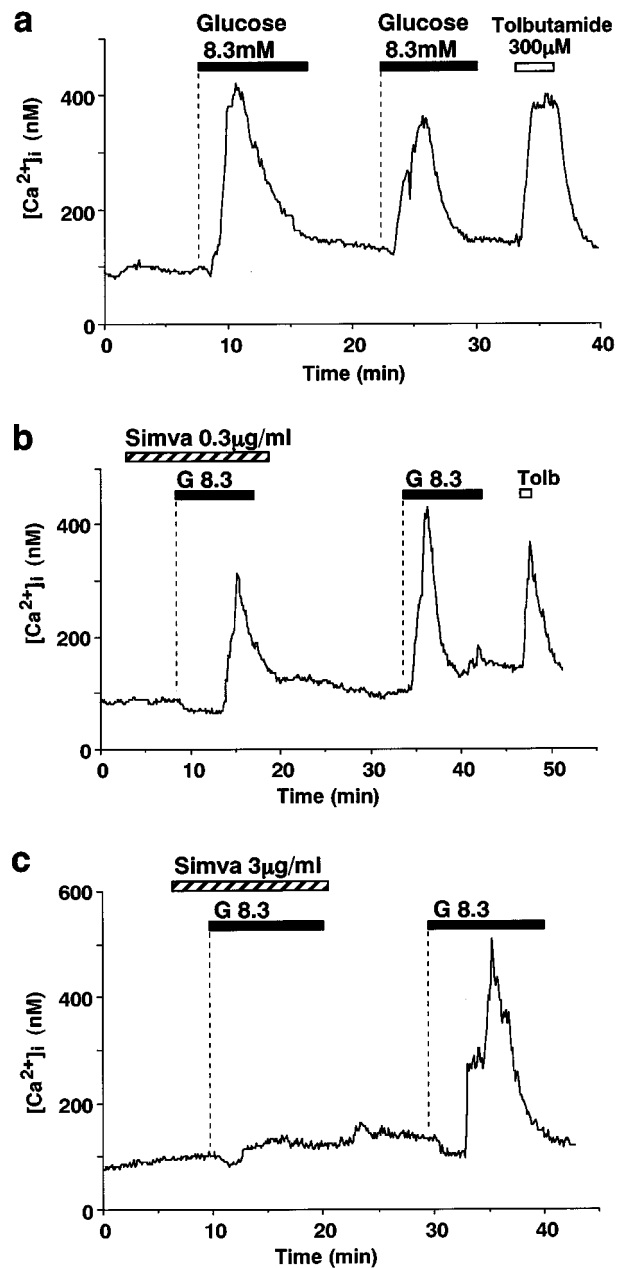


Figure 1 Glucose-induced first phase $[Ca^{2+}]_i$ increases and their inhibition by simvastatin in single β -cells. (a) A rise in glucose concentration from 2.8 to 8.3 mM, under superfusion conditions, evoked the first phase $[Ca^{2+}]_i$ increase in single rat pancreatic β -cells. The majority of β -cells responded to two sequential challenges by 8.3 mM glucose (G8.3) with increases in $[Ca^{2+}]_i$ with a similar pattern. The amplitude of the first phase $[Ca^{2+}]_i$ increase in response to the first challenge was either larger than or comparable to that in response to the second challenge. In the presence of simvastatin (Simva) at 0.3 μ g ml⁻¹ (b) and 3 μ g ml⁻¹ (c), the first phase $[Ca^{2+}]_i$ increase was reduced and abolished, respectively. After washing out simvastatin, the first phase $[Ca^{2+}]_i$ increase was restored in response to the second challenge with 8.3 mM glucose. Tolbutamide at 300 μ M (Tolb) induced rapid increases in $[Ca^{2+}]_i$ as seen in (a) and (b). The bars above the tracing indicate the periods of exposure to the agents specified. Dotted lines indicate beginning of exposure. The basal glucose concentration was 2.8 mM throughout measurements when not indicated otherwise. The results shown are representative of 14 cells in (a), six in (b) and ten in (c).

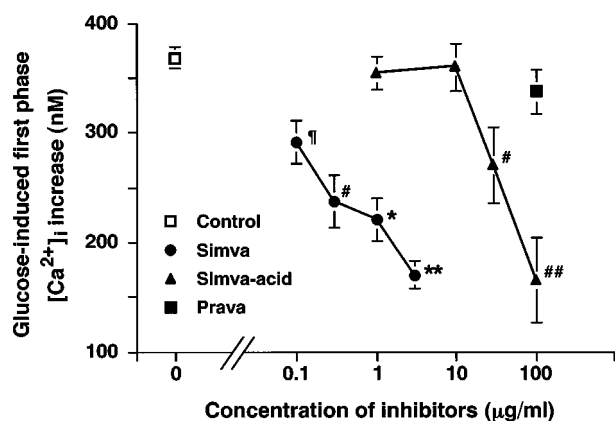


Figure 2 Concentration-response relationship for the inhibition of glucose-induced first phase $[Ca^{2+}]_i$ increases by HMG-CoA reductase inhibitors in single β -cells. The mean amplitude of the first phase $[Ca^{2+}]_i$ increase in response to 8.3 mM glucose was averaged and expressed as the mean \pm s.e. mean. The number of single β -cells examined was 127 for the control and 18–50 for each experimental condition. The mean amplitude of the first phase $[Ca^{2+}]_i$ increase was reduced by simvastatin at 0.1–3 $\mu\text{g ml}^{-1}$ and simvastatin-acid at 30–100 $\mu\text{g ml}^{-1}$. $\nabla P < 0.05$ for 0.1 $\mu\text{g ml}^{-1}$ simvastatin, $\# P < 0.02$ for 0.3 $\mu\text{g ml}^{-1}$ simvastatin and 30 $\mu\text{g ml}^{-1}$ simvastatin-acid, $\#\# P < 0.002$ for 100 $\mu\text{g ml}^{-1}$ simvastatin-acid, $* P < 0.001$ for 1 $\mu\text{g ml}^{-1}$ simvastatin, and $** P < 0.0001$ for 3 $\mu\text{g ml}^{-1}$ simvastatin vs the control.

amplitude of the first phase $[Ca^{2+}]_i$ increase was significantly reduced by 30 and 100 $\mu\text{g ml}^{-1}$ simvastatin-acid ($P < 0.01$ for 30 $\mu\text{g ml}^{-1}$ and $P < 0.002$ for 100 $\mu\text{g ml}^{-1}$) (Figure 2). Thus, simvastatin-acid inhibits the first phase $[Ca^{2+}]_i$ response to glucose in a concentration-dependent manner, but the inhibitory action is about two orders of magnitude less potent than simvastatin (Figure 2).

Pravastatin in a concentration range up to 100 $\mu\text{g ml}^{-1}$, when added to the superfusion solution, failed to affect the $[Ca^{2+}]_i$ response to 8.3 mM glucose (Figures 2 and 3c). The results indicated that the hydrophilic HMG-CoA reductase inhibitor was without an acute effect on the β -cell response to glucose.

Effects of simvastatin and pravastatin on the glucose-induced $[Ca^{2+}]_i$ oscillation in single β -cells

It has been suggested that $[Ca^{2+}]_i$ oscillations in islet β -cells are causally related to the pulsatile secretion of insulin (Gilon *et al.*, 1993; Hellman *et al.*, 1992; Bergsten, 1995) which plays an important role in the physiological control of glucose metabolism (O'Meara & Polonsky, 1994; Matthews *et al.*, 1983; O'Rahilly *et al.*, 1988). Therefore, the effects of HMG-CoA reductase inhibitors on the $[Ca^{2+}]_i$ oscillations were examined. Simvastatin at 0.3 $\mu\text{g ml}^{-1}$ partially and at 3 $\mu\text{g ml}^{-1}$ almost completely inhibited the $[Ca^{2+}]_i$ oscillations induced by 8.3 mM glucose, and they were either fully or partly reversible after washing out the inhibitor (Figure 4a). Simvastatin at 0.1 and 1 $\mu\text{g ml}^{-1}$ also partially inhibited the $[Ca^{2+}]_i$ oscillations (data not shown). In contrast, pravastatin in a concentration range up to 100 $\mu\text{g ml}^{-1}$ was without any appreciable effect (Figure 4b). Among 14 cells that exhibited $[Ca^{2+}]_i$ oscillations, only two cells (14%) continued to clearly oscillate during treatment with 1 $\mu\text{g ml}^{-1}$ simvastatin for about 15 min, and 13 cells (93%) oscillated after washing out this drug, showing a strong and reversible inhibition (Figure

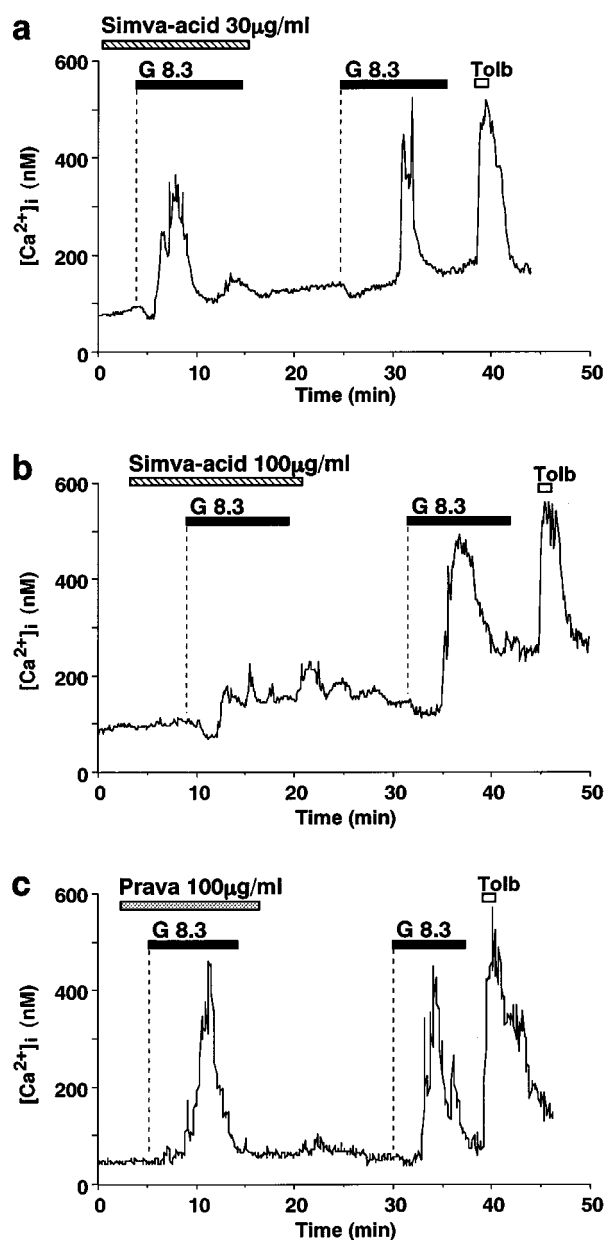


Figure 3 Simvastatin-acid, but not pravastatin, inhibits glucose-induced first phase $[Ca^{2+}]_i$ increases in single β -cells. Simvastatin-acid (Simva-acid) at 30 $\mu\text{g ml}^{-1}$ reduced (a) and at 100 $\mu\text{g ml}^{-1}$ abolished (b) the first phase $[Ca^{2+}]_i$ increase in response to 8.3 mM glucose (G8.3). After washing out simvastatin-acid, the first phase $[Ca^{2+}]_i$ increase was restored in response to the second challenge with 8.3 mM glucose. (c) Pravastatin (Prava) at 100 $\mu\text{g ml}^{-1}$ had no effects on the first phase $[Ca^{2+}]_i$ increase in response to 8.3 mM glucose. The results shown are representative of eight cells in (a), six in (b) and 17 in (c).

4c). In contrast, among 18 cells that exhibited $[Ca^{2+}]_i$ oscillations, 17 (94%) and 15 cells (88%) exhibited oscillations during treatment with and after washing out 100 $\mu\text{g ml}^{-1}$ pravastatin, respectively, in a manner similar to that before the drug treatment.

Effects of simvastatin and pravastatin on glucose-induced insulin secretion from islets

Insulin release from rat islets, under static incubation conditions, was stimulated with 8.3 mM glucose. The

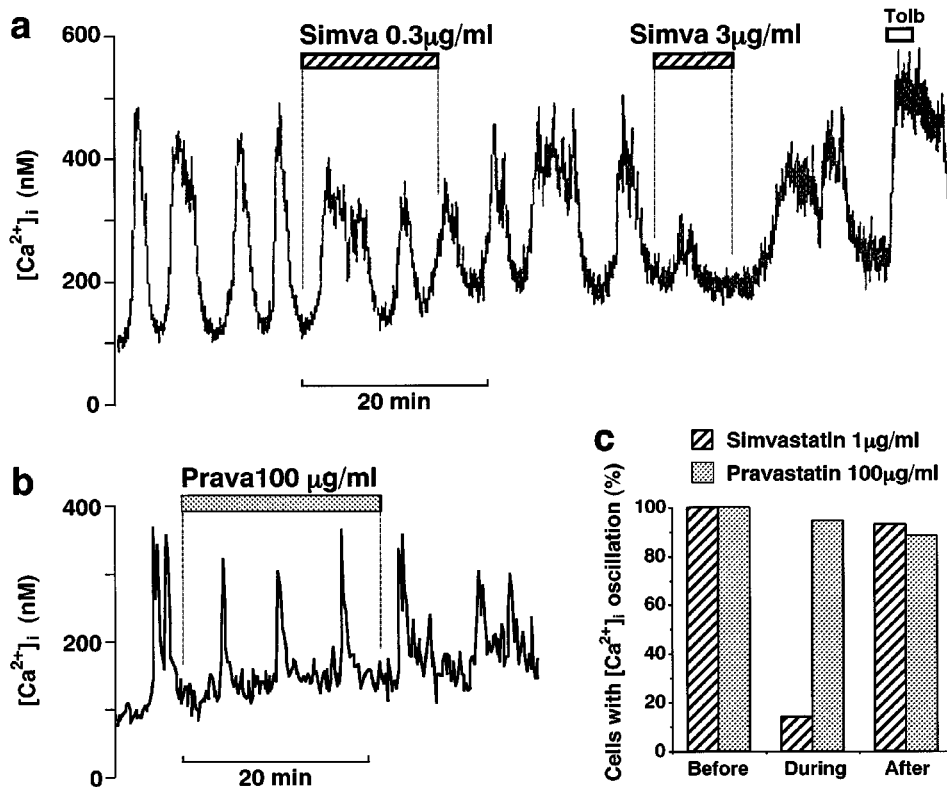


Figure 4 Simvastatin, but not pravastatin, inhibits glucose-induced $[Ca^{2+}]_i$ oscillations in single β -cells. (a) Simvastatin (Simva) at $0.3 \mu\text{g ml}^{-1}$ partially and at $3 \mu\text{g ml}^{-1}$ almost completely inhibited $[Ca^{2+}]_i$ oscillations induced by 8.3 mM glucose in a reversible manner. (b) Pravastatin (Prava) at $100 \mu\text{g ml}^{-1}$ had no effects on $[Ca^{2+}]_i$ oscillations. The results shown are representative of five cells in (a) and 15 in (b). (c) Effects of simvastatin and pravastatin on the incidence of $[Ca^{2+}]_i$ oscillations. Among the 14 cells exhibiting $[Ca^{2+}]_i$ oscillations in response to 8.3 mM glucose, two cells oscillated during treatment with $1 \mu\text{g ml}^{-1}$ simvastatin for about 15 min, and 13 cells oscillated after washing out this drug. In contrast, among the 18 oscillating cells, 17 and 15 cells oscillated during treatment with and after washing out $100 \mu\text{g ml}^{-1}$ pravastatin, respectively.

stimulated insulin release was significantly reduced in the presence of $0.3 \mu\text{g ml}^{-1}$ simvastatin ($P < 0.05$) (Figure 5). Simvastatin-acid at $30 \mu\text{g ml}^{-1}$, the concentration at which it moderately attenuated the first phase $[Ca^{2+}]_i$ response to glucose, mildly attenuated insulin release, though statistically not significant. In contrast pravastatin at $100 \mu\text{g ml}^{-1}$ failed to affect insulin release.

Effects of simvastatin and pravastatin on L-type Ca^{2+} channels in single β -cells

It has been shown that the glucose-induced increase in $[Ca^{2+}]_i$ in β -cells is due mainly, if not solely, to the Ca^{2+} influx through the L-type Ca^{2+} channels in the β -cell plasma membrane (Henquin, 1994; Ashcroft & Rorsman, 1989). Therefore, we investigated the effect of HMG-CoA reductase inhibitors on the Ca^{2+} currents of single pancreatic β -cells with the whole-cell voltage-clamp configuration. In the control external solution containing 2.8 mM glucose, a depolarizing pulse from a holding potential of -70 to 0 mV evoked a long-lasting inward current with an amplitude of about -70 pA (Figure 6Aa, B and C), the properties of which fit well with those of the current passing through the L-type Ca^{2+} channels in β -cells (Ashcroft & Rorsman, 1989). Upon exposure to $3 \mu\text{g ml}^{-1}$ simvastatin, the current decreased rapidly and markedly (Figure 6Ab and B), and upon washing out simvastatin the current was

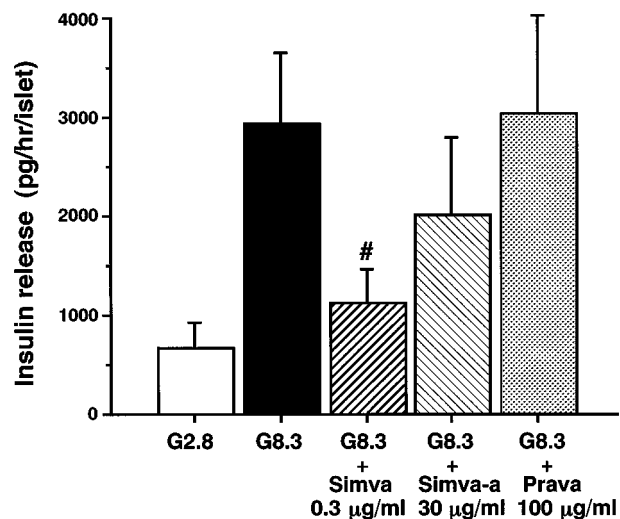


Figure 5 Simvastatin and simvastatin-acid, but not pravastatin, inhibit glucose-induced insulin secretion in islets. Insulin release from islets stimulated with 8.3 mM glucose, under static incubation conditions, was inhibited in the presence of $0.3 \mu\text{g ml}^{-1}$ simvastatin (Simva). Glucose-stimulated insulin release was mildly reduced by $30 \mu\text{g ml}^{-1}$ simvastatin-acid (Simva-a), but not significantly. Pravastatin (Prava) at $100 \mu\text{g ml}^{-1}$ was without effect on glucose-stimulated insulin release. The results are expressed as the mean \pm s.e. mean of six experiments. #, Significant difference ($P < 0.05$) vs the 8.3 mM glucose (G8.3) group and the pravastatin group.

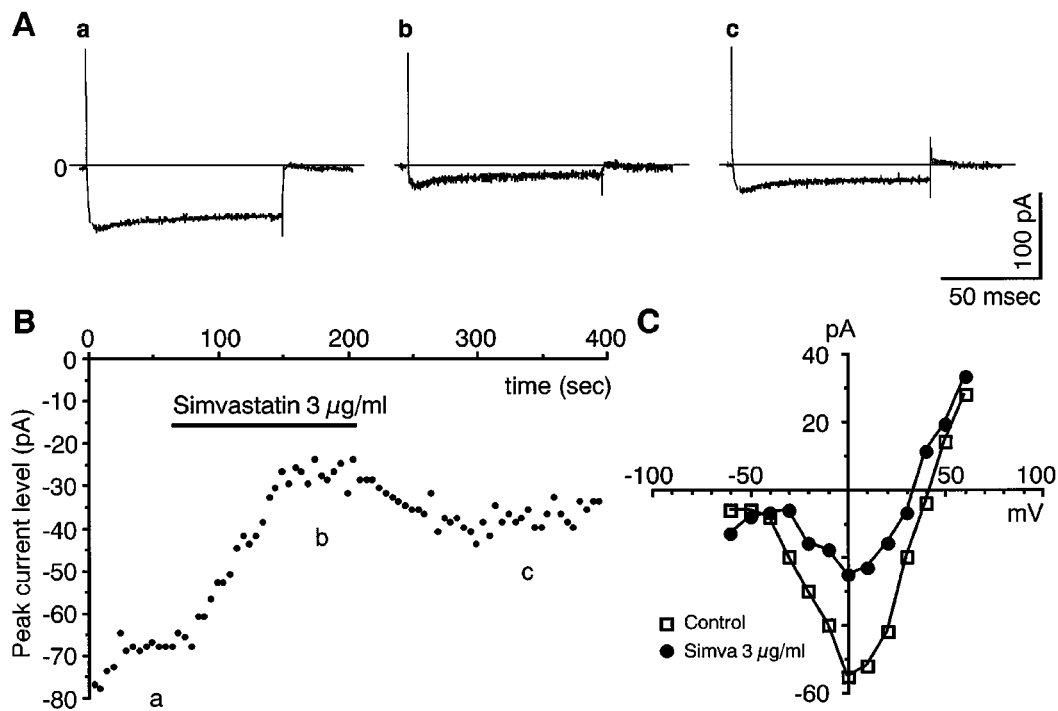


Figure 6 Simvastatin inhibits Ca^{2+} currents in single β -cells. (A) Whole-cell Ca^{2+} currents in a single β -cell depolarized to 0 mV from the holding potential of -70 mV before (a), during (b) and after exposure to $3 \mu g ml^{-1}$ simvastatin (c) under superfusion conditions. (B) Recording of the temporal change of the whole-cell Ca^{2+} currents. a, b and c, specify the time points at which the current traces in Aa, Ab and Ac were taken. (C) The current-voltage relationship of the peak Ca^{2+} current amplitude in the control and in the presence of $3 \mu g ml^{-1}$ simvastatin. The results shown are representative of five cells in (A) and (B) and three in (C).

partially restored (Figure 6Ac and B). Current-voltage relationships before and during exposure of β -cells to $3 \mu g ml^{-1}$ simvastatin are shown in Figure 6C. Simvastatin reduced the Ca^{2+} current amplitude without shifting the current-voltage relationship along the voltage axis. In contrast, pravastatin at $100 \mu g ml^{-1}$ was without effect on the Ca^{2+} current (data not shown).

Inhibition of arginine- and KCl-induced $[Ca^{2+}]_i$ increases in single cells by simvastatin

The Ca^{2+} influx through the L-type Ca^{2+} channels is thought to be the principal mechanism not only for the glucose- but also for the L-arginine- and KCl-induced release of insulin (Ashcroft & Rorsman, 1989). Arginine, a cationic amino acid, at relatively high concentrations (1–20 mM), directly depolarizes the β -cell plasma membrane when it is transported across it, resulting in a voltage-dependent activation of L-type Ca^{2+} channels and an increase in $[Ca^{2+}]_i$ (Henquin & Meissner, 1981; Blachier *et al.*, 1989; Yada, 1994; Smith *et al.*, 1997). KCl, *via* a direct depolarization, also induces an activation of L-type Ca^{2+} channels and a sustained increase in $[Ca^{2+}]_i$ (Yaekura *et al.*, 1996). The result that simvastatin inhibited the L-type Ca^{2+} channels urged us to examine whether this drug could inhibit the increases in $[Ca^{2+}]_i$ induced by arginine and KCl. Sequential administration of 10 mM arginine in brief pulses evoked a repetitive increase in $[Ca^{2+}]_i$, and in the presence of simvastatin the $[Ca^{2+}]_i$ response to arginine was markedly inhibited in a reversible manner (Figure 7a). A sustained increase in $[Ca^{2+}]_i$ was induced by 25 mM KCl and it was markedly reduced by simvastatin (Figure 7b). The inhibition started at about 20 s after administration of the drug.

Discussion

The novel findings of our study were: (1) Among the HMG-CoA reductase inhibitors, simvastatin was about two orders of magnitude more potent than simvastatin-acid in inhibiting glucose-induced $[Ca^{2+}]_i$ signalling in rat islet β -cells, whereas pravastatin was without effect; (2) Simvastatin inhibited glucose-induced insulin secretion from islets more potently than simvastatin-acid, whereas pravastatin was ineffective; (3) Simvastatin, but not pravastatin, inhibited L-type Ca^{2+} channels in β -cells; and (4) Simvastatin also inhibited the $[Ca^{2+}]_i$ increases induced by L-arginine and KCl in β -cells.

Only the HMG-CoA reductase inhibitors that attenuated the glucose-induced first phase $[Ca^{2+}]_i$ increases and $[Ca^{2+}]_i$ oscillations, i.e., simvastatin and simvastatin-acid, attenuated the glucose-induced insulin secretion, whereas pravastatin affected neither of these parameters. The rank order of potency for the inhibition of the $[Ca^{2+}]_i$ increase (simvastatin > simvastatin-acid >> pravastatin) was identical to that for the inhibition of insulin secretion. Simvastatin at $0.3 \mu g ml^{-1}$ partially inhibited both the $[Ca^{2+}]_i$ increase and insulin secretion, and the extent of inhibition was somewhat larger for insulin secretion, which may fit with the longer period of simvastatin treatment for insulin secretion (30 min) than for $[Ca^{2+}]_i$ (10–15 min). The increase in $[Ca^{2+}]_i$ is the principal signal that triggers insulin secretion (Wollheim & Sharp, 1981; Gilon *et al.*, 1993; Ammala *et al.*, 1993; Yada *et al.*, 1994; Henquin, 1994). These observations taken together suggest that the inhibition of the $[Ca^{2+}]_i$ increase accounts for the inhibition of insulin secretion.

The whole-cell patch-clamp study clearly demonstrated that simvastatin inhibits L-type Ca^{2+} channels in β -cells. This notion was further strengthened by the finding that simvastatin

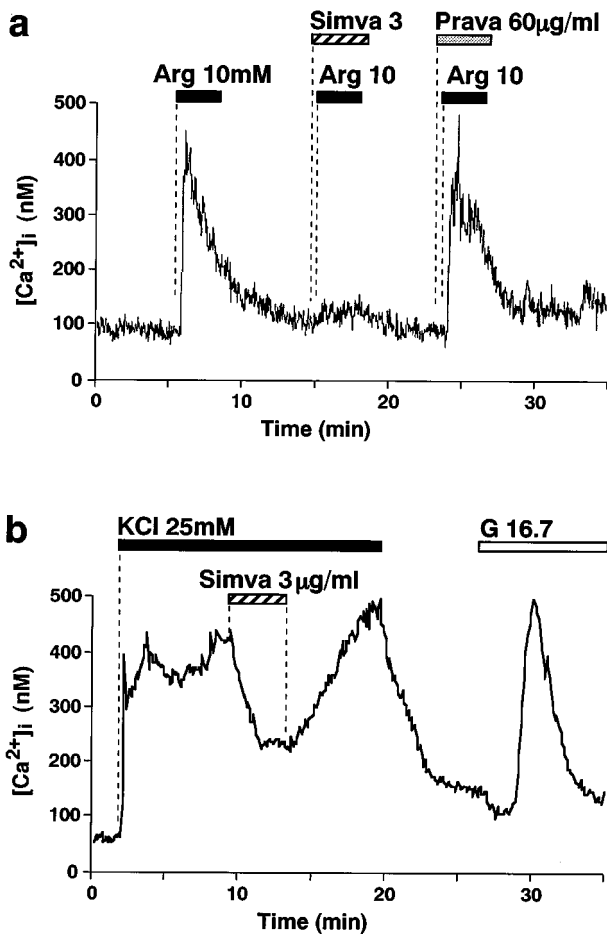


Figure 7 Simvastatin (Simva) inhibits $[Ca^{2+}]_i$ increases in response to L-arginine (Arg) and KCl in single β -cells. (a) An increase in $[Ca^{2+}]_i$ induced by 10 mM L-arginine was inhibited in the presence of $3 \mu\text{g ml}^{-1}$ simvastatin, but not $60 \mu\text{g ml}^{-1}$ pravastatin (Prava). (b) A sustained increase in $[Ca^{2+}]_i$ induced by 25 mM KCl was almost instantaneously inhibited by the addition of $3 \mu\text{g ml}^{-1}$ simvastatin, and it was restored upon washing out the drug. The glucose concentration was 8.3 mM in (a) and 2.8 mM in (b). G16.7: change to 16.7 mM glucose. The results shown are representative of five cells in (a) and 14 in (b).

inhibited the $[Ca^{2+}]_i$ increases induced by arginine and KCl, the insulin secretagogues that activate L-type Ca^{2+} channels and, consequently, increase $[Ca^{2+}]_i$ (Henquin & Meissner, 1981; Blachier *et al.*, 1989; Yada, 1994; Smith *et al.*, 1997; Yaekura *et al.*, 1996). The temporal profiles of the inhibition of Ca^{2+} currents by simvastatin and its recovery upon washout of the drug were similar to those of the inhibition of $[Ca^{2+}]_i$ increases (Figure 6B vs Figure 7b). Simvastatin inhibited both L-type Ca^{2+} channels and $[Ca^{2+}]_i$ increases while pravastatin had no effect on either; therefore, the two activities changed in parallel. The Ca^{2+} influx through L-type Ca^{2+} channels is thought to be the major route of glucose-induced $[Ca^{2+}]_i$ increases in β -cells (Henquin, 1994; Ashcroft & Rorsman, 1989). Accordingly, our results indicate that the inhibition of L-type Ca^{2+} channels is the major, if not the only, cause for the inhibition of $[Ca^{2+}]_i$ increases. Alternatively, it is possible that the inhibitory action of HMG-CoA reductase inhibitors is caused by the reduced level of mevalonate, that results from the inhibition of HMG-CoA reductase which catalyzes the formation of mevalonate from HMG-CoA (Alberts, 1988). However, in the presence of 100 μM mevalonic acid lactone for both preincubation (30 min) and experimental periods,

simvastatin inhibited the first phase $[Ca^{2+}]_i$ response to glucose in a manner similar to the control experiments without mevalonate (data not shown). The result that inhibition of Ca^{2+} currents and $[Ca^{2+}]_i$ increases started as soon as 10–20 s after administration of simvastatin suggests that inhibition of L-type Ca^{2+} channels and $[Ca^{2+}]_i$ increases is not mediated by the metabolic change that reduces mevalonate levels, but is due to a more direct interaction of the drug with the plasma membrane L-type Ca^{2+} channels.

Simvastatin at 0.1 – $3 \mu\text{g ml}^{-1}$, administered under superfusion conditions, concentration-dependently attenuated the glucose-induced first phase $[Ca^{2+}]_i$ increase and $[Ca^{2+}]_i$ oscillation, in a fully or partially reversible manner. The less lipophilic simvastatin-acid in this concentration range was without effect, but at the higher doses of 30 and 100 $\mu\text{g ml}^{-1}$, it attenuated the first phase $[Ca^{2+}]_i$ increases. Thus, simvastatin-acid was about two orders of magnitude less potent than simvastatin. The hydrophilic pravastatin at concentrations up to 100 μM affected neither the first phase $[Ca^{2+}]_i$ increase nor the $[Ca^{2+}]_i$ oscillation induced by glucose. The overwhelming potency of simvastatin over pravastatin has also previously been reported for worsening of segment shortening in the reperfused myocardium in dogs (Ichihara *et al.*, 1993) and for inhibition of proliferation of human smooth muscle cells (Negre-Arminou *et al.*, 1997). In our study and these reports, the inhibitory potencies of the HMG-CoA reductase inhibitors appeared to parallel their lipophilicities. It was shown that the partition coefficient between water and n-octanol, an indicator of lipophilicity, is 25118 for simvastatin, 75.8 for simvastatin-acid, and 0.34 for pravastatin (Serajuddin *et al.*, 1991), yielding a relative lipophilic ratio of about 70000:2000:1. This ratio fits well with the relative potency of the HMG-CoA reductase inhibitor for the inhibition of $[Ca^{2+}]_i$ increases and insulin secretion observed in the present study. Therefore, it is likely that the highly lipophilic simvastatin has a strong affinity for the cell membrane and, consequently, may also have an easy access to the intracellular space, and that these properties are related to the ability of simvastatin to effectively inhibit Ca^{2+} channels in the β -cell plasma membrane and, thereby, inhibit $[Ca^{2+}]_i$ and insulin responses to glucose. In contrast, hydrophilic pravastatin has a limited rate of access to the plasma membrane and the intracellular space, which likely accounts for the lack of immediate effects of this drug on Ca^{2+} channels, $[Ca^{2+}]_i$ increases and insulin secretion. In support of this idea, in β -cells which had been cultured for 1 day with a high concentration of pravastatin ($100 \mu\text{g ml}^{-1}$), the amplitude of the first phase $[Ca^{2+}]_i$ response to 8.3 mM glucose was mildly attenuated in comparison to that in the paired β -cells cultured under control conditions (T. Yada, unpublished data).

It has been reported that in healthy volunteers, after oral administration of simvastatin, the serum concentration of the sum of simvastatin and its active metabolites reaches a level around $0.01 \mu\text{g ml}^{-1}$ (Koga *et al.*, 1995). In our results, the concentration at which simvastatin exhibited an immediate and significant inhibition of glucose-induced $[Ca^{2+}]_i$ increase was $0.1 \mu\text{g ml}^{-1}$ and, therefore, one order of magnitude higher. However, since HMG-CoA reductase inhibitors are often used for a long period of several years or more (Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al.*, 1995; Byington *et al.*, 1995; Sacks *et al.*, 1996), the inhibitory effect might be evoked by simvastatin at lower concentrations when used chronically. Hypercholesterolaemia often occurs in diabetic patients. Since functional alterations of β -cells are known to be associated with type 2 diabetes (O'Meara & Polonsky, 1994; Porte & Kahn, 1995),

the possibility cannot be excluded that β -cells in some type 2 diabetic patients are more susceptible to the inhibition by lipophilic HMG-CoA reductase inhibitors. In type 2 diabetic patients with a reduced insulin secretory ability (Perley & Kipnis, 1967; Kosaka *et al.*, 1977; Pfeifer *et al.*, 1981), a further attenuation of the β -cell activity by simvastatin, even to a mild extent could have a pathophysiologically significant influence on glucose metabolism. It is also possible that the serum simvastatin reaches higher levels than $0.01 \mu\text{g ml}^{-1}$ in the patients with reduced rates of drug metabolism and/or excretion due to more or less impaired liver and/or kidney functions, which is the case in some patients with type 2 diabetes. A recent report (Neuvonen *et al.*, 1998) showed that when simvastatin was combined with the CYP3A4 inhibitor itraconazole, the serum concentration of simvastatin and simvastatin-acid increased one order of magnitude higher and exceeded $0.1 \mu\text{g ml}^{-1}$, the level at which the inhibition of β -cells were observed in the present study. It has also been reported that serum concentrations of fluvastatin, a HMG-CoA reductase inhibitor which has a similar lipophilicity to simvastatin-acid, reach a level as high as $0.25 \mu\text{g ml}^{-1}$ in healthy subjects (Appel *et al.*, 1995).

The effect of HMG-CoA reductase inhibitors on glucose metabolism and blood glucose level is an issue of great clinical importance. Impairment in insulin secretion and/or insulin action (insulin sensitivity) are considered to be the two major causes for the impaired glucose tolerance and type 2 diabetes. Regarding the effects of HMG-CoA reductase inhibitors on insulin action, it has been shown that lovastatin, a lipophilic HMG-CoA reductase inhibitor, disrupts the early events of insulin signalling, which include tyrosine phosphorylation of the insulin receptor β subunit and MAP kinase, association of the p85 subunit of PI-3 kinase with insulin receptor substrate-1, and activation of MAP kinase (Xu *et al.*, 1996; McGuire *et al.*, 1994). It has been reported that administration of simvastatin (Ohrvall *et al.*, 1995; Nielsen *et al.*, 1993) or pravastatin (Sheu *et al.*, 1994) in patients with hypercholesterolaemia does not improve or somewhat worsens insulin resistance despite their marked LDL-cholesterol-lowering

effect. Regarding the effects on blood glucose, it has been shown that plasma glucose concentrations from 08.00–16.00 h in response to breakfast and lunch were moderately but significantly higher after pravastatin treatment (Sheu *et al.*, 1994). A study in patients with type 2 diabetes and hyperlipidaemia has demonstrated that simvastatin treatment significantly elevated plasma glucose concentrations both at fasting and during an intravenous glucose tolerance test (IVGTT), which was accompanied by an increment of fasting insulin concentrations but not of insulin responses to IVGTT (Ohrvall *et al.*, 1995). This result may imply that the plasma insulin concentration increases to compensate for the impairment of insulin sensitivity but not to a sufficient extent to maintain blood glucose at a low level, thereby resulting in hyperglycaemia (Ohrvall *et al.*, 1995). Although the number of reports are limited, these *in vitro* and *in vivo* studies indicate reduction of insulin sensitivity by treatment with HMG-CoA reductase inhibitors. In addition, the present study revealed a novel action of simvastatin to directly inhibit the β -cell insulin secretory response to glucose. Thus, it appears that lipophilic HMG-CoA reductase inhibitors are able to impair secretion and may also affect insulin sensitivity and, consequently, could accelerate the pathogenesis and progression of type 2 diabetes.

In conclusion, our results reveal that simvastatin, the lipophilic HMG-CoA reductase inhibitor, has an ability to inhibit glucose-induced insulin secretion by inhibiting L-type Ca^{2+} channel and $[Ca^{2+}]_i$ signalling in pancreatic β -cells. Caution should be taken with this finding when HMG-CoA reductase inhibitors are used clinically, especially in patients with type 2 diabetes. Since L-type Ca^{2+} channels and $[Ca^{2+}]_i$ signalling regulate a variety of β -cell functions including insulin biosynthesis, possible effects of HMG-CoA reductase inhibitors on the β -cell functions other than secretion also need to be investigated.

This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan (to T.Y. and M.K.).

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