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Lovastatin Induces Relaxation and Inhibits L-Type Ca^{2+} Current in the Rat Basilar Artery

Andreas Bergdahl, Erik Persson, Per Hellstrand and Karl Swärd

Division of Molecular and Cellular Physiology, Department of Physiological Sciences, Lund University,
Biomedical Center, BMC F12, S-22184 Lund, Sweden

Abstract: Statins inhibit cholesterol biosynthesis and protect against ischaemic stroke. It has become increasingly apparent that the beneficial effects of statin therapy may extend beyond lowering of serum cholesterol. The present study was done to explore possible pleiotropic statin effects at the level of the cerebral vascular smooth muscle. Lovastatin, lovastatin acid, simvastatin and pravastatin, were added to segments of the rat basilar artery and effects on contraction and Ca^{2+} handling were examined. Pravastatin had no effect on contraction. Simvastatin, lovastatin, and, to a lesser degree, lovastatin acid, caused relaxation (IC_{50}=0.8, 1.9 and 22 µmol/l) of both intact and denuded arteries precontracted with 5-HT or high-K^+. This effect was not reversed by mevalonate, suggesting that it was not related to cholesterol or isoprenoid metabolism. Relaxation was associated with a reduction of the intracellular Ca^{2+} concentration measured with Fura 2 and with a reduced Mn^{2+} quench rate, suggesting a direct effect on ion channels in the smooth muscle cell membrane. Current measurements in isolated and voltage clamped basilar artery muscle cells demonstrated that both lovastatin and lovastatin acid inhibit L-type Ca^{2+} current. We propose that lipophilicity is an important factor behind the effects of statins on vascular tone and that Ca^{2+} current inhibition is the likely mechanism of action.

Statins, used for treatment of hypercholesterolaemia, reduce the relative risk of major coronary events and ischaemic stroke by ≈30% (Crouse *et al.* 1998; Maron *et al.* 2000). Inhibition of the cholesterol biosynthetic enzyme 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase in the liver is believed to be the primary mechanism of action, which leads to upregulation of liver low-density lipoprotein receptors and clearance of plasma low-density lipoprotein. Both hydrophobic and hydrophilic statins are available and this physicochemical property influences the tissue specificity. The hydrophilic statins do not pass extrahepatic cell membranes with ease but are actively transported into hepatic cells by organic anion transporters. Hydrophobic statins, on the other hand, cross lipid bilayers and may thus influence HMG-CoA reductase in extrahepatic tissues (Hamelin & Turgeon 1998; Ichihara & Satoh 2002; Liao 2002).

Despite significant risk reduction by statins in ischaemic stroke (Crouse *et al.* 1998), two large clinical trials failed to demonstrate a significant correlation between ischaemic stroke and serum cholesterol levels (Kannel *et al.* 1971; Multiple risk factor intervention trial 1982). HMG-CoA reductase not only provides the necessary building blocks for cholesterol, but also the isoprenoids farnesyl- and geranyl-geranyl-pyrophosphate, which are used for post-translational modification of Ras and Rho GTPases, respectively. Reduced isoprenylation impairs Ras and Rho activation and this has effects on smooth muscle cell proliferation and

migration (Liao 2002). It has been demonstrated that the expression of endothelial nitric oxide synthase is under the negative control of RhoA and that statins upregulate endothelial nitric oxide synthase in the cerebral vascular endothelium (Endres *et al.* 1998). This statin effect confers protection against ischaemic stroke (Endres *et al.* 1998; Liao 2002) and may thus resolve the apparent conundrum that ischaemic stroke, which does not correlate with serum cholesterol, is prevented by statins.

A further possible effect of diminished isoprenylation and impaired RhoA activation in cerebral vascular smooth muscle cells is reduced force generation as RhoA and its downstream effector Rho-associated kinase, play important roles in smooth muscle contraction (Uehata *et al.* 1997; Swärd *et al.* 2000; Fukata *et al.* 2001). Thus we conducted pilot experiments using lovastatin in a tissue culture model expecting to see effects after prolonged incubation, in parallel with the slow turnover of isoprenylation, requiring hours or days. Surprisingly, a rather rapid effect of lovastatin on arterial responsiveness was seen, and the experiments reported here were designed to confirm this effect and to establish a mechanism of action.

Materials and Methods

Animals and dissection. Female Sprague-Dawley rats weighing 250–300 g were sacrificed by cervical dislocation as approved by the regional ethics committee. After decapitation, the skull was cut open and the brain transferred to Ca^{2+}-free HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) buffered Krebs solution of the following composition (in mmol/l): NaCl, 135.5, KCl, 5.9, $MgCl_2$, 1.2, glucose, 11.6, HEPES, 11.6, pH 7.35. The basilar artery,

Author for correspondence: Karl Swärd, Department of Physiological Sciences, Lund University, BMC F12, S-22184 Lund, Sweden (fax +46 46 2224546, e-mail karl.sward@mphy.lu.se).

including 2 mm of each vertebral artery and the entire segment up to the posterior cerebral branches, was detached under a dissection microscope. The artery was subsequently cut into four segments of identical lengths (≈2 mm). In some experiments the endothelium was removed with a hair that was pulled through the lumen repeatedly. After denudation, 1 µmol/l acetylcholin failed to induce relaxation, verifying successful functional removal of the endothelium.

Force measurements. Basilar artery segments were mounted in a myograph (610M, Danish Myo Technology A/S, Aarhus, Denmark) using 40 µm stainless steel wire and stretched to a basal tension of 2 mN at 37°. After equilibration for 30 min. in HEPES buffered Krebs, containing 2.5 mmol/l Ca^{2+}, preparations were repeatedly contracted (7 min. contractions followed by 25 min. relaxation periods) with 1 µmol/l 5-HT or high-K$^+$ solution (obtained by isomolar substitution of NaCl for KCl) until stable contractions were attained. The effects of the drugs were then tested in pre- and post-treatment protocols and compared to the effect of vehicle (0.1–0.3% dimethyl sulfoxide or water). The dependence of the statin effect on cholesterol and isoprenoid metabolism was tested by pretreatment of tissue for 5 min. (1 mmol/l) or 1 hr (10 mmol/l) with hydrolysed mevalonate prior to drug addition.

Measurements of the intracellular Ca^{2+} concentration with Fura 2.

Four-mm long denuded segments of the basilar artery were mounted on glass capillaries and incubated for 3 hr at room temperature in the dark with 10 µmol/l Fura 2 AM, 0.05% pluronic F-127, and 2% dimethyl sulfoxide (DMSO). Measurements of the free intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) was then performed at room temperature as described (Gomez & Swärd 1997; Dreja *et al.* 2001). [Ca^{2+}]$_i$ is reported as the ratio of fluorescence for excitation at 340 and 380 nm, respectively. The Mn^{2+}-Fura 2 quench rate, a measure of Ca^{2+} influx, was determined as the slope of fluorescence decline during excitation at 360 nm after addition of 200 µmol/l of MnCl$_2$.

Cell isolation and patch-clamp. Muscle cells were isolated from the basilar artery and its primary branches as described (Gomez & Hellstrand 1995; Dreja *et al.* 2001). L-Type Ca^{2+} channel currents were measured, using 10 mmol/l Ba^{2+} as the charge carrier, by 200 msec. depolarising steps to +20 mV from a holding potential of −80 mV. The extracellular solution contained (in mmol/l): NaCl, 105, BaCl$_2$, 10, KCl, 5.4, MgCl$_2$, 1, TEA (tetraethyl ammonium chloride), 30, HEPES, 10, glucose, 5. pH was adjusted to 7.35 with NaOH. During experimentation solution was changed with a rapid superfusion system. The pipette solution contained (in mmol/l): CsCl, 140, MgCl$_2$, 1, TEA, 20, HEPES, 10, phosphocreatine, 10, ATP, 5, EGTA (ethylene glycol bis-(β-aminoethyl ether) N,N,N^1,N^1-tetraacetic acid), 1. CsOH was used to adjust pH to 7.2. Independent tests of the effects of lovastatin and lovastatin acid in the same cell were only obtained in 2 out of 17 patched cells. Accordingly, the statistical comparison of current density in fig. 5B is unpaired.

Chemicals. Pravastatin was a gift from Bristol-Myers Squibb-Scandinavia (Bromma, Sweden). Simvastatin was a gift from Merck Sharp and Dohme (Sollentuna, Sweden). Fura 2 AM and pluronic F-127 were from Molecular Probes (Eugene, OR, USA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). Stocks of lovastatin (also known as mevinolin) and mevalonate were prepared in DMSO and stored in the freezer. Lovastatin acid was obtained by hydrolysis in 250 mmol/l NaOH at 37° for 1 hr.

Statistics. Summarized data are expressed as means±S.E.M. Paired and unpaired statistical comparisons, as appropriate, were made with Student's t-test. The Bonferoni correction was used for multiple comparisons. P<0.05 was considered statistically significant.
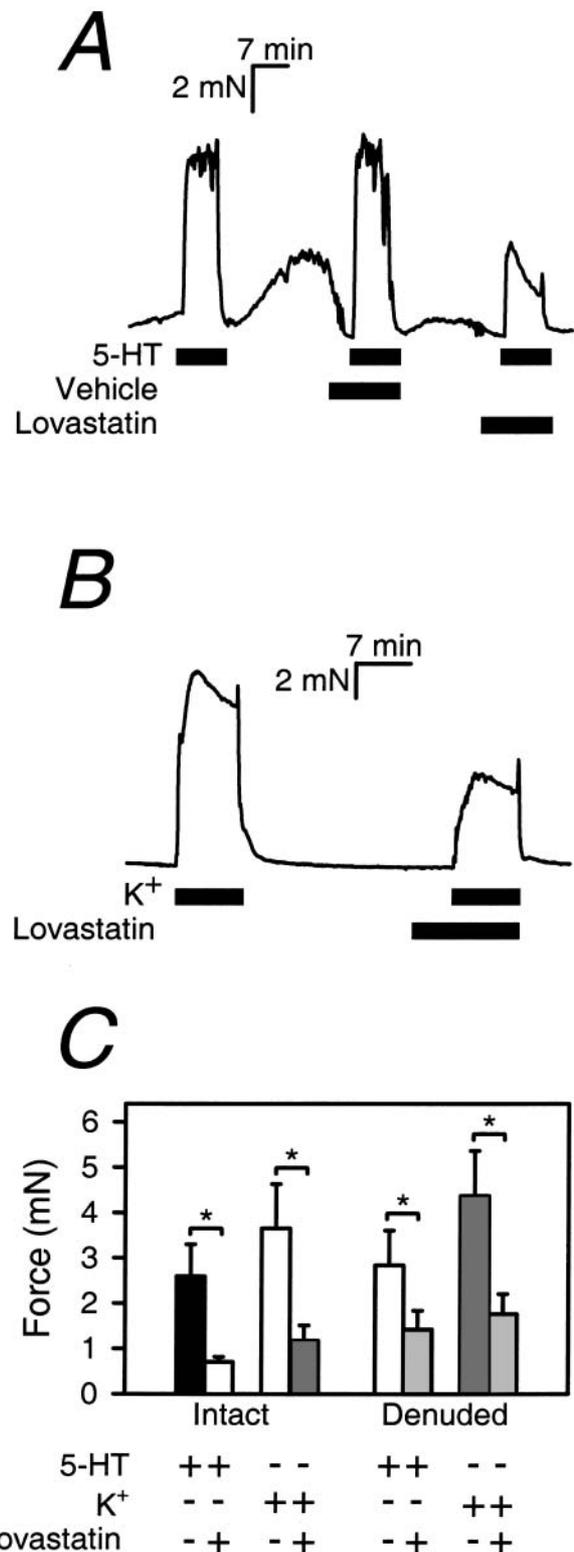
Fig. 1. Lovastatin inhibits contraction elicited by 5-HT and high-K$^+$ solution in intact and denuded rat basilar artery. Panels A and B show original traces of force in denuded segments contracted with 5-HT (1 µmol/l) and high-K$^+$ (140 mmol/l), respectively. Following the control contraction either vehicle (1‰ DMSO, A) or lovastatin (10 µmol/l, A and B) was added. Summarised data on basilar artery contraction in intact and denuded vessels are shown in panel C. * denotes P<0.05 versus control, n=3–4 for all.

A

B

C
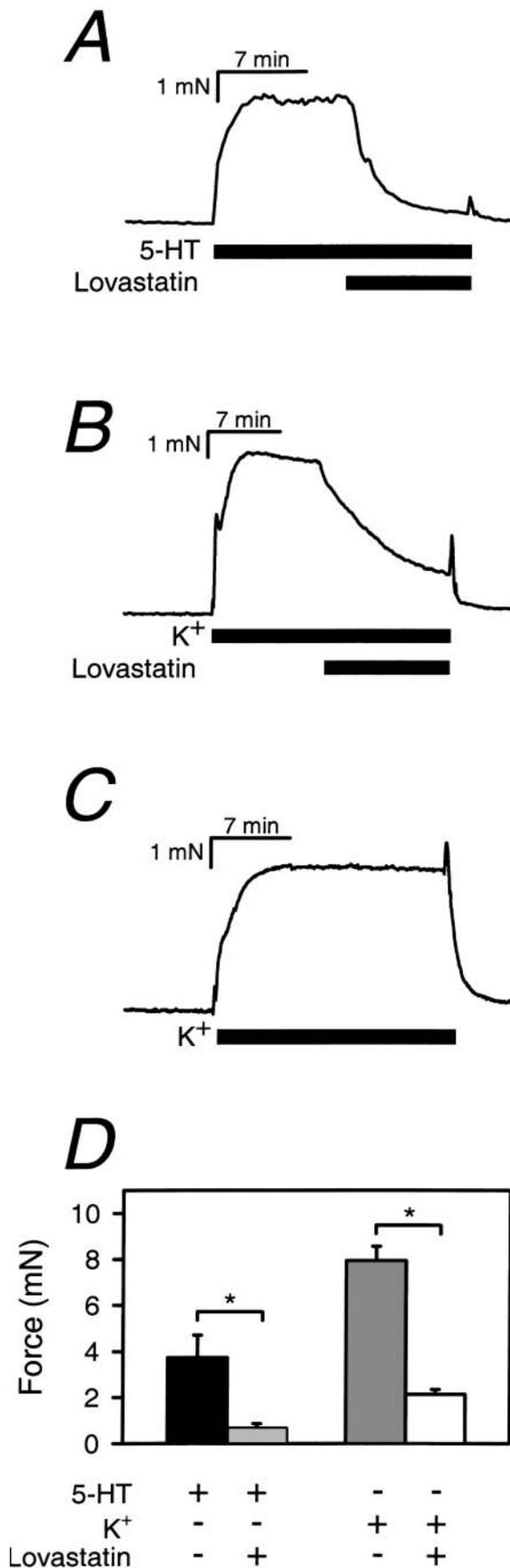

D

Fig. 2. The onset of lovastatin-induced relaxation in the denuded rat basilar artery is essentially immediate. Original records of contraction in response to 5-HT (1 µmol/l) and high-K^+ are shown in panels A–C. Lovastatin (10 µmol/l) was added during the stable (panel C) contractile plateau. Summarised data from experiments with this design are shown in panel D. * denotes $P < 0.05$, n=4 for all.

Results

Denuded basilar artery segments, mounted in a myograph, were contracted with either 5-HT (1 µmol/l) or high-K^+ solution (140 mmol/l) until stable contractions were obtained. Effects of lovastatin, lovastatin acid, pravastatin or vehicle were then tested. Drugs and vehicle were present 5 min. prior to, and during, contraction elicited by the same agent (fig. 1A and B). Neither lovastatin acid, pravastatin, nor vehicle had any significant effect on contraction when compared to the prior control contraction (10 µmol/l for all, n=3–4, data not shown). Lovastatin on the other hand, inhibited contraction elicited by both 5-HT and high-K^+ significantly (fig. 1A–C, 10 µmol/l, n=3–4). Lovastatin suppressed contraction to a similar extent in basilar artery segments with intact endothelium (compare bars in fig. 1C).

To test if there was a delay between drug addition and relaxation, denuded vessels were contracted and lovastatin was added during the stable plateau phase. As shown in fig. 2A and B, lovastatin caused essentially immediate relaxation. In controls, force was well maintained over the time-course of the experiment as shown in fig. 2C, and total relaxation (fig. 2D, n=4) corresponded reasonably well with that seen in the pretreatment protocol above. In experiments of similar design cumulatively increasing concentrations of simvastatin, lovastatin, lovastatin acid, pravastatin, and vehicle were added, resulting in dose-dependent
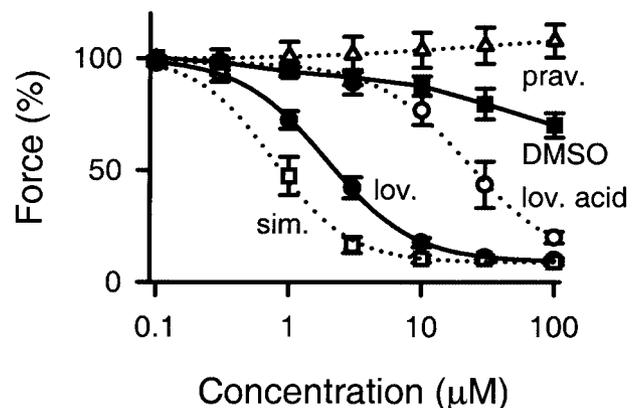
Fig. 3. Concentration-response relationships for statin-induced relaxation of the rat basilar artery. Denuded basilar arteries were contracted with high-K^+ solution, and lovastatin (lov.), simvastatin (sim.), lovastatin acid (lov. acid), pravstatin (prav.) or vehicle (dimethyl sulfoxide, DMSO) were added on top of the contractile plateau in a cumulative manner. Lines represent fits to the data of a logistic equation, yielding IC_{50} values of 0.8, 1.9 and 22 µmol/l. Means±S.E.M. are indicated. n=4.

Fig. 4. Lovastatin reduces intracellular free [Ca^{2+}] ([Ca^{2+}]$_i$) and Ca^{2+}-influx in the denuded basilar artery. Panel A shows an original record 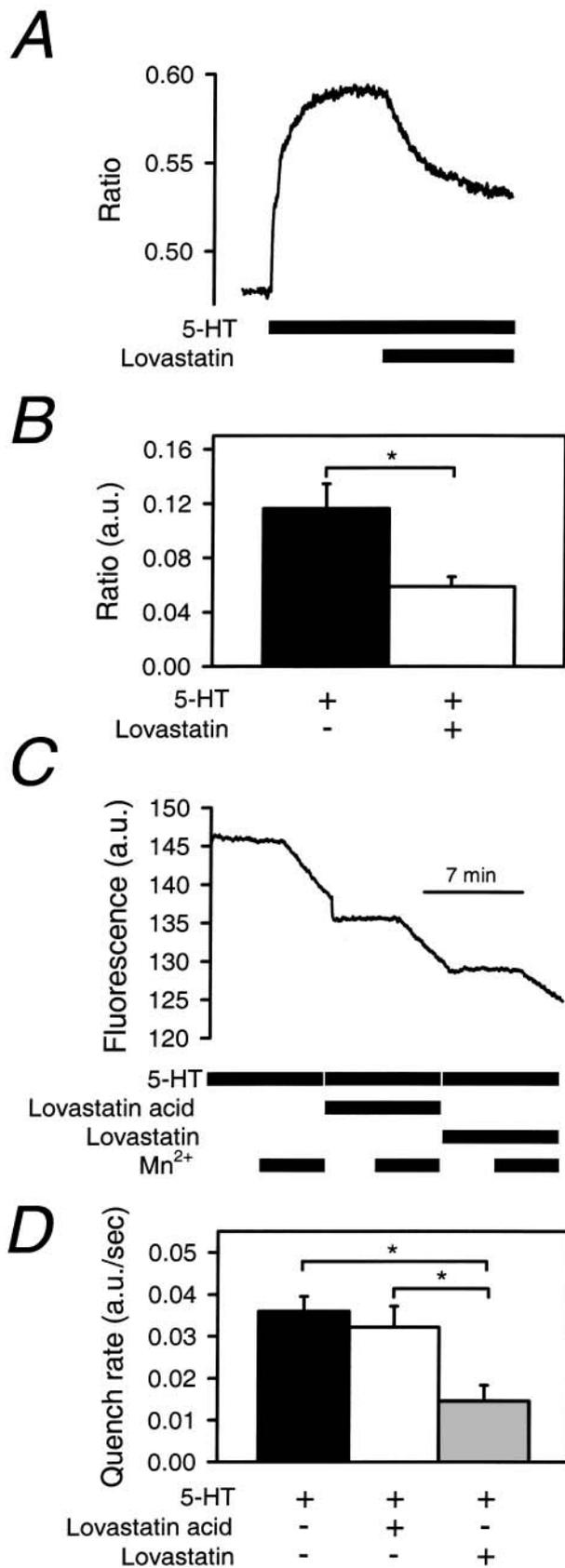of the ratio of Fura 2 fluorescence, for excitation at 340 and 380 nm respectively, which is a measure of [Ca^{2+}]$_i$. Stimulation with 5-HT (1 μmol/l) elicits a sustained increase in the ratio and subsequent infusion of lovastatin (10 μmol/l) leads to a prompt reduction. Summarised data from four experiments with this design are shown in panel B. Panel C shows an original record of fluorescence (given as arbitrary units (a.u.)) for excitation at 360 nm. Upon infusion of Mn^{2+} the fluorescence declines. The slope of this decline, i.e. the quench rate, is a measure of the Ca^{2+}-influx rate. The preparation was relaxed for 25 min. between each quench period and Mn^{2+} was added after 4 min. of stimulation with 1 μmol/l 5–HT in each cycle. Panel D shows summarised data on quench rates in controls and in the presence of lovastatin (10 μmol/l) or lovastatin acid (10 μmol/l). * denotes P<0.05. n=5 for comparisons in D.

relaxation (fig. 3, n=4 for all). The calculated IC$_{50}$ values were 0.8±0.1, 1.9±0.4 and 22±2 μmol/l for simvastatin, lovastatin and lovastatin acid, respectively. Pravastatin did not affect force at any concentration tested.

We tested if the effect of lovastatin could be reversed by preincubation with hydrolysed mevalonate. Neither 1 (5 min.) nor 10 mmol/l (1 hr) of this isoprenoid and cholesterol precursor suppressed the effect of lovastatin (n=4, data not shown). Collectively, these data show that lovastatin, independently of cholesterol and isoprenoid metabolism, and with a very rapid onset, suppresses contraction of cerebral vascular smooth muscle.

The ratio of Fura 2 fluorescence (a measure of the free intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$)) was then recorded in denuded basilar arteries to test if [Ca^{2+}]$_i$ turnover was affected. Stimulation with 5-HT resulted in a rapid and robust increase in the ratio and subsequent addition of lovastatin (10 μmol/l) reduced the ratio halfway to baseline (fig. 4A). Summarised data are shown in fig. 4B. Mn^{2+} quench of Fura 2 was used to estimate the Ca^{2+} influx rate. The fluorescence intensity at 510 nm, for excitation at 360 nm, in one experiment is shown in fig. 4C. Lovastatin (10 μmol/l), but not lovastatin acid (10 μmol/l), significantly inhibited the quench rate in the presence of 5-HT (fig. 4D), suggesting inhibition of a membrane Ca^{2+}-current.

Basilar artery smooth muscle cells were isolated and L-type currents recorded with Ba^{2+} as the charge carrier. Step depolarisation of the membrane from −80 to +20 mV resulted in ≈50 pA inward currents (fig. 5A). Rapid (≈1 sec.) change from normal extracellular solution (fig. 5A, trace a) to one containing either lovastatin (fig. 5A, 10 μmol/l, trace b) or lovastatin acid (fig. 5A, 10 μmol/l, trace c) caused rapid and reversible (not shown) reduction of inward current. Summarised data on the effect on L-type current is shown in fig. 5B.

Discussion

The present study demonstrates that lovastatin inhibits L-type current and reduces intracellular free [Ca^{2+}] and contraction in the cerebral circulation of the rat. Experiments
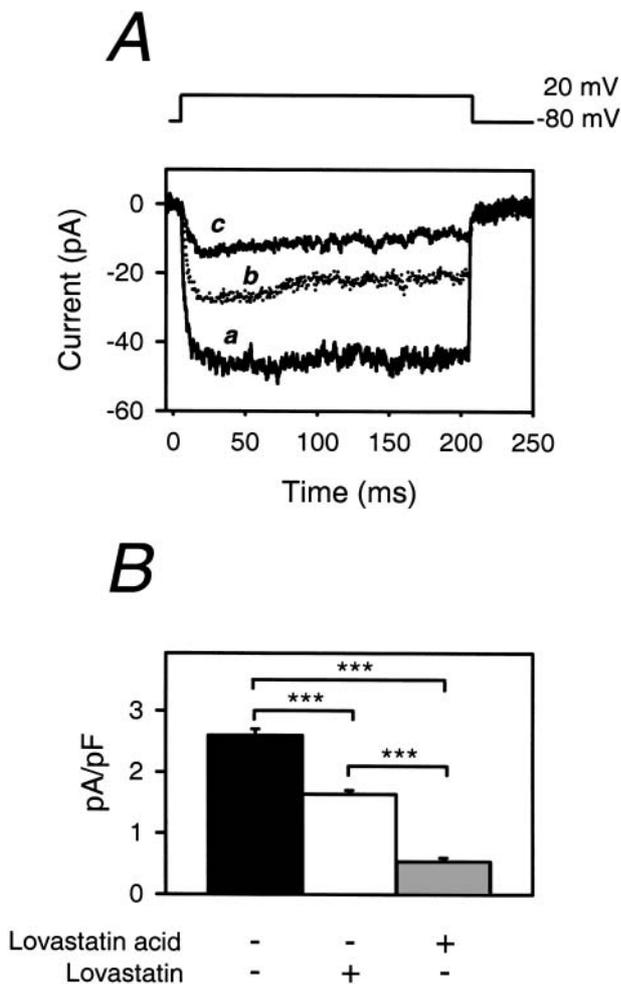
Fig. 5. Lovastatin and lovastatin acid inhibit L-type current in isolated basilar artery smooth muscle cells. Panel A shows original current traces from one basilar artery smooth muscle cell upon step depolarisation from -80 to $+20$ mV. Rapid change of the control extracellular solution (trace a in panel A) to one containing either lovastatin (trace b) or hydrolysed lovastatin (trace c) promptly reduces inward current. Summarised data on inward currents normalised to cell capacitance are shown in panel B. *** denotes P<0.001, n=17 for controls, 8 for lovastatin and 12 for the hydrolysate, respectively. The effect of both lovastatin and the hydrolysate in the same cell was tested twice, and the hydrolysate was more potent than the pro-drug on both occasions.

designed to reverse the effect of lovastatin with mevalonic acid or to mimic the effect with the water-soluble statin, pravastatin were negative. Basilar artery contraction elicited by membrane depolarisation and serotonin depend on the activation of L-type channels (eg. Alborch *et al.* 1995). The effects of lovastatin on Ca^{2+} current and contraction are thus entirely consistent, whereas the lower potency on contraction and Mn^{2+}-quench of lovastatin acid, which inhibited Ca^{2+} current more potently than lovastatin, is surprising. One possibility is that the statins act intracellularly and that cellular penetration in organised tissue is selectively reduced by hydrolysis.

Effects of statins on voltage-gated Ca^{2+} channels have

been reported by two other groups. Renaud *et al.* (1986) reported that lovastatin inhibits contractility and L-type current in cultured cardiomyocytes. This effect, at 0.1–1 µmol/l, appeared after 1–3 days of culture and was associated with reduced cellular cholesterol contents. Yada *et al.* (1999), on the other hand, demonstrated effects of the lipophilic simvastatin on L-type current in pancreatic β-cells, which was not reversed by mevalonic acid lactone. Comparing simvastatin with lovastatin acid and pravastatin, these authors reached the conclusion that the potency of Ca^{2+}-current inhibition correlates with increasing hydrophobicity. Interestingly, protection against ischaemic brain injury in animal models is similarly related to the hydrophobicicty of the statin used (Amin-Hanjani *et al.* 2001).

Inhibition of L-type current by statins may account for some of their reported effects in vascular smooth muscle. For instance, Tesfamariam *et al.* (1999) showed that atorvastatin and simvastatin inhibited aortic contraction and Ng *et al.* (1994) demonstrated reduced $[Ca^{2+}]_i$ and reduced Mn^{2+} quench in A10 smooth muscle cells treated with simvastatin. These studies, that of Yada *et al.* (1999), and the present work, have the finding in common that lipophilic statins are effective whereas the water-soluble statins are not. In contrast, while our study and that of Yada *et al.* (1999), argue that the effect of the lipophilic statins is independent of isoprenoid and cholesterol metabolism the studies by Tesfamariam *et al.* (1999) and Ng *et al.* (1994) demonstrate reversal by mevalonate, implicating either isoprenoids or cholesterol as important intermediaries.

Several published observations seem to refute the possibility that the rapid effects on tone and L-type current are due to reduced cholesterol content. Treatment of the rat tail artery with methyl-β-cyclodextrin reduced the arterial contents of cholesterol by 20% but was without any effect on contraction induced by depolarisation (Dreja *et al.* 2002). Similarly, Löhn *et al.* (2000) depleted cholesterol from basilar artery smooth muscle cells with cyclodextrin without observing any effects on L-type current amplitudes. Enrichment of cholesterol, on the other hand, leads to increased L-type current (Sen *et al.* 1992), but this may involve incorporation of cholesterol into membrane domains where it is not normally present. Thus, while effects on isoprenoid metabolism may not be entirely excluded, inhibition of L-type current by lovastatin appears to be independent of cholesterol biosynthesis.

Long-term pharmacological treatment targeting the cerebral vascular tone generally seems to offer protection against ischaemic brain damage. For instance, the reduced infarct volume and improved functional recovery obtained by L-arginine treatment after middle cerebral artery occlusion in the rat correlates closely with increased blood flow (Dalkara *et al.* 1994, Morikawa *et al.* 1994). Similarly, Limbourg *et al.* (2002) demonstrated protection against ischaemic stroke depending on enhanced cerebral blood flow with pharmacological levels (20 mg/kg intraperitoneally) of corticosteroids. In addition, attempts to increase cerebral blood flow with dihydropyridines offered signifi-

cant protection against ischaemic stroke (Gelmers *et al.* 1988), although this effect has subsequently been questioned (Horn *et al.* 2001).

A critical consideration is whether the plasma concentration of lovastatin ever reaches high enough levels to influence cerebrovascular L-type current (i.e. ≥ 0.3 μM). Reported (e.g. Neuvonen & Jalava 1996) peak (C_{max}) plasma concentrations of lovastatin on oral dosage in man range between 0.01–0.1 μmol/l and are thus at least three-times lower than the lowest effective concentration in our study. However, lovastatin is metabolised in the liver by the cytochrome P450 enzyme CYP3A4 in competition with other drugs. The combination of lovastatin with itraconazole, a CYP3A4 inhibitor, increased the plasma concentration of lovastatin 20–times (Neuvonen & Jalava 1996). Thus, certain oral drug combinations may lead to cerebrovascular Ca^{2+} current inhibition. Interestingly, the calcium antagonist diltiazem, often used in combination with statins, is also an effective blocker of CYP3A4. Furthermore, it cannot be excluded that human cerebral vessels, which are chronically exposed to statins, are somewhat more sensitive than rat vessels, as this situation allows longer time for cellular penetration.

In summary, we have shown that the lipophilic statins inhibit cerebrovascular tone and that lovastatin inhibits L-type current in a manner seemingly independent of both cholesterol and isoprenoid biosynthesis. Ca^{2+}-channel blockade by lipophilic statins may confound *in vitro* experiments in which the mevalonate pathway is probed. Provided that high enough levels of lipophilic statins are reached in the vicinity of the brain arterioles *in vivo*, this effect may tentatively play a role in the reported protection against ischaemic stroke.

Acknowledgements

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