Effects of atorvastatin and simvastatin on atrial plateau currents

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Abstract

Recent evidence has shown that the inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (statins) might exert antiarrhythmic effects both in experimental models and in humans. In this study we analyzed the effects of atorvastatin and simvastatin acid (SVA) on the currents responsible for the duration of the plateau of human atrial action potentials: hKv1.5, Kv4.3, and L-type Ca²⁺ (I_{Ca,L}). hKv1.5 and Kv4.3 currents were recorded in transfected Ltk⁻⁻ and Chinese hamster ovary cells, respectively, and I_{Ca,L} in mouse ventricular myocytes, using whole-cell patch-clamp. Atorvastatin and SVA produced a concentration-dependent block of hKv1.5 channels (IC₅₀ = 4.5 ± 1.7 μM and 5.7 ± 0.03 μM, respectively) and shifted the midpoint of the activation and inactivation curves to more negative potentials. Importantly, atorvastatin- and SVA-induced block was added to that produced by quinidine, a drug that blocks hKv1.5 channels by binding to their pore cavity. Atorvastatin and SVA blocked Kv4.3 channels in a concentration-dependent manner (IC₅₀ = 13.9 ± 3.6 nM and 7.0 ± 0.8 μM, respectively). Both drugs accelerated the inactivation kinetics and shifted the inactivation curve to more negative potentials. SVA (10 nM), but not atorvastatin, also blocked I_{Ca,L} producing a frequency-dependent block that, at 2 Hz, reached a 50.2 ± 1.5%. As a consequence of these effects, at nanomolar concentrations, atorvastatin lengthened, whereas SVA shortened, the duration of mouse atrial action potentials. The results suggest that atorvastatin and SVA alter Kv1.5 and Kv4.3 channel activity following a complex mechanism that does not imply the binding of the drug to the channel pore.

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1. Introduction

Statins, the inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are the most effective and best-tolerated drugs to treat elevated levels of low-density lipoprotein cholesterol (LDL-C). Results from both secondary and primary large-scale, multicenter, prevention trials have consistently demonstrated that statin therapy significantly reduces the risk of major coronary events (unstable angina pectoris, myocardial infarction, coronary death, and need for coronary revascularization), stroke and all-cause and cardiovascular mortality [1,2]. Although statins exert their major effects on coronary artery disease (CAD) by lowering LDL-C, a multitude of potentially cardioprotective effects have been ascribed to these drugs, largely based on in vitro and ex vivo data [3–5]. These pleiotropic actions of statins include improved endothelial function, modulation of autonomic function, stabilization of vulnerable plaques, and antioxidant, anti-inflammatory, antithrombotic, and cardioprotective effects [3–5].

Recent evidence has demonstrated that statins could be useful in the prevention of development and recurrences of atrial fibrillation (AF) in patients with or without CAD, an effect not observed with other lipid-lowering drugs [6,7]. These antiarrhythmic actions can be attributed, at least in part, to their pleiotropic effects. However, it could be possible that they exert some direct effects on transmembrane ion fluxes that may directly affect the electrophysiological properties of atrial muscle. Unfortunately, information on the putative effects of statins on cardiac ionic channels is nowadays scarce. Indeed, it has only been described that in rabbits treated with 5 mg/kg/day pravastatin, the drug exerted an antihypertrophic effect involving the opening of cardiac ATP-sensitive channels through the activation of the endothelial nitric oxide synthase (NOS) [8] and...
that, at concentrations higher than the therapeutic levels, lovastatin (1 μM) inhibited L-type calcium current \( (I_{\text{Ca,L}}) \) and uncoupled excitation from contraction in chicken cardiac cells [9].

In the human atria, action potential duration (APD) and refactoriness are determined by the balance between the \( I_{\text{Ca,L}} \) and several outward \( K^+ \) currents \([10,11]\). Two of these \( K^+ \) currents are the ultrarapid component of the delayed rectifier current, \( I_{\text{Kur}} \), which is exclusively present in the atria, and the 4-aminopyridine (4-AP) sensitive component of the transient outward current, \( I_{\text{to1}} \). Human cardiac \( I_{\text{Kur}} \) is carried by Kv1.5 channels, whereas \( I_{\text{to1}} \) is predominantly carried by Kv4.3 \( \alpha \)-subunits co-assembled with KChIPs, DPP6, and probably also KCNEs \( \beta \)-subunits \([10,11]\).

Atorvastatin and simvastatin are lipophilic, synthetic and fungal-derived statins, respectively \([12]\). Simvastatin structure exhibits a decalin ring (Type 1 statins), whereas atorvastatin is more lipophilic since it exhibits a fluorophenyl group (Type 2) \([13]\). Simvastatin, but not atorvastatin, is a \( \gamma \)-lactone produrg which is metabolized after oral ingestion to the open acid form (simvastatin acid, SVA), which is the active one. Statins share a common pharmacologic target, but they exhibit important intermolecular differences that contribute to their distinct efficacy, safety, and pleiotropic actions \([12]\). Since the acute effects of statins on hKv1.5, Kv4.3, and \( I_{\text{Ca,L}} \) currents are unknown, in the present study we have analyzed their effects on these currents that determine the height and duration of the plateau phase of human atrial action potentials (APs).

2. Methods

2.1. Isolation of myocytes

Single ventricular myocytes were obtained from hearts of male CD-1 mice (≈ 30 g) by dissociation using collagenase (type II, Worthington Biochemical Corporation Lakewood, NJ, USA) and protease (type XIV, Sigma Chemical Co. London, UK) as described \([14]\). Mice were heparinized (5000 UI/kg), anesthetized with ketamine and xilazine, and killed following the Guidelines for animal care and use of laboratory animals described in the Directive 86/609/EEC published in 1986 by the Council of the European Communities.

2.2. Cell culture

\( Ltk^- \) cells stably expressing hKv1.5 channels and Chinese hamster ovary (CHO) cells were grown in DMEM and Ham’s F12, respectively, with 10% fetal bovine serum in a 5% CO\(_2\) atmosphere \([14–17]\). The transient expression of Kv4.3 channels in CHO cells has been previously described \([15–17]\). The cells were transfected with the cDNA encoding Kv4.3 channels (3 μg) together with the cDNA encoding the CD8 antigen (0.5 μg) using Lipofectamine (Gibco, Grand Island, NY, USA). Before experimental use, cells were incubated with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450; Dynal, Norway). Most of the cells that were beaded also had channel expression \([15–17]\).

2.3. Solutions and drugs

\( Ltk^- \) and CHO cells were superfused with an “external solution” containing (mM): NaCl 130, KCl 4, CaCl\(_2\) 1, MgCl\(_2\) 1, HEPES 10 and glucose 10 (pH = 7.4 with NaOH). When measuring \( K^+ \) currents ventricular myocytes were superfused with this solution supplemented with tetrodotoxin 0.03 mM and CoCl\(_2\) 2 mM to inhibit \( I_{\text{Na}} \) and \( I_{\text{Ca,L}} \), respectively. The “internal solution” contained (mM): K-aspartate 80, KCl 4, KH\(_2\)PO\(_4\) 10, MgATP 5, phosphocreatine 3, HEPES 5, and EGTA 5 (pH = 7.2). To record \( I_{\text{Ca,L}} \) in ventricular myocytes the external solution contained (mM): NaCl 140, CsCl 5.4, CaCl\(_2\) 2.5, MgCl\(_2\) 0.5, HEPES 5.5, and glucose 11 (pH = 7.4), whereas the internal solution contained (mM): CsCl 130, TEA–Cl 20, EGTA 5, MgATP 5, and HEPES 10 (pH = 7.2). Atorvastatin calcium (Pfizer Inc., New York, NY, USA) was dissolved in methanol to yield 0.01 M stock solutions. Simvastatin (Merck and Co., Whitehouse Station, NJ, USA) was activated following manufacturer’s instructions to obtain 0.001 M stock solutions of SVA that were used in the following 2 h. Control solutions contained the same solvent concentration as the test solution.

2.4. Recording techniques for APs

APs were recorded at 34 °C in mice left atria superfused with Tyrode’s solution (mM: NaCl 137, KCl 5.4, MgCl\(_2\) 1.0, CaCl\(_2\) 1.8, NaH\(_2\)PO\(_4\) 0.42, NaHCO\(_3\) 11, and glucose 10), bubbled with 95% O\(_2\)/5% CO\(_2\), and driven at 3 Hz using conventional microelectrode techniques \([15]\).

2.5. Recording techniques for ionic currents

Currents were recorded at 22–24 °C using the whole-cell configuration of the patch-clamp with Axopatch 200B amplifiers and pClamp 9.0 software (Axon Instruments, Foster City, CA, USA). hKv1.5 and Kv4.3 currents were sampled at 2 and 4 kHz, respectively, and filtered at half the sampling frequency. Pipettes were pulled from Narishige (GD1; Narishige Co Ltd, Tokyo, Japan) borosilicate capillary tubes using a programmable patch micropipette puller (P-2000, Sutter Instruments Co., Novato, CA, USA) and were heat polished with a microforge (MF-83, Narishige). Micropipette resistance was kept <3.5 MΩ when filled with the internal solution and immersed in the external solution. Maximum hKv1.5 and Kv4.3 current amplitudes averaged 1.3 ± ± 0.1 nA (n = 24) and 1.9±0.4 nA (n = 18), respectively. Access resistance and cell capacitance were 4.1±0.3 MΩ and 11.7±0.6 pF in \( Ltk^- \), and 3.6±0.3 MΩ and 11.6±1.6 pF in CHO cells, respectively. Typically ≈80% of capacitance and series resistance were compensated, obtaining mean uncompensated access resistances of 2.6±0.3 MΩ and 1.9±0.3 MΩ for \( Ltk^- \) and CHO cells, respectively. Mean capacitance, access resistance, and uncompensated access resistance in myocytes were 138±13 pF, 4.0±0.6 MΩ, and 1.7±0.4 MΩ (n = 8), respectively. Thus, no significant voltage errors (<5 mV) due to micropipettes series resistance were expected. Superfusion with atorvastatin or SVA
did not change the cell capacitance of Ltk−, CHO, or ventricular cells.

2.6. Pulse protocols and analysis

In Ltk− and CHO cells the holding potential was maintained at −80 mV and the cycle time for any protocol was 10 s to avoid accumulation of inactivation and/or block. The protocol to obtain current–voltage relationships consisted of 250 ms (Kv4.3) or 500 ms (hKv1.5) pulses that were imposed in 10 mV increments between −80 and +60 mV for hKv1.5 and between −90 and +50 mV for Kv4.3. Deactivating hKv1.5 “tail” currents were recorded on return to −40 mV. The activation curves of hKv1.5 channels were constructed by plotting tail current amplitudes as a function of the membrane potential of the preceding pulse.

To obtain the inactivation curves of hKv1.5 and Kv4.3 channels a two-step protocol was used. The first 10 s (hKv1.5) or 250 ms (Kv4.3) conditioning pulse from −80 mV to potentials between −80 and +60 mV (Kv4.3) or between −90 and +50 mV (Kv4.3) was followed by a test pulse to +60 mV (hKv1.5) or to +50 mV (Kv4.3). Inactivation curves were constructed by plotting the current amplitude obtained with the test pulse, as a function of the voltage command of the conditioning pulse. To describe the time course of current activation and/or inactivation upon depolarization, as well as the tail currents upon repolarization, an exponential analysis was used [14–17].

To obtain the IC50 (concentration of drug that produces the half maximum blockade) and the Hill coefficient, nH, the fractional block obtained at various drug concentrations [D] was fitted to the equation:

\[ f = 1/\{1 + (IC_{50}/[D])^{n_H}\} \]  

(1)

In mouse ventricular myocytes, outward K+ currents were recorded in cells previously superfused with 4-AP (50 μM) by applying 250-ms pulses from −80 mV to potentials ranging from −90 to +50 mV. Under these conditions, IKur would be inhibited [14], remaining a transient current that rapidly activated and decayed to a constant level at the end of the pulse that is mainly composed by I01,1, carried through Kv4.3 channels [14,18]. In another group of experiments, after a 200-ms prepulse to +40 mV to inactivate the transient component, 250-ms pulses to +50 mV were applied. Using this protocol a slowly inactivating delayed rectifier outward K+ current (IKslow), composed of IKslow1, encoded by hKv1.5 channels, and IKslow2, encoded by Kv2.1 channels [18], was obtained. Since Kv2.1 currents do not inactivate, the inactivating component was considered as a result of the decay of the current carried by Kv1.5 channels, and the current amplitude was measured as the difference between the peak current and the current at the end of the pulse. Using this procedure, Kv1.5 current amplitude is somewhat underestimated, and the blockade values should be considered as approximated since it is not easy to accurately determine the participation of each component in the total current amplitude [18].

The holding potential of ventricular myocytes when recording I_{Ca,L} was maintained at −80 mV and the cycle time for any protocol was 10 s. The protocol to obtain current–voltage relationships consisted of 500-ms pulses that were imposed in 5 mV increments between −25 and +45 mV. The I_{Na} was inactivated by the application of 50-ms prepulse to −30 mV.

2.7. Statistical methods

Data obtained in the absence and the presence of drug were compared in a paired manner. For comparisons at a single voltage, differences were analyzed using the Student’s t-test. To analyze block at multiple voltages, two-way analysis of variance was used, followed by Newman–Keuls test. Results were expressed as mean±SEM. A P-value <0.05 was considered as significant.

3. Results

3.1. Effects of atorvastatin and SVA on hKv1.5 currents

The inhibition constant values of HMG-CoA reductase described for atorvastatin and SVA are 8 nM and 11 nM, respectively [13]. Therefore, we decided to explore the effects of atorvastatin and SVA at 10 nM. Fig. 1 shows hKv1.5 currents elicited by 500-ms pulses from −80 to +60 mV in the absence, presence, and after washout of atorvastatin (panel A) and SVA (panel B). Atorvastatin reduced the current at the end of the pulse by 26.3±3.5% (n=10, P<0.01), an effect reversible upon washout. In contrast, SVA produced a negligible effect reducing the current at the end of the pulses by 5.8±3.0% (n=6, P>0.05). Inset in Fig. 1A shows the time course of induction and relief of block when a cell was superfused with atorvastatin-containing solution. The effects reached steady state after 4–6 min of superfusion being reversible upon washout with drug-free solution during 8–10 min. These results suggest that the effects of atorvastatin were not mediated by an effect on the lipid content of the membrane since it is unlikely that in 4–6 min the drug can produce a membrane cholesterol synthesis inhibition that can recover within 10 min of drug washout. Furthermore, identical time course of washin and washout was observed when a blocking concentration of SVA was tested (8 μM, not shown).

Neither atorvastatin (n=10) nor SVA (n=8) modified the time course of tail currents elicited upon repolarization to −40 mV after pulses to +60 mV (Table 1). The concentration dependence of the atorvastatin and SVA blockade measured at the end of pulses to +60 mV is shown in panels C and D, respectively. The atorvastatin-induced block smoothly increased as the drug concentration increased, yielding IC_{50} values of 3.6±2.3 μM (n=1) and 4.5±1.7 μM (n=2±0.02) (Fig. 1C). In contrast, SVA-induced block markedly increased in a narrow range of concentrations and the IC_{50}
averaged 2.5±0.7 μM (n_H = 1) and 5.7±0.03 μM (n_H = 9.1±0.4) (Fig. 1D).

To test the putative frequency dependence of the inhibition produced by atorvastatin and SVA, 16-pulse trains to +60 mV at 1, 2 (200-ms pulses) or 5 Hz (100-ms pulses) were applied. Trains were separated from each other by 2-minute intervals. Peak current amplitude decreased under control conditions because of the accumulation of the slow inactivation (Table 2). In the presence of atorvastatin a certain amount of block was apparent from the first depolarization applied (“tonic block”). Thereafter, during the train the blockade increased until a steady state was achieved (frequency-dependent block). Tonic and frequency-dependent block are summarized in Table 2. Data demonstrated that atorvastatin induced a blockade which was increased with the repetitive stimulation. In contrast, blockade in the presence of SVA was not apparent even at fast rates of stimulation.

Figs. 2A and B show hKv1.5 currents recorded by applying 500-ms pulses from −80 to +60 mV and the tail currents obtained upon repolarization to −40 mV in the absence, presence, and after washout of 10 nM atorvastatin (A) and 10 nM SVA (B). In A, the inset shows hKv1.5 current amplitude at +60 mV as a function of time from a representative experiment. The arrows indicate the beginning and the end of superfusion with atorvastatin-containing solution. Reduction of current amplitude at the end of pulses to +60 mV plotted as a function of atorvastatin (C) and SVA (D) concentration. Each point represents the mean±SEM of ≥6 experiments.

Fig. 1. Effects of atorvastatin and SVA on hKv1.5 currents. hKv1.5 currents recorded by applying 500-ms pulses from −80 to +60 mV and the tail currents obtained upon repolarization to −40 mV in the absence, presence, and after washout of 10 nM atorvastatin (A) and 10 nM SVA (B). In A, the inset shows hKv1.5 current amplitude at +60 mV as a function of time from a representative experiment. The arrows indicate the beginning and the end of superfusion with atorvastatin-containing solution. Reduction of current amplitude at the end of pulses to +60 mV plotted as a function of atorvastatin (C) and SVA (D) concentration. Each point represents the mean±SEM of ≥6 experiments.
in duration) at potentials ranging from −80 to +60 mV. Atorvastatin shifted the $F_h$ to more negative potentials without modifying the slope (Table 1). Fractional block demonstrated that the blockade increased in the voltage range of channel inactivation, reaching a maximum at −20 mV of 35.7 ± 7.0% (P < 0.05 vs. blockade at −80 mV).

3.2. Effects of atorvastatin and SVA on Kv4.3 currents

Figs. 3A and B show Kv4.3 currents recorded by applying 250-ms pulses to +50 mV in the absence, the presence, and after washout of 10 nM atorvastatin and 10 nM SVA, respectively. As in hKv1.5 channels effects of SVA on Kv4.3 channels were negligible. Kv4.3 currents rose rapidly to a peak ($\tau_{act} = 1.1 ± 0.1$ ms at +50 mV, n = 15) and then inactivated following a biexponential process. Since atorvastatin significantly accelerated the current inactivation (Table 1), the blockade was measured as the reduction in the total charge crossing the membrane, estimated from the integral of the current at +50 mV. The IC$_{50}$ values in the presence of atorvastatin were 13.9 ± 3.6 nM ($n_H = 0.2 ± 0.03$) and 81.0 ± 4.7 nM ($n_H = 1$) (Fig. 3C). Concentration-dependent effects of SVA were measured using also the decrease in the charge as an index of block and the IC$_{50}$ values obtained were 6.2 ± 2.7 μM ($n_H = 1$) and 7.0 ± 0.8 μM ($n_H = 5.7 ± 3.3$) (Fig. 3D).

To test the frequency dependence of the inhibition produced by atorvastatin and SVA, 16-pulse trains to +50 mV at 1, 2 (200-ms pulses) or 5 Hz (100-ms pulses) were applied. Trains were separated from each other by 2-minute intervals. In the presence of atorvastatin, a slight, but significant, increase of block was observed when cells were stimulated repetitively. Tonic and frequency-dependent block are summarized in Table 2. In contrast, 10 nM SVA did not block Kv4.3 channels even after repetitive stimulation.

Table 1

<table>
<thead>
<tr>
<th>Current</th>
<th>Frequency (Hz)</th>
<th>Decrease in control (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Atorvastatin (10 nM)</td>
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<tr>
<td></td>
<td></td>
<td>Tonic block (%)</td>
</tr>
<tr>
<td>hKv1.5</td>
<td>1</td>
<td>13.4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30.6 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>45.6 ± 0.5</td>
</tr>
<tr>
<td>Kv4.3</td>
<td>1</td>
<td>8.7 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18.0 ± 5.5</td>
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<tr>
<td></td>
<td>5</td>
<td>47.6 ± 7.8</td>
</tr>
<tr>
<td>Ca,L</td>
<td>0.5</td>
<td>12.3 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5.6 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.4 ± 1.5</td>
</tr>
</tbody>
</table>

Data are mean ± SEM of >6 experiments. *P < 0.05 vs. frequency-dependent block at 1 Hz. §P < 0.05 vs. frequency-dependent block at 0.5 and 1 Hz.
amplitude and shifted the $V_{1/2}$, without modifying the slope (Table 1). The blockade significantly increased as the fraction of the inactivated Kv4.3 channels increased, reaching a maximum at $-30$ mV of $67.4 \pm 8.7\%$ ($n=6$, $P<0.05$ vs. blockade at $-90$ mV).

### 3.3. Involvement of antioxidant actions and nitric oxide (NO) in the effects of atorvastatin and SVA

The concentration–response curves of the effects of atorvastatin and SVA on hKv1.5 and Kv4.3 are quite unusual.
Fig. 3. Effects of atorvastatin and SVA on Kv4.3 currents. Kv4.3 currents in the absence, presence, and after washout of 10 nM atorvastatin (A) and 10 nM SVA (B). Reduction of total Kv4.3 charge crossing the membrane at +50 mV plotted as a function of atorvastatin (C) and SVA (D) concentration. Mean charge–voltage relationships in the absence and presence of atorvastatin (E) and SVA (F). In E and F, the squares represent the ratio between the charge crossing the membrane in the presence and the absence of drug. Normalized inactivation curves in the absence and presence of atorvastatin (G) and SVA (H). *P < 0.05 vs. control data. Each point represents the mean±SEM of ≥6 experiments.
One putative explanation for these results could be that the blockade was not produced by a “specific interaction” between the drug and the channel protein following the law of mass action, but by an “unspecific” mechanism.

It has been demonstrated that some effects induced by statins can be associated to their antioxidant properties and to their ability to increase the production of NO by the up-regulation of the endothelial NO. Therefore, in a first group of experiments we studied the effects of atorvastatin on hKv1.5 and Kv4.3 in the presence of the lipophilic antioxidant probucol (Medichem, Spain) to determine whether they were mediated by their antioxidant actions. hKv1.5 (Fig. 4A) and Kv4.3 (Fig. 4B) currents were recorded under control conditions, in the presence of 5 μM probucol alone and plus 10 nM atorvastatin. Probucol decreased hKv1.5 current amplitude at the end of the pulse by 37.1±6.3% (n=10). Superfusion of atorvastatin plus probucol resulted in a further reduction of the current (relative to the blockade obtained with probucol alone) that reached 31.8±8.6% (n=5). Since 5 μM probucol decreased the Kv4.3 charge at +50 mV by 82.0±4.5% (n=6) a lower concentration was used. Superfusion with 1 μM probucol alone reduced Kv4.3 charge by 19.5±3.4% (n=10), whereas superfusion of atorvastatin (Fig. 4B) plus probucol resulted in a further reduction (relative to the blockade obtained with probucol alone) of 78.8±8.3% (n=5). These effects were not significantly different from those obtained in the absence of probucol, suggesting that atorvastatin inhibited the hKv1.5 and Kv4.3 currents through a mechanism independent of their antioxidant properties. Furthermore, it should be stressed that identical results were obtained when vitamin C (200 μM), a more hydrophilic antioxidant, was used or when the effects of a blocking concentration of SVA (8 μM) were tested in the presence of probucol (not shown).

Figs. 4C and D show hKv1.5 and Kv4.3 currents under control conditions and in the presence of the NOS inhibitor L-NAME-methyl ester (L-NAME, 500 μM) alone and plus 10 nM atorvastatin to determine the involvement of NO in their blocking effects. L-NAME did not significantly modify hKv1.5 currents (4.2±1.8% n=5, at the end of pulses to +60 mV), and addition of atorvastatin (Fig. 4C) to L-NAME produced an inhibition of the current relative to that produced by L-NAME of 27.7±6.1% (n=5). L-NAME slightly decreased the Kv4.3 charge crossing the membrane at +50 mV (11.6±2.6%, n=10). Superfusion of L-NAME plus atorvastatin (Fig. 4D) further decreased the charge crossing the membrane by 36.4±2.1% (n=5). These effects were not different from those induced by atorvastatin in the absence of L-NAME (P>0.05). Identical conclusion was reached when the effects of 8 μM SVA were tested in the presence of L-NAME and compared with those obtained in the absence of L-NAME (not shown).

3.4. Effects of the combination of quinidine with atorvastatin or SVA

Molecular determinants of the binding site for drugs that block Kv1.5 channels have been extensively studied. Recent results suggest that there is a common binding site for all these blockers, located in the pore of the channel and formed by residues of the S6 segment that face towards the pore. As it has been mentioned above, the concentration–response curve of the effects of atorvastatin and SVA on hKv1.5 channels might suggest that they produce an unspecific perturbation of the cell membrane instead of a specific interaction with a receptor located into the channel protein. To test whether the atorvastatin and SVA effects are specific, in the next group of experiments we tested the effects of the combination of each statin with quinidine, a well known hKv1.5 blocker. If the combination produced less block than the more potent drug alone, the competition for a common binding site at the pore would be confirmed. Figs. 4E and F show current traces obtained in control conditions and in the presence of 10 μM quinidine when 500-ms pulses to +60 mV were applied. Quinidine decreased the peak outward current and induced a subsequent exponential decline of the current during the depolarizing pulse, so that the current amplitude at the end of the pulse decreased by 41.8±6.1% (n=12). Fig. 4E shows the current traces obtained when, after the superfusion with quinidine, the cell was superfused with the combination of quinidine 10 μM plus atorvastatin 10 nM. As can be observed, in the presence of quinidine, atorvastatin produced a further decrease of the current, the blockade reaching a 71.9±3.9% (P<0.05, n=6). This result clearly demonstrated that the blockade produced by atorvastatin was completely added to the blockade produced by quinidine. Similar results were obtained when the effects of the combination of 10 μM quinidine plus increasing concentrations of atorvastatin were tested (inset in Fig. 4E). Moreover, this additive effect was also observed when atorvastatin was first superfused.

Fig. 4F shows the results obtained by the combination of quinidine plus a blocking concentration of SVA (8 μM, that reduced the current by 75.6±3.8%, n=6). Addition of SVA in the presence of quinidine almost abolished the current at the end of the pulses, the blockade reaching a 91.2±3.1% (P<0.05, n=6). As in the case of atorvastatin, the additive effects produced by the combination were also observed when SVA was first superfused.

All these results suggest that atorvastatin and SVA do not block hKv1.5 channels by interacting with the quinidine binding site.

3.5. Effects of atorvastatin on native K+ currents

To test whether the effects of atorvastatin that we described so far in cloned channels appeared in native currents, in the next group of experiments we studied the effects of atorvastatin in enzymatically dissociated mouse ventricular myocytes which exhibit robust Ito and Ikur generated by Kv4.3 and Kv1.5 channels, respectively. Fig. 5A shows outward K+ currents recorded in mouse ventricular myocytes by applying 250-ms pulses from −80 to +50 mV under control conditions and in the presence of 50 μM 4-AP alone and plus 10 nM atorvastatin. 50 μM 4-AP selectively inhibited Ikur [10,14], leaving a transient current.
Fig. 4. Effects of atorvastatin on hKv1.5 and Kv4.3 channels in the presence of probucol and L-NAME. (A) hKv1.5 currents under control conditions and after superfusion of 5 μM probucol alone and plus 10 nM atorvastatin. (B) Kv4.3 currents under control conditions and after superfusion of 1 μM probucol alone and plus atorvastatin. (C) hKv1.5 currents under control conditions and after superfusion of 500 μM L-NAME alone and plus 10 nM atorvastatin. (D) Kv4.3 currents under control conditions and after superfusion of L-NAME alone and plus atorvastatin. hKv1.5 currents elicited by applying 500-ms pulses from −80 to +60 mV under control conditions, after superfusion of 10 μM quinidine alone or in combination with 10 nM atorvastatin (E) and 8 μM SVA (F). The inset in panel E shows the concentration dependence of the effects of atorvastatin alone (same data as that in Fig. 1C) and of the combination of a fixed dose of quinidine (10 μM) plus increasing concentrations of atorvastatin.
that rapidly activated and decayed to a constant level at the end of the pulse. Superfusion of atorvastatin plus 4-AP further reduced the current amplitude by 42.0 ± 5.8% (n = 5) without modifying the inactivation kinetics (τfC = 21.0 ± 2.3 ms and τsC = 178.4 ± 29 ms, vs. τfAT = 20.1 ± 5.6 ms and τsAT = 199.4 ± 30 ms, P > 0.05). We next studied the effects of atorvastatin on IKur. A 200-ms prepulse to +40 mV to inactivate IKur was applied followed by a 250-ms pulse to +50 mV, eliciting a slowly inactivating outward K+ current (see inset in panel B) composed of at least two components, where only IKur, carried by Kv1.5 channels, exhibits slow inactivation [14]. Atorvastatin inhibited the current (measured as the difference between the peak current and the current at the end of the pulse) elicited by the pulse to +50 mV by 38.9 ± 9.4% (n = 4), without modifying the time course of current decay (τC = 244.2 ± 34.7 ms vs. τAT = 252.1 ± 25.2 ms, P > 0.05) (Fig. 5B). Finally, it should be stressed that the blocking effects produced by 8 μM VnKv1.5 and Kv4.3 were also reproduced in both IKur and IKur (not shown).

3.6. Effects of atorvastatin and SVA on ICa,L

Since the plateau phase duration is the result of a delicate balance between outward K+ and ICa,L currents, we also studied the effects of atorvastatin and SVA on ICa,L recorded in mouse ventricular myocytes. Fig. 6A illustrates the stability of the current when recorded over a prolonged period of time in 6 cells. Atorvastatin 10 nM did not modify the current amplitude (1.9 ± 2% of inhibition at 0 mV, n = 6, P > 0.05) at any of the voltages tested (Figs. 6B and C). Furthermore, increasing the frequency of stimulation by applying trains of 150-ms pulses at 0.5, 1, and 2 Hz did not significantly increase the blockade (Table 2). In contrast, 10 nM SVA significantly reduced the ICa,L at potentials positive to −10 mV (Fig. 6D), the blockade at 0 mV being 12.3 ± 1.8% (n = 6, P < 0.05) (Fig. 6E). These effects were completely reversible after washout (current amplitude after washout was 94.0 ± 3.5% of the control amplitude). In Fig. 6F the blockade at 0 mV when applying pulses at 0.1 Hz was plotted as a function of the SVA concentration, the IC50 being 3.9 ± 0.2 μM (n1 = 1) and 4.1 ± 0.1 μM (n2 = 0.8 ± 0.02). We also explored the possible frequency dependence of this blockade. SVA at 10 nM produced a marked frequency-dependent ICa,L block that reached a 50.2 ± 1.5% when trains of pulses at 2 Hz were applied (Fig. 6G and Table 2).

3.7. Effects of atorvastatin and SVA on mouse atrial APs

For identifying the putative significance of the effects of atorvastatin and SVA on IKur, IKur, and ICa,L in shaping the cardiac AP in the atria of an species that exhibit both Kv1.5 and Kv4.3 channels, we next studied the effects of both statins on the AP characteristics recorded in multicellular mouse atrial preparations. Figs. 7A and B show APs recorded using microelectrodes techniques in the absence and presence of 10 nM atorvastatin and 10 nM SVA, respectively. Atorvastatin and SVA did not modify the resting membrane potential (RPc = −79.0 ± 1.5 mV) or the AP amplitude (APAc = 104.6 ± 1.8 mV) (P > 0.05, n = 10). Atorvastatin slightly, but significantly, lengthened the APD90 (38.6 ± 3.2 ms vs. 48.6 ± 4.7 ms, n = 6, P < 0.05), whereas SVA, at the same concentration, significantly shortened the APD50 (10.4 ± 1.6 ms vs. 7.3 ± 0.9 ms), and APD90 (42.3 ± 2.3 ms vs. 35.3 ± 2.6 ms) (n = 4, P < 0.05) (Fig. 7B). Concentration-dependent effects of atorvastatin and SVA on APD measured at 20, 50, and 90% of repolarization in mouse atrial preparations are plotted in Figs. 7C and D, respectively. Atorvastatin significantly prolonged the APD90 at concentrations ≥ 1 nM.

Fig. 5. Effects of atorvastatin in native IKur and IKur currents. (A) Outward K+ currents recorded in mouse ventricular myocytes elicited by applying 250-ms pulses from −80 to +50 mV under control conditions and after superfusion of 50 μM 4-AP alone and plus 10 nM atorvastatin. (B) Outward K+ currents elicited by applying 250-ms pulses to +50 mV, after a prepulse to +40 mV, in the absence and presence of atorvastatin. The inset shows the outward K+ current recorded by applying a 250-ms prepulse to +40 mV to inactivate the IKur followed by the test pulse to +50 mV.
Fig. 6. Effects of atorvastatin and SVA on \( I_{Ca,L} \). (A) Normalized mean \( I_{Ca,L} \) amplitude at 0 mV as a function of time to demonstrate the current stability during the recordings. \( I_{Ca,L} \) recorded in mouse ventricular myocytes elicited by applying 500-ms pulses to potentials between −25 and +45 mV after a 50-ms prepulse to −30 mV to inactivate \( I_{Na} \), in the absence and presence of 10 nM atorvastatin (B) and 10 nM SVA (D). \( I_{Ca,L} \) current–voltage relationships in the absence and presence of atorvastatin (C) and SVA (E). * \( P < 0.05 \) vs. control. (F) Reduction of \( I_{Ca,L} \) at 0 mV as a function of the SVA concentration. (G) Ratio of peak \( I_{Ca,L} \) amplitude in the presence and the absence of SVA (10 nM) as a function of the applied pulse when trains at 0.5, 1, and 2 Hz were applied. § \( P < 0.05 \) vs. frequency-dependent block at 0.5 and 1 Hz. In panels C, E, F, and G each point represents the mean ± SEM of ≥6 experiments.
On the other hand, SVA, at micromolar concentrations at which $I_{\text{Ca,L}}, I_{\text{Kur}}$, and $I_{\text{to}}$ block was apparent, did not modify the APD.

4. Discussion

In this study we demonstrated that atorvastatin and SVA inhibited hKv1.5 and Kv4.3 currents and that this inhibition was reproduced in native $I_{\text{Kur}}$ and $I_{\text{to}}$. Moreover, SVA, but not atorvastatin, inhibited $I_{\text{Ca,L}}$ at concentrations at which it did not block these $K^+$ channels. As a consequence of its effects on plateau currents, atorvastatin lengthened, whereas SVA shortened, the APD in mouse atria. The results also suggest that atorvastatin and SVA produced a complex blockade of these $K^+$ channels, suggesting a novel mechanism of action. Independently of the mechanism underlying the observed effects, the present study is, to our knowledge, the first demonstration of the blockade of cardiac hKv1.5 and Kv4.3 channels by atorvastatin and of $I_{\text{Ca,L}}$ by SVA at nanomolar concentrations.

Atorvastatin-induced block of hKv1.5 and Kv4.3 channels increased almost linearly in the wide range of concentrations tested, without reaching saturation. On the contrary, concentration–response curves of SVA on hKv1.5 and Kv4.3 were extremely steep. The Hill equation assumes a simple binding reaction between the drug and its receptor following the law of mass action. However, it is accepted that the $n_H$ is an “interaction coefficient”, which reflects cooperativity among putative binding sites at the receptor [21]. Following this assumption, our results could suggest that there is more than one binding site for SVA, both on hKv1.5 and on Kv4.3 channels, positively coupled among them, whereas there is only one binding site in Ca$^{2+}$ channels. In the same way, there may be more than one binding site for atorvastatin negatively coupled among them, both in hKv1.5 and Kv4.3 channels. It was accepted that channel blocking drugs interact with some residues, highly conserved among K$^+$ channels, located in the S6 domain and near the base of the pore helix. In hKv1.5 channels most of these residues face towards the channel pore [19]. This implies that there is a single drug binding site on each hKv1.5 channel which is common for most of the blockers so far described. However, other residues, which do not face the pore, are also important for determining the potency of block of drugs like AVE0118 [22]. The existence on hKv1.5 channels of residues that determine the drug binding and that do not face the central cavity of the pore allows the speculation about the presence of several binding sites on each channel. Our results demonstrated that in hKv1.5 channels the atorvastatin- and SVA-induced block was added to the quinidine-induced block, a result that suggests that atorvastatin and SVA do not bind to the quinidine binding site on hKv1.5 channels, which is assumed to be located at the pore [20]. Therefore, the effects produced by atorvastatin and SVA on K$^+$ channels could be interpreted either as the result of their interaction with several coupled non described binding sites at the channel level or as a result of an unspecified perturbation of the membrane that alters the channel behavior reducing the ion efflux.

In favor of the latter hypothesis is the fact that both SVA and atorvastatin are highly lipophilic drugs ($\log P \approx 5–6$) that...
become easily embedded into the membrane having overlapping locations in the hydrocarbon core adjacent to the phospholipid headgroups [12]. Moreover, the blocking effects could be attributed to an effect on the lipid content of the membrane. However, atorvastatin and simvastatin decrease the HMG-CoA reductase activity in L cells only after incubation with the drugs for 18 h [23]. Thus, it seems very difficult to attribute the effects of both drugs to a change on the lipid content of the membrane since it is unlikely that in 4–6 min these drugs can produce a membrane cholesterol synthesis inhibition that can recover within 10 min of drug washout. Moreover, in our experiments the membrane capacitance did not change, as it would be expected if the lipid composition of the membrane (which modulates the dielectric constant, and thus, the capacitance [24,25]) had changed. Therefore, it could be concluded that the non-specific perturbation of the membrane seems a very unlikely mechanism for being responsible for the atorvastatin and SVA effects, even when a modification of the cholesterol enrichment of the lipid rafts surrounding the channels cannot be ruled out [26]. Furthermore, under our experimental conditions, neither the antioxidant effects nor the ability to increase the production of NO exhibited by statins were involved in the effects.

Besides the data suggesting that atorvastatin induced block increases with the activation and inactivation of the Kv4.3 and hKv1.5, channels, there are data that strongly suggest a direct interaction of the statins with the channel proteins. Indeed, atorvastatin produced a frequency-dependent block of hKv1.5 and Kv4.3 channels and SVA of Ca2+ channels. Frequency-dependent block has classically been explained on the basis of a direct interaction of the drug with the channel protein when the channel transits through its open and/or inactivated state. Very recently it has been demonstrated that toluene, a volatile organic compound (VOC), also produces a frequency-dependent block of Nav1.5 channels expressed in oocytes [27]. It is interesting to note that the effects of VOCs (like toluene, 1,1,1-trichloroethylene, and perchloroethylene) and volatile anesthetics (like halothane and isoflurane) used to be attributed to their ability to perturb the fluidity of the plasma membrane. In contrast, currently existing evidence indicates that the voltage- and time-dependent modifications produced by VOCs and volatile anesthetics exhibit molecular determinants that suggest a specific interaction with transmembrane segments of Na+ [28], Ca2+ [29–31], and K+ [32–34] channel proteins. In fact, frequency-dependent block of Nav1.4 produced by toluene is determined by its binding to the F1579 residue located in the S6 segment [28]. Furthermore, the concentration dependence of the effects of atorvastatin and SVA on hKv1.5 and Kv4.3 currents is similar to the concentration dependence exhibited by several VOCs in Ca2+ currents measured in PC12 cells [29]. In fact, perchloroethylene exhibited a very steep, whereas 1,1,1-trichloroethylene an almost linear concentration–response curve. Therefore, channel blocking properties of atorvastatin and SVA are very similar to those exhibited by very lipophilic drugs like VOCs and volatile anesthetics, suggesting a common blocking mechanism which involves a specific interaction with the channel protein.

4.1. Clinical implications

Statins are the most prescribed agents for treating hypercholesterolemia because of their efficacy in reducing LDL-C. Furthermore, they are also useful in the management and prevention of cardiovascular diseases in normocholesterolemic patients, due to their pleiotropic actions [4,5]. However, most of the pleiotropic effects of atorvastatin and SVA are produced in vitro at concentrations between 1 and 10 μM [5,35,36], while free peak plasma concentrations reached after administration of 20–80 mg atorvastatin or 40 mg simvastatin are 0.8–12 nM and 6 nM (SVA concentrations), respectively [37,38]. Therefore, the present results demonstrate that, at the same concentration at which atorvastatin inhibits the HMG-CoA reductase, it also decreases hKv1.5 and Kv4.3 currents. Furthermore, SVA, at the HMG-CoA inhibitory concentration, produces a frequency-dependent block of the ICa,L.

Experimental and clinical studies have demonstrated that statins could be useful in the prevention of development and recurrences of AF, through mechanisms independent of the reduction in serum cholesterol levels [6,7,39,40]. In fact, in some of these studies the antiarrhythmic effects were apparent after a very short period of administration [6,39]. Simvastatin prevented AF promotion in dogs by attenuating the APD and refractoriness abbreviation and the downregulation of the Cav1.2 α-subunit expression produced after 7 days of atrial tachypacing [40]. It has been proposed that the control of intracellular Ca2+ concentration, which is likely altered in the face of rapid rates, would help to prevent the Ca2+-dependent gene transcription responsible for the electrical remodeling [41]. Therefore, it is possible that the frequency-dependent reduction in ICa,L produced by SVA might contribute to the attenuation in Cav1.2 downregulation and to the prevention of the electrical remodeling observed with simvastatin in the dog model of AF [40]. On the other hand, our results indicated that atorvastatin, as a consequence of its hKv1.5 and Kv4.3 blocking properties, prolongs mouse atrial AP. Therefore, it would be of great interest to analyze the effects of atorvastatin in human atrial APs. Particularly because, even when in mouse atria Kv1.5 and Kv4.3 channels are present, the mouse atrial AP do not recapitulate the shape and properties of human APs because of the presence of several K+ channels not expressed in human myocardium [11]. Data on human atrial preparations and in animal models demonstrated that blockers of IKur and Ito may have a high clinical potential in the treatment of AF [42,43] because they prolong the refractoriness in the remodeled atria with minor effects in ventricular repolarization. Furthermore, recent data suggested that, in human atria, the blockade of IKur and Ito would increase the height and duration of the plateau [43], a prolongation which is critical for the termination of the periodic activity of sustained, high frequency, functional reentrant sources (rotors) that can be responsible for AF [44]. Therefore, we conclude that atorvastatin and SVA may modify human atrial plateau currents, producing a mild prolongation of the plateau phase, in the case of atorvastatin,
and a decrease in the Ca$^{2+}$ entry into the myocyte, in the case of SVA. However, caution should be exerted before using the present results to explain the possible antiarrhythmic actions of atorvastatin and simvastatin, particularly when considering that the current analysis has been performed at room temperature and using a low frequency of stimulation for avoiding the accumulation of block and/or current inactivation. Moreover, here we describe the acute effects produced by atorvastatin and SVA on ion channels involved in the plateau phase, whereas the possible effects produced after long period of treatment were not analyzed. Thus, further studies are needed to determine the putative clinical implications of these results and if the novel mechanism of hKv1.5 and Kv4.3 block produced by atorvastatin and SVA can translate into therapeutical advantages or disadvantages.

5. Nomenclature

Footnotes

HMG-CoA reductase 3-Hydroxy-3-methylglutaryl coenzyme A reductase
LDL-C Low-density lipoprotein cholesterol
CAD Coronary artery disease
AF Atrial fibrillation
NOS Nitric oxide synthase
$\frac{I_{\text{Ca,L}}}{I_{\text{Ca,L}}} $ L-type calcium current
APD Action potential duration
$\frac{I_{\text{Kr}}}{I_{\text{Kr}}} $ Ultrarapid delayed rectifier K$^+$ current
4-AP 4-Aminopyridine
$\frac{I_{\text{to}}}{I_{\text{to}}} $ Transient outward current
KChIP Kv channel-interacting proteins type 2
DPP6 Dipeptidyl-aminopeptidase-like protein 6
SVA Simvastatin acid
AP Action potential
CHO Chinese hamster ovary cells
DMEM Dulbecco’s Modified Eagle Medium
HEPES N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]
EGTA Ethylene glycol-bis(b-amino ethyl ether)N,N,N’,N’-tetraacetic acid
TEA Tetraethylammonium
IC$_{50}$ Concentration of drug that produces the half maximum blockade
$\eta_{\text{H}} $ Hill coefficient
$\frac{I_{\text{K,slow}}}{I_{\text{K,slow}}} $ Slowly inactivating delayed rectifier outward K$^+$ current
SEM Standard error of the mean
$V_{\text{h}} $ The midpoint of the activation/inactivation curve
$k $ Slope factor for the activation/inactivation curve
$\tau $ Time constant
VOCs Volatile organic compounds

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