

RESEARCH PAPER

Statins and fenofibrate affect skeletal muscle chloride conductance in rats by differently impairing CIC-1 channel regulation and expression

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Background and purpose: Statins and fibrates can produce mild to life-threatening skeletal muscle damage. Resting chloride channel conductance (gCl), carried by the CIC-1 channel, is reduced in muscles of rats chronically treated with fluvastatin, atorvastatin or fenofibrate, along with increased resting cytosolic calcium in statin-treated rats. A high gCl, controlled by the Ca²⁺-dependent protein kinase C (PKC), maintains sarcolemma electrical stability and its reduction alters muscle function. Here, we investigated how statins and fenofibrate impaired gCl.

Experimental approach: In rats treated with fluvastatin, atorvastatin or fenofibrate, we examined the involvement of PKC in gCl reduction by the two intracellular microelectrodes technique and CIC-1 mRNA level by quantitative real time-polymerase chain reaction. Direct drug effects were tested by patch clamp analysis on human CIC-1 channels expressed in human embryonic kidney (HEK) 293 cells.

Key results: Chelerythrine, a PKC inhibitor, applied *in vitro* on muscle dissected from atorvastatin-treated rats fully restored gCl, suggesting the involvement of this enzyme in statin action. Chelerythrine partially restored gCl in muscles from fluvastatin-treated rats but not in those from fenofibrate-treated rats, implying additional mechanisms for gCl impairment. Accordingly, a decrease of CIC-1 channel mRNA was found in both fluvastatin- and fenofibrate-treated rat muscles. Fenofibric acid, the *in vivo* metabolite of fenofibrate, but not fluvastatin, rapidly reduced chloride currents in HEK 293 cells.

Conclusions and implications: Our data suggest multiple mechanisms underlie the effect of statins and fenofibrate on CIC-1 channel conductance. While statins promote Ca²⁺-mediated PKC activation, fenofibrate directly inhibits CIC-1 channels and both fluvastatin and fenofibrate impair expression of mRNA for CIC-1.

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Abbreviations: 9-AC, anthracene-9-carboxylic acid; CPP, 2-p-(chlorophenoxy)-propionic acid; EDL, extensor digitorum longus; EMG, electromyographic; gCl, chloride channel conductance; gK, potassium conductance; HEK, human embryonic kidney; PKC, protein kinase C; r_m , membrane resistance; RMP, resting membrane potential

Introduction

Statins (HMG CoA reductase inhibitors) are widely used to reduce plasma cholesterol levels, and several clinical trials have established the benefit of these drugs in the prevention of coronary heart disease (Davidson and Robinson, 2007).

Recent findings have also shown that statins may have cholesterol-independent beneficial effects during inflammatory syndromes, Alzheimer's disease and cancer, that may expand their clinical utility (Weitz-Schmidt, 2002; Stuve *et al.*, 2003; Demierre *et al.*, 2005). However, statin therapy can be severely limited by adverse effects involving many tissues, especially skeletal muscle. Cerivastatin, marketed in the United States in 1998, was subsequently withdrawn because of a high rate of rhabdomyolysis-induced deaths associated with the drug. Although rhabdomyolysis is very uncommon, clinical use of these medications is more frequently associated with a variety of less severe adverse effects on muscle including myalgia, cramps and weakness that arise during long-term

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use (Thompson *et al.*, 2003). However, the mechanism determining muscle damage is still not clear, although some investigators have implicated certain consequences of inhibiting cholesterol biosynthesis, such as depletion of ubiquinone or of small GTP-binding proteins involved in myocyte maintenance (Vaughan and Gotto, 2004).

Fibrates are another important class of lipid-lowering drugs useful for the management of dyslipidemia. By acting on the peroxisome proliferator-activated receptor (PPAR)- α , mainly expressed in tissues that metabolize fatty acids, these drugs lower serum triglyceride levels and raise high-density lipoprotein-cholesterol (Cannon, 2008). As with statins, fibrate monotherapy is associated with a risk of myopathy (Davidson *et al.*, 2007) and this risk is enhanced when these agents are given together. However, whereas gemfibrozil can increase plasma concentration of the commonly prescribed statins, fenofibrate has less influence on the pharmacokinetics of statins (Cannon, 2008).

We have previously found that lipophilic statins can affect skeletal muscle function by reducing the resting chloride channel conductance (gCl) in rats, through a mechanism independent of their ability to lower endogenous cholesterol (Pierno *et al.*, 2006). We have also demonstrated that chronic treatment with clofibrate reduces resting gCl (Conte Camerino *et al.*, 1984). A large gCl, carried by the ClC-1 chloride channel, is important for muscle function as it stabilizes resting membrane potential (RMP) and helps to repolarize the membrane after action potentials (Aromataris and Rychkov, 2006). Loss of function mutations in ClC-1 cause myotonia congenita, an inherited condition characterized by delayed skeletal muscle relaxation after voluntary contraction (Koch *et al.*, 1992; Jentsch *et al.*, 2005). Resting gCl strictly depends on ClC-1 chloride channel expression and regulation. A Ca²⁺- and phospholipid-dependent protein kinase C (PKC) controls ClC-1 channel activity and activation of PKC closes the channel (Rosenbohm *et al.*, 1999), reducing gCl (Bryant and Conte Camerino, 1991; De Luca *et al.*, 1994). In fast-twitch muscles, the chloride channel is maintained in a dephosphorylated state allowing high resting gCl to preserve muscle membrane potential stability. In contrast, slow-twitch, anti-gravity muscles, such as soleus muscle, are characterized by a lower gCl, due to both a lower expression of the ClC-1 channel and different regulation by PKC (Pierno *et al.*, 2007). Indeed, the use of pharmacological tools, such as chelerythrine, a PKC inhibitor, suggests that a higher basal activity of this enzyme in soleus muscle is responsible for the phosphorylation and closure of a fraction of ClC-1 channels at rest. We previously demonstrated that statins also affect the excitation–contraction coupling mechanism (Pierno *et al.*, 1999a). Accordingly, recent studies confirmed that statins either chronically administered or applied acutely *in vitro* produce a mitochondria-mediated increase of resting cytosolic calcium in intact muscle cells (Sirvent *et al.*, 2005; Liantonio *et al.*, 2007).

To address the myotoxic potential of statins and fibrates, we investigated the molecular mechanisms by which these drugs reduce resting gCl. We treated rats chronically with fluvastatin (5 mg·kg⁻¹ and 20 mg·kg⁻¹), atorvastatin (10 mg·kg⁻¹) or fenofibrate (60 mg·kg⁻¹) and then analysed the effects of drug treatments on the signalling pathways related to PKC-

mediated gCl regulation and on ClC-1 gene expression. We also evaluated the direct effects of drugs by measuring macroscopic gCl in native skeletal muscle fibres or chloride current from human ClC-1 channels expressed in human embryonic kidney (HEK) 293 cells. Our experiments have demonstrated different mechanisms responsible for drug-induced reduction of gCl. The activation of Ca²⁺-dependent PKC was involved in statin-induced gCl decline, whereas fenofibrate was able to reduce gCl by a direct action on ClC-1. A concomitant decrease in ClC-1 mRNA expression occurred in both fluvastatin- and fenofibrate-treated animals and probably contributed to the reduced gCl.

Methods

Animal care and treatments

All experiments were conducted in accordance with the Italian Guidelines for the use of laboratory animals, which conforms with the European Community Directive published in 1986 (86/609/EEC). Adult male Wistar rats (Charles River Laboratories, Italy) were used for chronic and acute studies. The animals, weighing 300–350 g at the beginning of the treatment, were housed individually in rat appropriate cages in an environmentally controlled room and fed with 30 g·day⁻¹ of a commercial rodent chow (Charles River, 4RF21) in addition to tap water *ad libitum*. Five groups of five rats each were chronically treated with 20 mg·kg⁻¹ of fluvastatin, 5 mg·kg⁻¹ of fluvastatin, 10 mg·kg⁻¹ of atorvastatin, 60 mg·kg⁻¹ of fenofibrate and with the vehicle (carboxymethylcellulose, CMC) used to dissolve the drugs. Fluvastatin (Lescol, Novartis), atorvastatin (Torvast, Pfizer) and fenofibrate (Lipsin, Caber) dissolved in CMC (0.5%) were administered to the different groups of animals orally via an esophageal cannula, once a day, for 2 months (Pierno *et al.*, 2006). During treatment, all rats were weighed weekly and vital parameters (general health conditions, water and food consumption) were recorded. Rats treated with the highest dose of fluvastatin (20 mg·kg⁻¹) exhibited a slight decrease in body weight gain, compared with control and all other treated animals. This effect, related to a lower food intake (25 g·day⁻¹ vs. 30 g·day⁻¹), was probably due to a non-muscle effect. Skeletal muscle performance was evaluated daily by testing each rat for the righting reflex (the ability of the rat to straighten itself on four legs when turned on its back) which, when abnormal, is a sign of myotonia. In all the animals, the righting reflex evaluated during the entire treatment period was normal. Drug and channel nomenclature conforms to the British Journal of Pharmacology Guide to Receptors and Channels (Alexander *et al.*, 2008).

Electromyographic recordings

Electromyographic (EMG) technique was used to evaluate the presence of muscular abnormalities due to drug treatment or myotonic-like signs (Heller *et al.*, 1982). EMG traces were recorded in all rats in each experimental condition at the beginning and every 2 weeks until the end of treatment by using the Biopack MP100 acquisition system (EMG 100C, Biopack Systems Inc., Santa Barbara, CA, USA). EMG activity

was measured by two thin concentric needle electrodes inserted in the gastrocnemius muscle of rats anaesthetized with pentobarbital (60 mg·kg⁻¹, i.p., supplemented if required). Insertional activity was initiated by slight movement of the electrode within the muscle and the presence of spontaneous activity (not due to the hind-limb movement) was evaluated. EMG activity was monitored continuously over a 3- to 4-min period starting from the insertion of the electrodes. A qualitative estimate of myotonic-like activity was based on various parameters such as the voltage excursion, the frequency and the duration of the burst. EMG activity was also recorded in three rats acutely injected with anthracene-9-carboxylic acid (9-AC) (5 mg·kg⁻¹ i.p.) (Sigma Aldrich, Italy) a well-known blocker of ClC-1 channel conductance and, because of this, a myotonia-inducer (Kwieciński *et al.*, 1988). We used 9-AC as a reference compound for observing myotonia discharges on EMG recordings, with the aim of comparing the effects of long-term treatments with statins or fenofibrate.

Recordings of skeletal muscle gCl by two-intracellular microelectrode technique: in vivo and in vitro studies

The extensor digitorum longus (EDL) and soleus muscles were dissected from treated and control animals under urethane anesthesia (1.2 g·kg⁻¹ i.p.). The preparations were immediately placed in a 25 mL muscle bath, maintained at 30°C and perfused with normal or chloride-free physiological solution (gassed with 95% O₂ and 5% CO₂; pH = 7.2–7.3) (Bryant and Conte Camerino, 1991). The normal physiological solution had the following composition (in mmol·L⁻¹): NaCl 148, KCl 4.5, CaCl₂ 2.0, MgCl₂ 1.0, NaHCO₃ 12.0, NaH₂PO₄ 0.44 and glucose 5.55. The chloride-free solution was prepared by equimolar replacement of methylsulphate salts for NaCl and KCl and nitrate salts for CaCl₂ and MgCl₂. The contralateral EDL muscles were also surgically removed and frozen in liquid nitrogen until mRNA analysis.

As previously detailed (Bryant and Conte Camerino, 1991; De Luca *et al.*, 1994; Pierno *et al.*, 2007), by means of standard two-intracellular-microelectrode technique, RMP, cable parameters, component conductances and excitability characteristics of treated and untreated muscle fibres were measured in current-clamp mode. Cable parameters, in both normal and chloride-free solutions, were calculated from the electrotonic potential elicited by square-wave hyperpolarizing current pulse (100 ms duration) injected by the current electrode. The membrane voltage responses were monitored by the voltage electrode at two distances from the current electrode. The current pulse generation, the acquisition of the voltage records and the calculation of the fibre constants were performed in real time under computer control as described elsewhere (Bryant and Conte Camerino, 1991). From the experimentally determined values of input resistance, space and time constant, and from an assumed myoplasmic resistivity, of 125 Ω·cm, fibre diameter, membrane resistance (*r_m*) and membrane capacitance were calculated (Bryant and Conte Camerino, 1991). The reciprocal of *r_m* from each fibre in normal physiological solution was assumed to be *g_m* and the same parameter measured in chloride-free solution was considered to be the potassium conductance (gK). The mean

gCl was estimated as the mean *g_m* minus the mean gK (Bryant and Conte Camerino, 1991).

For *in vitro* studies atorvastatin calcium salt (Synfine Laboratories, Richmond Hill, Canada), fluvastatin sodium salt (Calbiochem, Milan, Italy), simvastatin (Tocris Bioscience, Bristol, UK) and fenofibric acid, the *in vivo* metabolite of fenofibrate (Sigma Aldrich, Milan, Italy), were dissolved in dimethyl sulphoxide and tested on isolated EDL muscle dissected from control rats. Fenofibric acid was obtained by hydrolysis of fenofibrate. Resting gCl was measured before and 30 min after addition of increasing concentrations of the different hypolipidemic drugs. The concentration-response curves obtained were well fitted with a first-order binding function:

$$gCl = \min + (1 - \min) / (1 + ([\text{drug}] / IC_{50})^h),$$

where IC₅₀ (μmol·L⁻¹) is the half-maximum inhibitory concentration, *h* is the slope factor and min is the minimal gCl.

Chelerythrine (Tocris Bioscience) 1 μmol·L⁻¹ was applied *in vitro* on EDL muscles dissected from control and chronically treated rats. To study if chelerythrine prevented the reduction of gCl induced acutely by hypolipidemic drugs, we pre-incubated EDL control muscles with chelerythrine for 1 h and then measured gCl 30 min after hypolipidemic drug addition.

Real-time quantitative polymerase chain reaction (PCR)

For each muscle sample, total RNA was isolated by using Trizol reagent. Total RNA (3 μg) was used for reverse transcription. Synthesis of cDNA was performed by using random hexamers (annealed 10 min, 25°C) and Superscript II reverse transcriptase (Invitrogen-Life Technologies, Carlsbad, CA, USA) incubated at 42°C for 50 min. We assayed expression of ClC-1 mRNA using a pre-designed TaqMan assay (Assay ID Rn00565736_ml; Applied Biosystems, Foster City, CA, USA) and a custom assay for β-actin (primer and probes sequences available on request). Triplicate reactions were carried out in parallel for each individual muscle sample. The results were compared with a gene-specific standard curve and normalized to expression of the housekeeping gene β-actin in the same samples. The template used for determining standard curves consisted of plasmid DNA containing the expected target sequence evaluated by Spectrophotometer (ND-1000 Nano-Drop, Wilmington, DE, USA) (Rogers *et al.*, 2002; Lundquist *et al.*, 2005).

Whole-cell patch-clamp recordings of hClC-1 chloride currents

Full-length hClC-1 constructs were subcloned into the mammalian expression vector pRc/CMV as previously described (Rosenbohm *et al.*, 1999). The HEK 293 cells used in the study were from stocks maintained in this laboratory. They were cultured in standard Dulbecco's modified Eagle's medium with penicillin/streptomycin (DMEM+P/S) before and after transfection. The cells were passaged in a 100 mm dish about 24 h before transfection. Cell density for transfection was about 60–80%. The HEK 293 cells were cotransfected with 4 μg of plasmid DNA encoding the chloride channel and lower amount of plasmid DNA encoding the CD8 receptor,

using the calcium phosphate co-precipitation method (Desaphy *et al.*, 2001). About 15 h after transfection (full confluence was reached), the cells were washed with DMEM+P/S, divided in small 60 mm dishes and maintained in DMEM+P/S until patch-clamp (36–72 h after transfection). The cells were washed several times with recording solution before patch-clamping.

For patch-clamp recording, successfully transfected cells were identified using Dynal microbeads coated with anti-CD8 antibody (Dynal AS, Oslo, Norway). Whole-cell CIC-1 chloride currents were recorded at room temperature (20–22°C) using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA). Voltage clamp protocols and data acquisition were performed with PClamp 9.2 software (Axon Instruments) through a 12-bit A-D/D-A interface (Digidata 1322A, Axon Instruments). The external solution contained (mmol·L⁻¹): 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES and 5 glucose, and the pH was set to 7.4 with NaOH. The pipette solution contained (mmol·L⁻¹): 130 CsCl, 2 MgCl₂, 5 EGTA and 10 HEPES, and the pH was adjusted to 7.4 with CsOH. With such solutions, pipettes made from Corning 7052 glass (Garner Glass, Claremont, CA, USA) had a resistance of 1–3 MΩ. Currents were low-passed filtered at 5 kHz (–3 dB) by the amplifier and digitized at 10–20 kHz. Series resistance and whole-cell capacitance were almost fully compensated for by the amplifier circuitry and no run-down was observed during the experiments.

Statistical analyses

Data are expressed as mean ± SEM. The estimates for SEM and number of fibres (*n*) of gCl were obtained from the variance of gm and gK, assuming no covariance, and from the number of fibres sampled for gm and gK respectively (Bryant and Conte Camerino, 1991). Analysis of variance (ANOVA) was used to evaluate statistical differences between treated and control groups followed by Bonferroni's *t*-test or Tukey's post-test (only for real-time PCR). Comparison of means from each treated rat and the related control group was evaluated by unpaired Student's *t*-test.

Results

To determine the cellular and molecular mechanisms responsible for diminished muscle membrane gCl, we used several *ex vivo* and *in vitro* approaches. We will first present the results of the *in vivo* studies followed by our findings from *in vitro* and molecular experiments.

In vivo studies

Effects of drug treatment on EMG activity of gastrocnemius muscle The gastrocnemius muscle of control rats showed normal electrical activity characterized by rapid and brief bursts occurring only during voluntary movements and followed by long periods of rest during muscle inactivity. We recorded slight spontaneous electrical activity in one out of 10 animals treated with fluvastatin at both doses, in two out of 10 animals treated with atorvastatin and in two out of 10 rats

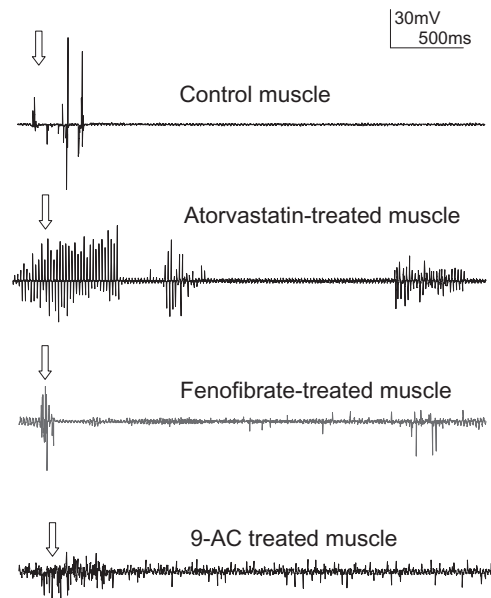


Figure 1 Example of EMG activity recorded from the gastrocnemius muscle of control rats and rats treated with atorvastatin (10 mg·kg⁻¹) or fenofibrate (60 mg·kg⁻¹) anaesthetized with pentobarbital. In the control animal, repetitive discharges occurred with needle insertion (normal insertional activity indicated by the arrow) and were followed by a long period of muscle inactivity. Repetitive discharges with variable frequency and amplitude were apparent and persisted for a few ms in an atorvastatin-treated rat. Additional bursts could be elicited by minimal movement of the needle *in situ*. This effect was observed in two of 10 treated rats. In fenofibrate-treated rats spontaneous discharges lasting a few seconds appeared after rat movement. This effect was observed in two of 10 treated rats. In comparison, the EMG activity of rats treated with 9-AC showed higher activity than in atorvastatin and fenofibrate-treated rats, lasting several seconds. This effect was observed in three out of three animals. Calibration bar, 30 mV, 500 ms. 9-AC, anthracene-9-carboxylic acid; EMG, electromyographic.

treated with fenofibrate. Figure 1 illustrates a representative EMG trace recorded from an atorvastatin-treated rat in which brief high-frequency repetitive discharges, lasting 500 ms, appeared after muscle movement. In the fenofibrate-treated rat, we also observed high-frequency repetitive discharges that persisted for 4 s, at most. However, these EMG abnormalities found in statin and fenofibrate-treated rats appeared only after 7–8 weeks of treatment. In comparison, as previously documented (Heller *et al.*, 1982; Kwieciński *et al.*, 1988), animals acutely treated with 9-AC exhibited the expected myotonic-like discharges with spontaneous high-frequency electrical activity recorded starting 1 h after the injection, and lasting several seconds (>5 s) after voluntary movement (Figure 1). These recordings were more dramatic than those observed in animals treated with hypolipidemic drugs.

Ex vivo studies

Effects of statins and fibrate chronic treatment on muscle gCl: involvement of PKC As previously demonstrated (Pierno *et al.*, 2006), gCl was reduced by 20–35% in EDL muscles of rats chronically treated with the different drugs, compared with the gCl in muscles from control animals (Figure 2). In contrast, gCl measured in soleus muscle was unchanged (control

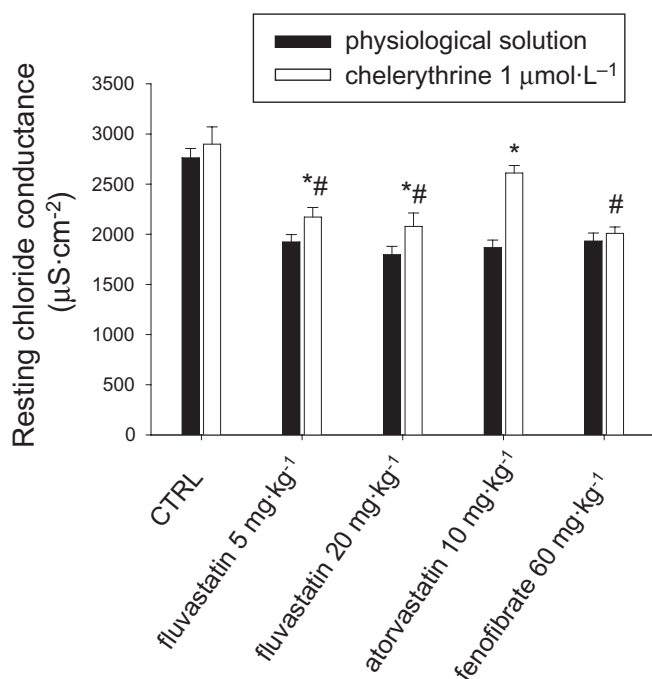


Figure 2 Effects of chelerythrine application *in vitro* on the chloride channel conductance (gCl) of extensor digitorum longus muscle of fluvastatin (5 mg·kg⁻¹ or 20 mg·kg⁻¹), atorvastatin (10 mg·kg⁻¹) and fenofibrate (60 mg·kg⁻¹)-treated and control (CTRL) rats. Each bar represents the mean \pm SEM of gCl measured in muscle fibres in the presence of chelerythrine (1 μ mol·L⁻¹) with respect to the value recorded in the absence of the compound (physiological solution). The means have been calculated from 25–35 fibres of 3–5 rats. *Significantly different with respect to the value measured in the absence of chelerythrine (by Student's *t*-test) ($P < 0.05$ or less). #Significantly different with respect to control value measured in physiological solution (by Student's *t*-test) ($P < 0.001$).

1511 \pm 178 μ S·cm⁻², $n = 27$; 20 mg·kg⁻¹ fluvastatin 1832 \pm 99 μ S·cm⁻², $n = 25$; 10 mg·kg⁻¹ atorvastatin 1441 \pm 83 μ S·cm⁻², $n = 29$). We analysed the involvement of PKC in the reduction of gCl by using the PKC inhibitor chelerythrine. As observed previously (Pierno *et al.*, 2007), the *in vitro* application of chelerythrine to EDL muscle of rats treated with the vehicle induced a slight increase of gCl (Figure 2) because of the high proportion of CIC-1 channels in a dephosphorylated state. When chelerythrine (1 μ mol·L⁻¹) was applied to EDL muscle dissected from rats treated with 5 mg·kg⁻¹ or 20 mg·kg⁻¹ fluvastatin, gCl was partially restored towards the control value, increasing by 13% and 15%, with respect to the value measured before the application of the PKC inhibitor (Figure 2). Chelerythrine significantly increased gCl of muscles from atorvastatin-treated rat by 40%, reaching the value measured in control rats. In contrast, chelerythrine was unable to antagonize the reduction of gCl induced by fenofibrate treatment (Figure 2).

Effects of chronic treatment with fluvastatin and fenofibrate on CIC-1 mRNA expression Because the reduction of gCl in fluvastatin-treated rats was not completely attributable to an increase of PKC activity, as observed for atorvastatin treatment, we examined the effects of fluvastatin on CIC-1 gene expression. We also investigated CIC-1 mRNA levels in

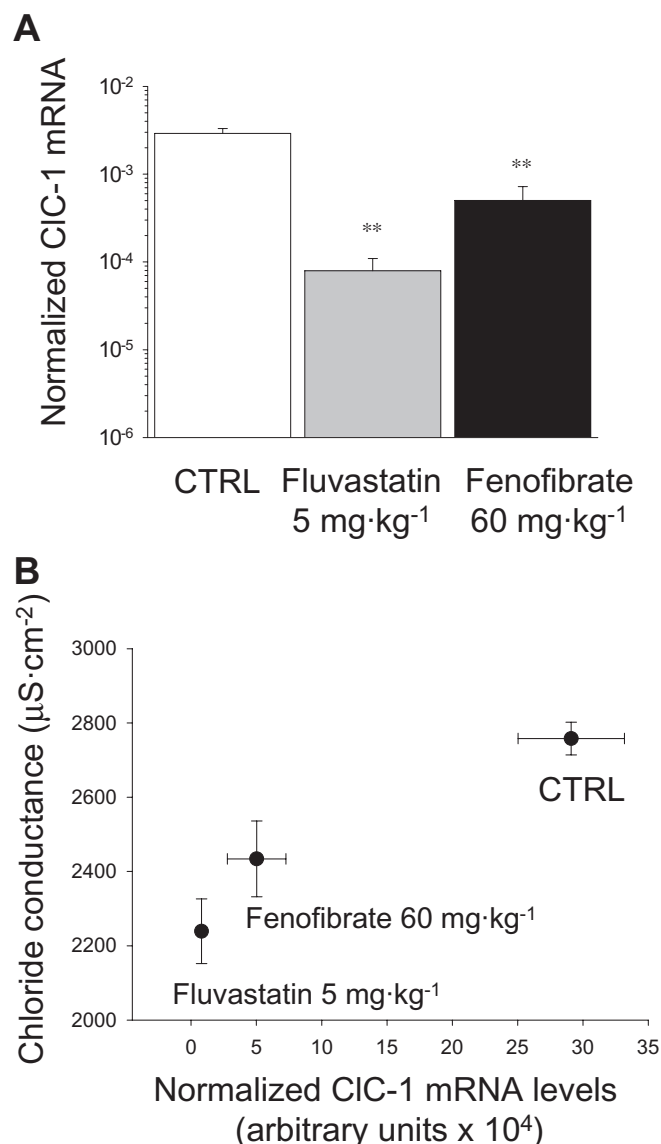


Figure 3 Effects of fluvastatin and fenofibrate treatment on muscle CIC-1 mRNA expression. (A) Normalized CIC-1 mRNA level measured by real time-polymerase chain reaction in extensor digitorum longus (EDL) muscle of 3–4 fluvastatin (5 mg·kg⁻¹) and fenofibrate (60 mg·kg⁻¹)-treated rats with respect to control rats. The level of CIC-1 were normalized to β -actin which was constant in all muscle preparations. **Significantly different compared with control (CTRL) ($P < 0.05$, one-way ANOVA test followed by Tukey's post-test). (B) Relationship between CIC-1 mRNA expression and resting gCl measured in the contralateral EDL muscles of the same animals. gCl, chloride channel conductance.

fenofibrate-treated animals in which PKC is not involved in reducing gCl. The results obtained by real-time quantitative (RT)-PCR analysis demonstrated a reduction of CIC-1 mRNA in EDL muscle of fluvastatin-treated rats, even at the lower dose (5 mg·kg⁻¹) (Figure 3A). Also fenofibrate-treated rats showed lower expression of CIC-1 mRNA than control rats (Figure 3A). The relationship between CIC-1 mRNA levels and gCl is illustrated in Figure 3B. In particular, to better evaluate the individual response to drug treatment, in this figure we plotted the gCl measured only in the contra-lateral EDL

muscles of the same fluvastatin (5 mg·kg⁻¹) or fenofibrate-treated animals in which the CIC-1 mRNA amount had been determined. In these animals, gCl was significantly reduced by 16% and 20%, respectively, slightly different from the data in Figure 2 probably because of variability among individual animals encountered with *in vivo* pharmacological treatments. The degree of reduction in CIC-1 mRNA expression is greater than the respective decrease in gCl and below we discuss potential explanations for this phenomenon.

In vitro studies

Effects of *in vitro* application of fluvastatin, atorvastatin and fenofibric acid on muscle gCl When tested *in vitro* on EDL muscle fibres, fluvastatin reduced gCl in a concentration-dependent manner, with a 30% reduction at the higher concentration tested (50 μmol·L⁻¹, Figure 4A). Atorvastatin tested at the same concentrations was slightly less effective in reducing gCl (50 μmol·L⁻¹, 2183 ± 97 μS·cm⁻², *n* = 13; control, 2736 ± 45 μS·cm⁻², *n* = 24). However, from the concentration-response curve, similar values of IC₅₀ (half-maximal inhibiting concentration) were calculated (fluvastatin: IC₅₀ = 9.2 ± 3.3 μmol·L⁻¹; *h* = 1.33 ± 0.6; minimum = 0.645 ± 0.06. atorvastatin: IC₅₀ = 8.6 ± 7.2 μmol·L⁻¹; *h* = 0.73 ± 0.4; minimum = 0.746 ± 0.07). Chelerythrine completely prevented the reduction of gCl induced by either fluvastatin (Figure 4B) or atorvastatin (2183 ± 97 μS·cm⁻², *n* = 13 and 2982 ± 61 μS·cm⁻², *n* = 20, in the absence and in the presence of chelerythrine respectively). Fenofibric acid, the metabolite of fenofibrate responsible for the *in vivo* drug effect, concentration-dependently reduced gCl (100 μmol·L⁻¹, 2430 ± 79 μS·cm⁻², *n* = 23; 500 μmol·L⁻¹, 1964 ± 121 μS·cm⁻², *n* = 28; no drug, 2745 ± 80 μS·cm⁻², *n* = 23). As observed for *in vivo* treatment, chelerythrine did not prevent the reduction of gCl induced by the *in vitro* application of fenofibric acid (data not shown).

Atorvastatin at the higher concentration tested (50 μmol·L⁻¹) did not modify resting gCl of soleus muscle, being 1540 ± 65 μS·cm⁻², *n* = 17 and 1552 ± 47 μS·cm⁻², *n* = 21; before and after addition of the compound. In contrast, the *in vitro* application of fenofibric acid (500 μmol·L⁻¹) to soleus muscle markedly reduced gCl from 1478 ± 74 μS·cm⁻², *n* = 16 of control to 908 ± 52 μS·cm⁻², *n* = 24, respectively, strongly corroborating the results obtained *in vivo* and giving additional support to the hypothesis of the direct block of the channel with minor involvement of PKC.

Effects of statins and fenofibrate on human CIC-1 channel expressed in HEK 293 cells To confirm the possible direct effect of drugs on the chloride channels responsible for the gCl, fenofibric acid, fluvastatin and simvastatin (500 μmol·L⁻¹) were tested *in vitro* in HEK cells expressing human hCIC-1 channels. We used the more lipophilic simvastatin as a reference compound. Whole-cell chloride currents were recorded before and 5 min after drug application. Neither fluvastatin (Figure 5A,B) nor simvastatin (not shown) had any effect on chloride current amplitude. In contrast, as observed for the structurally related 2-p-(chlorophenoxy)-propionic acid (CPP) derivatives (Aromataris *et al.*, 1999; Liantonio *et al.*, 2003), fenofibric acid caused a rapid and marked decrease of inward hCIC-1 currents and acceleration of current deactivation

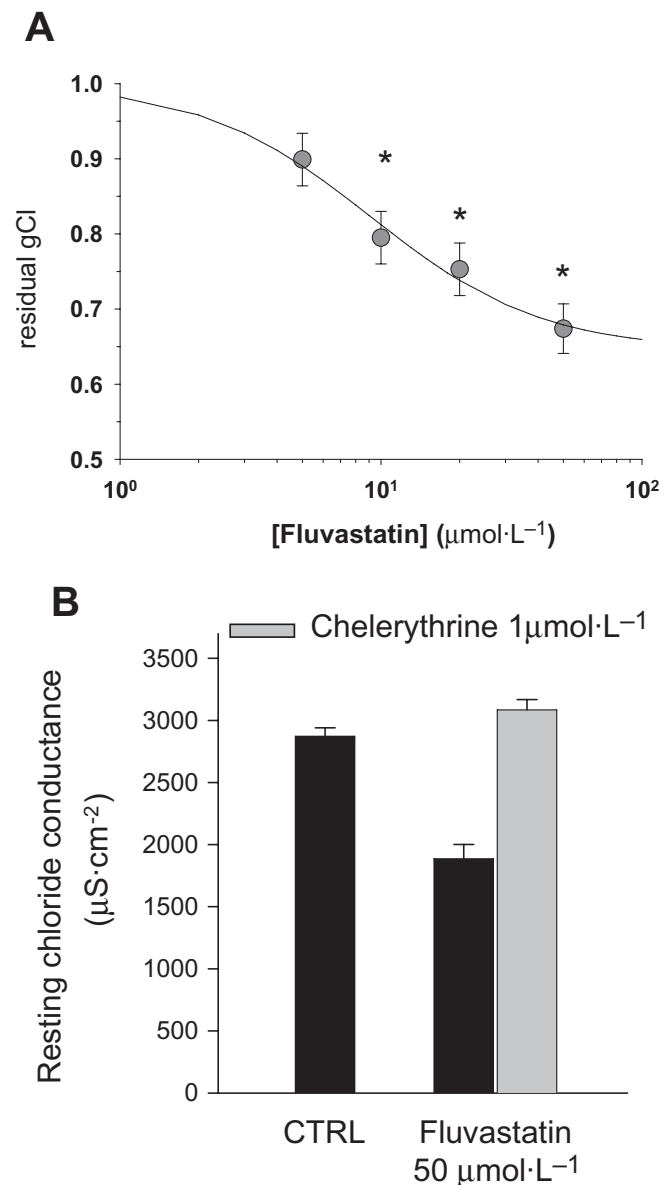


Figure 4 Effects of acute application of fluvastatin (A) on resting chloride channel conductance (gCl) measured in untreated rat extensor digitorum longus muscle. The dose-response relationship was constructed by calculating the residual gCl at different concentrations of fluvastatin. Each data point was calculated as the mean ± SEM of gCl from 27 to 60 fibres of 5–7 rats. *Significantly different with respect to control (*P* < 0.05 or less) by Student's *t*-test. In (B) is shown the effect of chelerythrine (1 μmol·L⁻¹) application in muscle bath containing 50 μmol·L⁻¹ of fluvastatin. The protein kinase C inhibitor completely prevented the reduction of gCl induced by the statin by shifting gCl towards the control (CTRL) value.

(Figure 5C,D). These data strongly suggest that, like the CPP derivatives, fenofibric acid is capable of interfering with CIC-1 gating by interacting with an intracellular binding site (Pusch *et al.*, 2000).

Discussion

The finding that hypolipidemic drugs, such as fenofibrate, clofibrate and lipophilic statins, reduced *in vivo* the resting

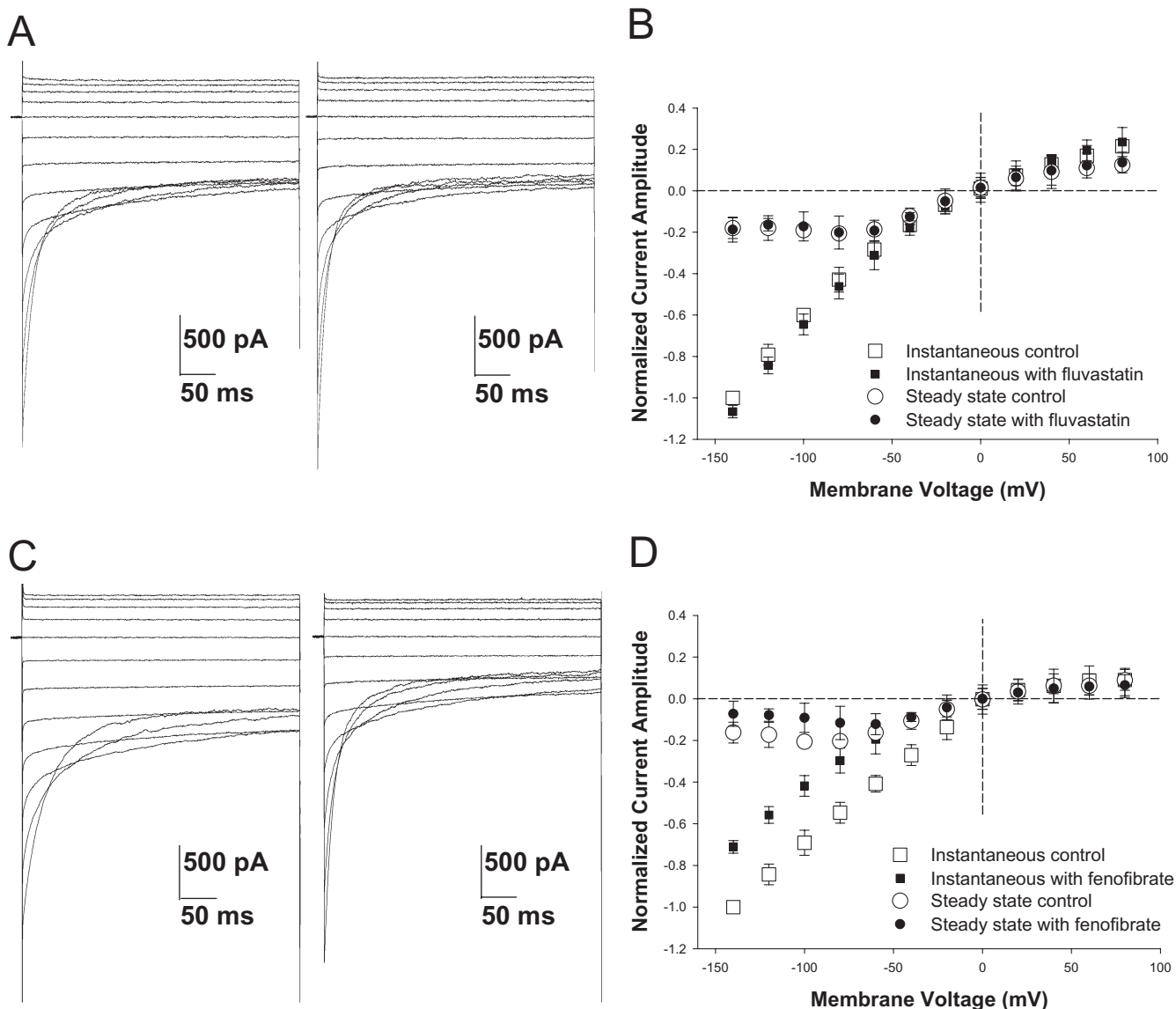


Figure 5 Effects of *in vitro* application of fluvastatin ($500 \mu\text{mol}\cdot\text{L}^{-1}$) and fenofibrate ($500 \mu\text{mol}\cdot\text{L}^{-1}$) on heterologously expressed hCIC-1 channel. Whole-cell patch-clamp chloride currents were elicited from a holding potential of 0 mV by 400 ms test pulses ranging from -140 to $+80$ mV in 20-mV increments. Current was recorded in the absence (A, C left panels), then presence of $500 \mu\text{mol}\cdot\text{L}^{-1}$ fluvastatin (a, right panel) or $500 \mu\text{mol}\cdot\text{L}^{-1}$ fenofibrate (C, right panel). Normalized current-voltage relationships were obtained for instantaneous currents, measured at the beginning of the test pulse, and steady state currents, measured at the end of test pulse, in control and in presence of $500 \mu\text{mol}\cdot\text{L}^{-1}$ fluvastatin (B) or $500 \mu\text{mol}\cdot\text{L}^{-1}$ fenofibrate (D). Each data point is the mean \pm SEM from four cells (fluvastatin) or five cells (fenofibrate). In each cell, the current amplitude was normalized with respect to current value measured at -140 mV in control condition. Fluvastatin had no effect, whereas fenofibrate significantly reduced inward chloride currents.

chloride conductance (g_{Cl}) in rat skeletal muscle has been already established (Conte Camerino *et al.*, 1984; Pierno *et al.*, 1995; Pierno *et al.*, 2006). We previously showed that only simvastatin, but not pravastatin, was able to affect g_{Cl} , an effect we attributed to the greater lipophilic properties of the former. Nevertheless, statins of more recent generation, such as fluvastatin and atorvastatin, were more potent than simvastatin in reducing g_{Cl} , independently of lipophilic status. Here, we attempted to clarify the molecular mechanisms by which statins and fibrate inhibit g_{Cl} .

We first investigated the effects of the hypolipidemic drugs on the metabolic pathway responsible for the regulation of

CIC-1 channels, the main determinant of sarcolemmal g_{Cl} , which involves the calcium-dependent PKC. The high g_{Cl} typical of fast-twitch muscles is sustained by a low basal activity of PKC that maintains CIC-1 in a dephosphorylated, active state (De Luca *et al.*, 1994; Pierno *et al.*, 2007). *In vitro* PKC activation by phorbol esters concentration-dependently reduced the g_{Cl} , while PKC inhibition by chelerythrine produced little or no effect. We reported here a new finding: the reduction of skeletal muscle g_{Cl} induced by statins is mediated by the activation of the PKC pathway. When chelerythrine was applied *in vitro* to skeletal muscle from fluvastatin and atorvastatin-treated rats, the g_{Cl} was restored towards

that of control, suggesting that these drugs, by activating PKC, were likely to have increased phosphorylation and closure of the CIC-1 chloride channel. We argue that this is a rapid effect specific to muscle because acute *in vitro* application of statins to muscle fibres reduced the gCl and this reduction was totally prevented by prior incubation with chelerythrine, whereas no effect of statins was found on heterologously expressed CIC-1 channels. This hypothesis is further supported by the observation that statin treatment had no effect, either *in vivo* or when applied *in vitro*, on the gCl in the slow-twitch soleus muscle, a muscle type characterized by a higher basal PKC activity, than that found in the fast-twitch muscle (Pierno *et al.*, 2007) and that in contrast, fenofibrate markedly reduced gCl in this muscle. One possible mechanism for PKC activation by statins may be elevated intracellular Ca^{2+} concentrations. Indeed, we have demonstrated that *in vivo* administration as well as acute *in vitro* application of either fluvastatin or atorvastatin increased resting intracellular Ca^{2+} concentration in EDL muscle fibres, through a mechanism that involves the sequential release from mitochondria and then sarcoplasmic reticulum (Liantonio *et al.*, 2007). Thus, the modification of calcium handling mechanisms induced by these drugs may account for a rapid, calcium-dependent, PKC activation. However, other mechanisms may contribute to *in vivo* effects, that is, increased expression of PKC through PPAR activation (Martin *et al.*, 2001; Inoue *et al.*, 2002).

Although PKC activation contributes to the action of both statins, chelerythrine did not fully reverse the reduction of gCl induced by fluvastatin, suggesting that additional events might contribute to the reduction of gCl. Also chelerythrine had no effect on the gCl reduction induced by fenofibrate. This is in accord with the lack of change in cytosolic calcium and mechanical threshold for contraction after fenofibrate treatment (Pierno *et al.*, 2006; Liantonio *et al.*, 2007). Thus, fenofibrate acts through a different mechanism. Patch clamp studies showed that fenofibric acid directly reduced the CIC-1 chloride current. The correlation between chemical structure and blocking properties between fenofibric acid and clofibrac acid (Liantonio *et al.*, 2003) lead us to envisage a direct effect of fenofibrate in blocking this channel. In contrast, neither fluvastatin nor simvastatin modifies CIC-1 currents after incubation not exceeding 5 min, again corroborating the key role of PKC in statin-induced gCl modification.

Because the reduction of gCl in fluvastatin-treated animals was not completely antagonized by chelerythrine and was not due to a direct block of the channel, a modification of CIC-1 expression was hypothesized. Statins and fibrates can modify the expression of various genes through activation of PPAR (Martin *et al.*, 2001) or GTP-ase protein inhibition (Habib *et al.*, 2007). Data obtained by quantitative RT-PCR demonstrated a significant reduction of CIC-1 mRNA in fluvastatin as well as in fenofibrate-treated rats with respect to controls. Although the reduction of CIC-1 mRNA is proportionally larger than the decrease of gCl, similar apparent discrepancies have been described in other conditions. For instance, in heterozygous ADR^{+/-} mice, in which one CIC allele is disrupted, the mRNA for CIC-1 channels was reduced to 50% of that measured in wild-type mice (Chen *et al.*, 1997) but gCl was normal, suggesting that post-translational modulation

can compensate to some extent for a reduction in CIC-1 mRNA level, at least in pathogenic conditions. A similar mechanism may account for the disproportional changes in CIC-1 mRNA expression and gCl in the drug-treated rats. The measurement of gCl gives a direct assessment of protein function that plays a crucial role in controlling muscle excitability and, secondarily, muscle contractility. Our results demonstrate that gCl is reduced in drug-treated rats and that this effect is, at least partly, related to reduced expression of CIC-1 mRNA. More direct effects on protein function may also contribute to the reduction of gCl, in a manner specific to each drug.

In conclusion, the reduction of gCl we measured in statin and fenofibrate chronically treated rats was not greater than 30%, even at doses much higher than those clinically used. It is unlikely that myotonia-like phenomena will occur in this situation, because myotonia is normally characterized by stronger reduction of gCl (more than 50% of the normal value) (Pusch, 2002). Accordingly, the EMG activity recorded on fluvastatin, atorvastatin and fenofibrate-treated animals showed signs of altered excitability characterized by brief bursts of AP following normal movement-induced electrical activity, which were different from those seen during 9-AC acutely induced myotonia but closer to those recorded in humans during muscle cramping or fasciculation (Costa *et al.*, 2005; Serrao *et al.*, 2007). Taking into account the already observed lack of histological damage (Pierno *et al.*, 2006), it is feasible that the reduction of gCl observed can be responsible for the mild adverse effects of hypolipidemic drugs, such as myalgia and cramps. The EMG alteration was observed only after 7–8 weeks of treatment but we can not exclude occurrence at earlier stages, as these phenomena may be episodic in nature and their frequency most probably increased with duration of treatment. Our data provide a molecular link between the reduction of gCl induced by statins and fenofibrate and the pathogenesis of skeletal muscle side effects. Figure 6 and Figure S1 summarizes all the results obtained in this study and hypothesizes the sequence of events involved in the mechanism determining muscle CIC-1 channel impairment. It should be considered that physiopathological conditions in which there is an increase of PKC activity, such as exercise (Rose *et al.*, 2004; Ort *et al.*, 2007) or aging (De Luca *et al.*, 1994; Pierno *et al.*, 2007), statin therapy should be continuously monitored. Higher rates of adverse side effects occur in aged patients, in which gCl can be affected due to development of sarcopenia (Pierno *et al.*, 1999b). The use of compounds able to reduce muscle PKC activity should be considered to prevent skeletal muscle side effects that may arise during hypolipidemic drug therapy.

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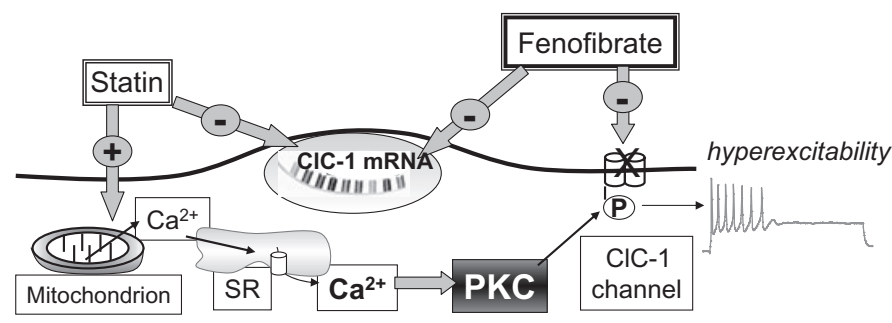


Figure 6 Diagram of the mechanisms by which statins and fenofibrate affect resting chloride channel conductance (gCl), carried by CIC-1 channels in skeletal muscle. It is known that protein kinase C (PKC) activation is responsible for the phosphorylation and closure of CIC-1 channel producing a reduction of gCl together with an increase of sarcolemma excitability (De Luca *et al.*, 1994; Pierno *et al.*, 2007). As described in the text, statins affect CIC-1 channel regulation by increasing PKC activity via calcium-release from mitochondria and sarcoplasmic reticulum (SR) (Liantonio *et al.*, 2007). Fluvastatin also reduced CIC-1 mRNA expression. Fenofibrate acted by directly blocking CIC-1 channels and by reducing its expression.

Conflict of interest

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The mechanisms by which fluvastatin and fenofibrate affect resting chloride conductance, sustained by ClC-1 channels in rat skeletal muscle (fast-twitch-phenotype) fibres.

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