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RESEARCH PAPER

Niflumic acid inhibits chloride conductance of rat skeletal muscle by directly inhibiting the CLC-1 channel and by increasing intracellular calcium

A Liantonio[1], V Giannuzzi[1], A Picollo[2], E Babini[2], M Pusch[2] and D Conte Camerino[1]

[1]*Unità di Farmacologia, Dipartimento Farmacobiologico, Facoltà di Farmacia, Università di Bari, Bari, Italy and* [2]*Centro Nazionale delle Ricerche (CNR), Istituto di Biofisica, CNR, Genova, Italy*

Background and purpose: Given the crucial role of the skeletal muscle chloride conductance (gCl), supported by the voltage-gated chloride channel CLC-1, in controlling muscle excitability, the availability of ligands modulating CLC-1 are of potential medical as well as toxicological importance. Here, we focused our attention on niflumic acid (NFA), a molecule belonging to the fenamates group of non-steroidal anti-inflammatory drugs (NSAID).
Experimental approach: Rat muscle Cl^- conductance (gCl) and heterologously expressed CLC-1 currents were evaluated by means of current-clamp (using two-microelectrodes) and patch-clamp techniques, respectively. Fura-2 fluorescence was used to determine intracellular calcium concentration, $[Ca^{2+}]_i$, in native muscle fibres.
Key results: NFA inhibited native gCl with an IC_{50} of 42 μM and blocked CLC-1 by interacting with an intracellular binding site. Additionally, NFA increased basal $[Ca^{2+}]_i$ in myofibres by promoting a mitochondrial calcium efflux that was not dependent on cyclooxygenase or CLC-1. A structure-activity study revealed that the molecular conditions that mediate the two effects are different. Pretreatment with the Ca-dependent protein kinase C (PKC) inhibitor chelerythrine partially inhibited the NFA effect. Therefore, in addition to direct channel block, NFA also inhibits gCl indirectly by promoting PKC activation.
Conclusions and Implications: These cellular effects of NFA on skeletal muscle demonstrate that it is possible to modify CLC-1 and consequently gCl directly by interacting with channel proteins and indirectly by interfering with the calcium-dependent regulation of the channel. The effect of NFA on mitochondrial calcium stores suggests that NSAIDs, widely used drugs, could have potentially dangerous side-effects.
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Keywords: niflumic acid; skeletal muscle; CLC-1 chloride channel; calcium homeostasis; structure-activity study; macroscopic chloride conductance

Abbreviations: 9-AC, 9-anthracene carboxylic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; CPP, 2-p-(chlorophenoxy) propionic acid; CsA, cyclosporine A; DPC, 2-(phenylamino)benzoic acid; EDL, Exstensor digitorum longus; FFA, flufenamic acid; gCl, chloride conductance; MCFA, meclofenamic acid; MFA, mefenamic acid; NFA, niflumic acid; NSAID, non-steroidal anti-inflammatory drugs; PKC, Ca-dependent protein kinase C; PTP, permeability transition pore; SR, sarcoplasmic reticulum; TFA, tolfenamic acid

Introduction

In skeletal muscle, chloride conductance (gCl) is carried mostly by the CLC-1 channel and accounts for 80% of the membrane conductance (gm) at rest. Changes in membrane potential in response to excitatory currents are strictly dependent on this electrical parameter. The pivotal role of gCl in regulating muscle excitability is illustrated by the hyperexcitability observed in myotonia congenita and myotonic dystrophy, muscular diseases that decrease the function of CLC-1 either owing to mutations in the *CLCN1* gene (Koch *et al*., 1992) or to aberrant splicing of CLC-1 RNA (Mankodi *et al*., 2002), respectively. Furthermore, the protective effect of muscle acidification in depolarized muscle fibres is largely attributable to a reduction of gCl (Pedersen *et al*., 2004, 2005).

Given the crucial role of gCl in muscle physiology, the availability of specific ligands that modulate CLC-1 could be of potential medical as well as toxicological importance. Among the few available, relatively high affinity ligands for CLC-1, 2-p-(chlorophenoxy)propionic acid (CPP) derivatives and 9-anthracene carboxylic acid (9-AC) are the most well

Correspondence: Professor D Conte Camerino, Section of Pharmacology, Dept. of Pharmacobiology, Faculty of Pharmacy, University of Bari, Via Orabona 4, I-70125 Bari, Italy. E-mail: conte@farmbiol.uniba.it
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characterized. S(−)−CPP blocks native gCl with micromolar affinity (Conte Camerino *et al*., 1988; De Luca *et al*., 1992) by interfering with CLC-1 channel gating (Aromataris *et al*., 1999), acting from the intracellular side (Pusch *et al*., 2000; Liantonio *et al*., 2003). The inhibitory binding site of CPP derivatives and 9-AC has been mapped out in considerable detail on the CLC-1 protein (Estévez *et al*., 2003). Furthermore, CLC-1 has also been shown to be a target of therapeutic molecules with different clinical uses such as statins (Pierno *et al*., 1995), taurine (Pierno *et al*., 1998; Conte Camerino *et al*., 2004) and non-steroidal anti-inflammatory drugs (NSAID) (Astill *et al*., 1996). In searching for new modulators of gCl, we focused our attention on niflumic acid (NFA), a molecule belonging to the fenamates group of NSAID. The mechanism of its anti-inflammatory action is based on inhibition of cyclooxygenases; this results in antipyretic, analgesic and anti-inflammatory effects (Vane and Botting, 1998). In addition to these effects on prostaglandin synthesis, fenamates affect a variety of ion channels. They act as inhibitors of calcium-activated chloride channels (White and Aylwin, 1990; Large and Wang, 1996; Qu and Hartzell, 2001; Hartzell *et al*., 2005), cystic fibrosis transmembrane conductance regulator chloride channels (CFTR) (McCarty *et al*., 1993), non-selective cation channels (Gögelein *et al*., 1990) and voltage-gated potassium channels (Lee and Wang, 1999), and enhance calcium-activated and KCNQ2/Q3 potassium channels (Ottolia and Toro, 1994; Peretz *et al*., 2005). NFA, in particular, directly modulates chloride channels belonging to the CLC family such as CLC-1 (Astill *et al*., 1996) and CLC-K (Wangemann *et al*., 1986; Liantonio *et al*., 2006). In addition, more importantly, NFA is also able to induce changes in the intracellular calcium concentration, $[Ca^{2+}]_i$, in neurons, smooth muscle cells, gastric mucosal cells and a mouse mandibular cell line, by affecting intracellular calcium stores (Poronnik *et al*., 1992; Lee *et al*., 1996; Cruickshank *et al*., 2003; Tomisato *et al*., 2004). We considered this effect potentially useful for exploring the calcium-dependent mechanisms of gCl regulation. Indeed, CLC-1 is biochemically regulated by a G-protein-coupled phosphorylation reaction cascade. For example, the phosphorylation of CLC-1 by Ca-dependent protein kinase C (PKC) decreases channel activity in native muscle fibres (De Luca *et al*., 1998) and in heterologous expression systems (Rosenbohm *et al*., 1999). It was recently hypothesized that the phosphorylation of CLC-1 is also involved in channel trafficking of CLC-1 to the sarcolemmal membrane (Papponen *et al*., 2005). Thus, intracellular calcium homeostasis through PKC activity could play a key role in modulating native gCl. In support of this hypothesis, a relationship between gCl and $[Ca^{2+}]_i$ has been shown to exist. For example, pathophysiological conditions of skeletal muscle with a reduced value of gCl, such as ageing (De Luca *et al*., 1994) or muscular dystrophy (De Luca *et al*., 2003), are characterized by elevated $[Ca^{2+}]_i$ (Fraysse *et al*., 2004, 2006), whereas an increase of gCl, an event that occurs in a disuse condition such as microgravity (Pierno *et al*., 2002), is associated with a decrease in $[Ca^{2+}]_i$ (Fraysse *et al*., 2003). Thus, a molecule capable of interfering with the biochemical pathways that regulate CLC-1 channel activity could reveal the physiological significance of these regulatory systems in modulating native gCl and could be of potential therapeutic interest.

In accordance with previous findings (Astill *et al*., 1996), we showed that NFA blocks gCl of rat muscle fibres as well as CLC-1 expressed in oocytes. Additionally, NFA produces a mitochondria-dependent increase in $[Ca^{2+}]_i$ in muscle fibres. By testing the effects of several NFA derivatives and using pharmacological tools, we were able to differentiate the molecular conditions required to mediate the two different effects (on CLC-1 and on $[Ca^{2+}]_i$) as well as to gain insight into the mechanism of action of NFA on skeletal muscle. The ability of NFA to block CLC-1 directly and indirectly through inhibition of Ca-dependent phosphorylation by PKC, suggests that NFA could be considered to be a member of a new class of inhibitors of native muscle gCl. However, at the same time, the results have raised awareness of possible side effects of these widely used drugs.

Methods

Animal care and surgery

Animal care was consistent with the Italian guidelines for the use of laboratory animals and the European Community Directive published in 1986 (86/609/EEC). Adult male Wistar rats (Charles River Laboratories, Calco, Italy) were used for the experiments. Animals had free access to food and water, and were maintained at a constant room temperature (22–24°C) and exposed to a light cycle of 12 h per day. Exstensor digitorum longus (EDL) muscle was removed from the animals under deep anaesthesia with urethane (1.2 g kg^{-1} body weight) and the preparations were promptly used for electrophysiological experiments.

Measurements of macroscopic Cl^- conductance on native muscle fibres

Soon after the biopsy, the EDL muscle was stretched to about 1.5 times its resting length on a 3-mm plastic rod in a temperature-controlled muscle chamber at 30°C and perfused with a physiological solution (see below) in the absence and presence of the test compounds (De Luca *et al*., 1992). The macroscopic Cl^- conductance (gCl) of EDL muscle fibres was calculated from the cable parameters, and in particular from the membrane resistance (Rm), measured by standard cable analysis with the two intracellular microelectrode technique. In brief, a voltage-sensitive microelectrode (3 M KCl) was used to measure the membrane potential and the voltage deflection (electrotonic potential) monitored, at two distances (0.5 mm and about 1 mm), in response to a hyperpolarizing square wave current pulse passed through a second electrode (2 M Kcitrate). Current pulse generation, acquisition of the voltage records and calculation of fibre constants (fibre diameter, membrane capacitance and Rm) were carried out under computer control, as described in detail previously (De Luca *et al*., 1998). In each fibre, the total gm was 1/Rm in the normal physiological solution, whereas potassium conductance (gK) was 1/Rm in the Cl^--free physiological solution. The mean

gCl was calculated as the mean gm minus the mean gK. The data are expressed as mean ± s.e.m. The s.e.m. for gCl was calculated as described previously (Green and Margerison, 1978). As in the case of CPP derivatives that modulate native gCl by acting from the intracellular side (De Luca *et al*., 1992; Liantonio *et al*., 2003), recordings were started after 15–20 min of incubation with NFA and other compounds, to be sure that a steady state for the drug effect was reached. The concentration–response relationship for NFA was fitted with the following equation: % block of $gCl = 100/(1 + ([drug]/IC_{50})^n)$ where [drug] is the concentration of NFA tested, IC_{50} is the concentration of drug needed to block gCl by 50% and n is the slope of the curve.

Fluorescence measurements of resting intracellular Ca^{2+} ions
Small bundles of 5–10 EDL muscle fibres arranged in a single layer were dissected lengthwise, tendon to tendon and incubated for 2 h at 25°C in normal physiological (NP) solution containing 5 μM of fura-2 acetoxymethyl ester (fura-2 AM, Molecular Probes-Invitrogen, Italy), the membrane-permeant form of the probe, mixed to 0.05% (v/v) Pluronic F-127 (Molecular Probes). After the loading, muscle fibres were washed with normal physiological solution and mounted in a modified RC-27NE experimental chamber (Warner Instrument Inc., Hamden, CT, USA) on the stage of an inverted Eclipse TE300 microscope (Nikon, Japan) with a ×40 Plan-Fluor objective (Nikon, Japan). The mean sarcomere length was set to 2.5–2.7 μm. Fluorescence measurements were made using a QuantiCell 900 integrated imaging system (Visitech International Ltd, Sunderland, UK) as described previously (Fraysse *et al*., 2003, 2004).

During the experiments, pairs of background subtracted images of fura-2 fluorescence (510 nm) after excitation at 340 and 380 nm were acquired and ratiometric images (340/380 nm) were calculated for each muscle fibre preparation using QC2000 software. Subsequently, fluorescence ratio values were converted to the resting cytosolic calcium [Ca^{2+}] (nM), after a calibration procedure, using the equation: $[Ca^{2+}]_i = (R - R_{min})/(R_{max} - R) * K_D * \beta$ where R is the ratio of fluorescence excited at 340 nm to that excited at 380 nm; K_D is affinity constant of fura-2 for calcium, which was taken as 145 nM (Molecular Probes); β is a parameter according to Grynkiewicz *et al*. (1985) and was determined experimentally *in situ* in ionomycin-permeabilized muscle fibres, as described previously (Fraysse *et al*., 2003). Briefly, R_{min} and R_{max} were determined in muscle fibres incubated in Ca^{2+}-free NP solution containing 10 mM ethylene glycol bis(β-aminoethyl ether)- *N,N,N′,N′*-tetraacetic acid (EGTA) and in NP solution, respectively. To check fibre integrity, preparations were stimulated by the application of depolarizing 100 mM K^+ solution. Fibres that did not respond by producing calcium transients were discarded.

The concentration–response relationship for NFA was fitted with the following equation: $\Delta[Ca^{2+}]_i = \Delta[Ca^{2+}]_i\ max/(1 + ([drug]/EC_{50})^n)$ where $\Delta[Ca^{2+}]_i$ max is the maximal NFA-induced increase in $[Ca^{2+}]_i$, [drug] is the NFA concentration, EC_{50} is the concentration of drug needed to produce 50% of the maximal effect, and n is the slope of the curve.

Expression of CLC-1 in Xenopus laevis *oocytes*
Expression of the human CLC-1 channel in *Xenopus* oocytes was determined as described previously (Pusch *et al*., 2000). Patch-clamp measurements were obtained at 18 ± 1°C using the inside-out configuration with an EPC-7 amplifier (HEKA, Lambrecht, Germany) and the following pulse protocol: from a holding potential of 0 mV, after a prepulse to 60 mV for 100 ms, the voltage was stepped to various test values (from −140 to 80 mV in 20 mV increments) for 200 ms and followed by a constant voltage pulse to −100 mV to record the tail current. For solution exchange, inside-out patches were introduced into ~0.5 mm wide perfusion tubes. Changing between tubes required about 10 s. Apparent dissociation constants, K_D, were determined by calculating the ratio of the steady-state current in the absence and presence of the drug and fitting the ratios at a fixed voltage by the use of the equation: $I(c)/I(0) = 1/(1 + c/K_D)$ where c is the concentration. Errors in figures are indicated as s.e.m.

Solutions and chemical compounds
For measurements in native rat skeletal muscle fibres, the normal physiological solution contained (in mM): NaCl 148, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1, NaH_2PO_4 0.44, $NaHCO_3$ 12, glucose 5.5. The pH of all solutions was adjusted to 7.3–7.4 by bubbling them with 95% O_2/5% CO_2. The calcium-free solution had the same composition of the normal physiological solution excepted that $CaCl_2$ was omitted and 10 mM of EGTA was added. The Cl^--free solution was made by equimolar substitution of methylsulphate salt for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$.

For patch-clamp measurements on heterologous, expressed CLC-1 the following solutions were used: intracellular solution (in mM), *N*-methyl-D-glucamine-chloride 100, $MgCl_2$ 2, HEPES 10, EGTA 2 at pH 7.3; extracellular solution, *N*-methyl-D-glucamine-chloride 100, $MgCl_2$ 5 and HEPES 10 at pH 7.3.

All chemicals cited above and ionomycin, caffeine, ruthenium red, thapsigargin, oligomycin, cyclosporine A (CsA), indomethacin and meloxicam, NFA, flufenamic acid (FFA), mefenamic acid (MFA), meclofenamic acid (MCFA), tolfenamic acid (TFA), 2-(phenylamino)benzoic acid (DPC), were purchased from Sigma (St Louis, MO, USA). All remaining NFA derivatives were synthesized in our laboratory according to procedures described previously (Liantonio *et al*., 2006).

Statistical analysis
Significance levels were calculated using Student's unpaired *t*-test.

Results

Effect of NFA on macroscopic gCl of rat skeletal muscle fibres and on heterologously expressed CLC-1

NFA inhibits native gCl. NFA inhibited native gCl in a concentration-dependent manner with an IC_{50} of ~42 μM (Figure 1). At each concentration tested the maximal

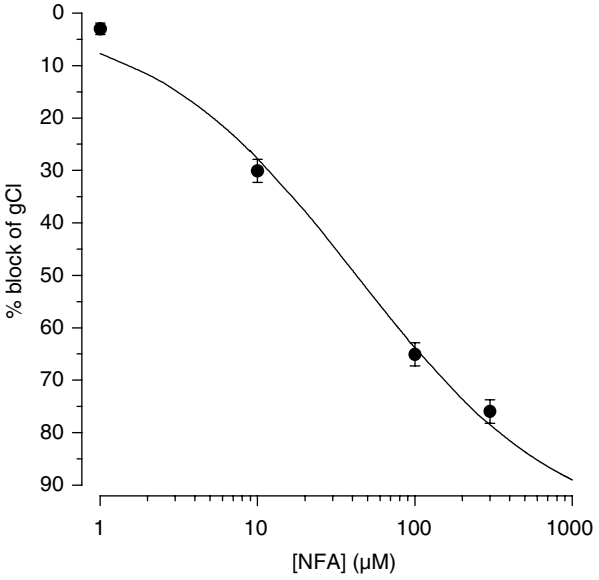

Figure 1 Concentration–response curves for the inhibition of gCl of native rat skeletal muscle fibres by NFA. The mean value of gCl obtained after application of each concentration (15–32 fibres from two to three preparations) has been normalized to the related mean value of gCl recorded in the absence of drug. Thus each point represents the normalized percent inhibition of gCl (vertical lines show s.e.m.) in the presence of drug. The solid line represents the fit obtained as described in Methods with an IC_{50} value of $\sim 42\,\mu$M and an apparent Hill coefficient of 0.66.

blocking effect was reached after 10–15 min of drug incubation and block was reversible upon wash out. Application of NFA at 100 μM produced a 67% block of gCl, reducing it from the control value of 2700 ± 164 to $891 \pm 113\,\mu S\,cm^{-2}$ ($P < 0.05$).

NFA inhibits heterologously expressed CLC-1. To determine whether NFA blocks native gCl by acting intra- or extracellularly, we characterized the effect of NFA on CLC-1 expressed in *Xenopus* oocytes. In two-electrode voltage-clamp recordings, we observed only a slight current reduction after extracellular drug application in the concentration range 10–200 μM. Higher concentrations, that is $\geqslant 500\,\mu$M, were required to produce appreciable inhibition after 10–15 min of drug incubation (data not shown). In contrast, when applied from the intracellular side in inside-out patch-clamp recordings, NFA showed an elevated affinity toward CLC-1 causing a rapid and marked decrease of steady-state currents at 100 μM (Figure 2a). Within the time of solution exchange (around 10 s; see Methods), the effect was practically instantaneous. Similar to other organic acids (Pusch *et al*., 2000, 2002; Liantonio *et al*., 2003), the block was dose-dependent (see Figure 2b, which shows the dose–response curve at -100 mV) and more pronounced at negative voltages, with apparent K_D values of 22 ± 2 and $143 \pm 18\,\mu$M at -100 and 60 mV, respectively. These data unequivocally indicate that NFA is capable of interfering with CLC-1 gating by interacting with an intracellular

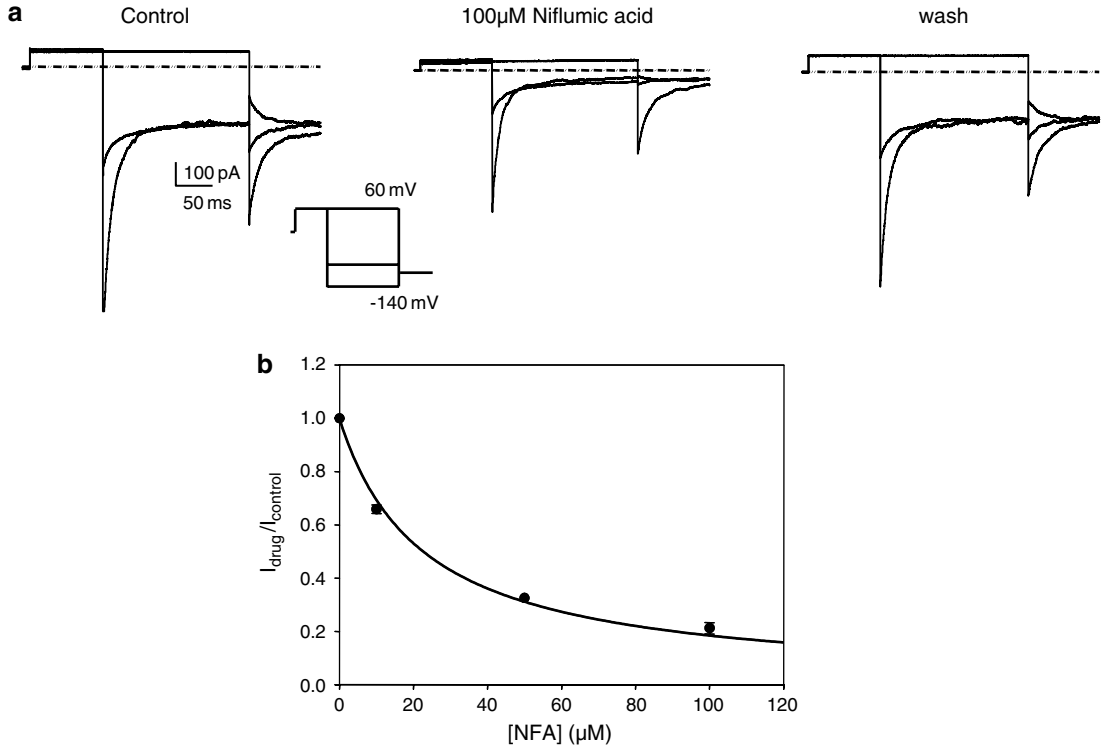

Figure 2 Effect of internally applied NFA on heterologously expressed CLC-1. (**a**) The inside-out patch-clamp traces were elicited using the pulse-protocol described in Methods. For clarity, as depicted in the inset, only the current traces corresponding to -140, -80 and 60 mV are shown. The scale bars apply to the entire figure. (**b**) Dose–response relationship of the block at -100 mV. The solid line is fit to the equation described in Methods with a K_D value of $22 \pm 2\,\mu$M.

binding site, similar to CPP derivatives (Pusch *et al*., 2000; Estévez *et al*., 2003; Liantonio *et al*., 2003).

Endogenous background currents were generally much smaller ($<1\,\mu$A) than those induced after expression of CLC-1 (around 5–10 μA for the two-electrode voltage-clamp experiments), indicating that it is unlikely that the effect of NFA observed after expression of CLC-1 is due to its action on endogenous currents. However, as NFA is known to inhibit the endogenous Ca^{2+} activated chloride current in *Xenopus* oocytes (White and Aylwin, 1990; Large and Wang, 1996; Qu and Hartzell, 2001; Hartzell *et al*., 2005), to exclude any interference of endogenous currents with our results, we applied NFA (200 μM) to non-injected oocytes. NFA slightly reduced the small ($<1\,\mu$A) currents seen in non-injected oocytes (data not shown), unequivocally showing that all the described effects are due to the NFA-mediated CLC-1 blockade.

Effect of NFA on intracellular calcium homeostasis of native rat skeletal muscle fibres

NFA increases $[Ca^{2+}]_i$ in rat skeletal muscle fibres. In agreement with previous studies (Fraysse *et al*.,. 2003, 2006), resting $[Ca^{2+}]_i$ of fast-twitch EDL myofibres was in the 20–30 nM concentration range. Application of 100 μM NFA led to an increase of $[Ca^{2+}]_i$ from 23.7 ± 3.9 to 121 ± 14 nM characterized by a slow rising phase reaching a plateau after 10 min of incubation (Figure 3a). When the drug was removed, $[Ca^{2+}]_i$ slowly returned to near the basal level (Figure 3a). The increase of $[Ca^{2+}]_i$ produced by NFA was concentration dependent, with a half-maximal effect at 100 μM (Figure 3b). Therefore in further investigations, we routinely applied NFA and related compounds at 100 μM.

Investigation of the source of calcium release. Removal of external Ca^{2+} in the bath solution did not abolish the NFA-induced $[Ca^{2+}]_i$ rise; it produced an increase of 97 ± 9.1 nM, a value not significantly different from that obtained in the presence of extracellular calcium ($P>0.35$) (Figure 3c).

These results strongly suggest that NFA induces release of Ca^{2+} from an internal store. We therefore attempted to identify the relevant intracellular source. Initially, we investigated the possible involvement of the sarcoplasmic reticulum (SR) by using thapsigargin, which inhibits the Ca^{2+}-ATPase pump responsible for sequestering Ca^{2+} in the SR and hence depletes the store by irreversibly preventing its refilling. After a treatment with 10 μM thapsigargin, application of 40 mM caffeine still produced an increase in $[Ca^{2+}]_i$ (Figure 4a), showing that the SR stores were initially filled with Ca^{2+}. However, as expected, a second application of caffeine in the continued presence of thapsigargin did not produce a successive $[Ca^{2+}]_i$ transient, indicating that the SR was indeed depleted of Ca^{2+}. When 100 μM NFA was successively applied, an increase of $[Ca^{2+}]_i$ was observed with a similar intensity to that obtained with fibres not treated with thapsigargin (Figure 4a and d). By using ruthenium red, we further supported this result. Indeed, pretreatment with ruthenium red, an inhibitor of the SR-Ca^{2+} release channel (RyR), at a concentration (5 μM) that completely prevented 40 mM caffeine effects (Figure 4b), did not affect the NFA-induced $[Ca^{2+}]_i$ increase (Figure 4b and d). Taken together, these data indicate that NFA mobilizes Ca^{2+} from an intracellular source that is not the SR.

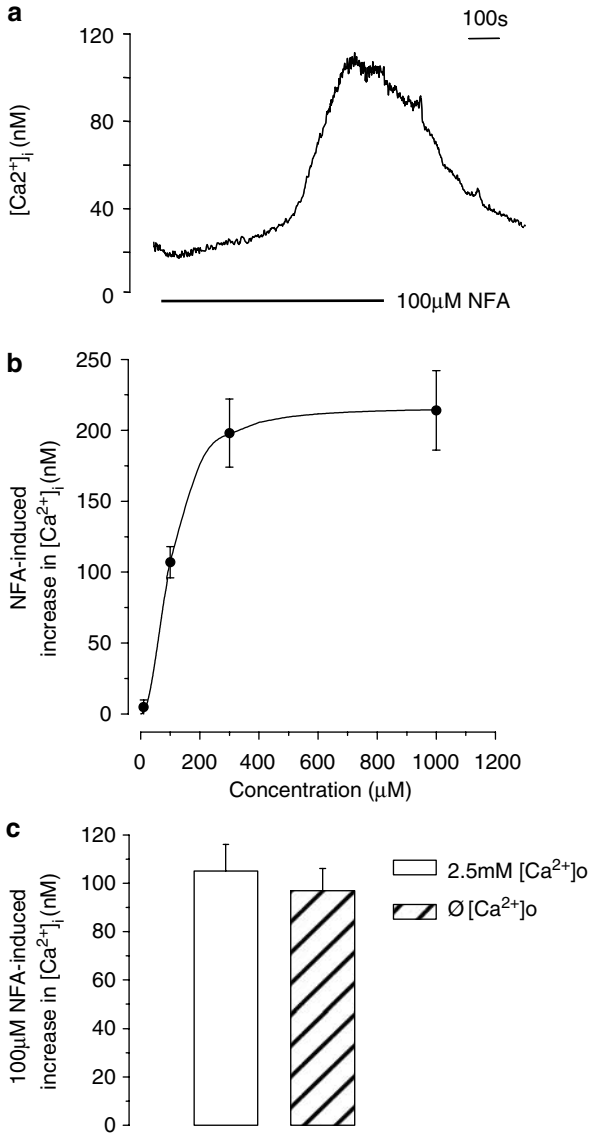

Figure 3 Effect of NFA on $[Ca^{2+}]_i$ of native rat skeletal muscle fibres. (**a**) Typical calcium increase induced by application of 100 μM NFA on Fura-2-loaded myofibres of rat EDL muscle. (**b**) Dose–response relationship for the effect of NFA on $[Ca^{2+}]_i$. Data are shown as mean and s.e.m. and each point is representative of 15–24 fibres; the solid line represents the fit obtained as described in Methods with an IC_{50} value of $\sim$100 μM; (**c**) the increase in $[Ca^{2+}]_i$ induced by 100 μM NFA measured in the presence of external calcium (normal Ringer solution) or in the absence of external calcium ($\varnothing$ Ca^{2+}). The bars indicate the mean$\pm$s.e.m. from 15 to 20 fibres.

In the mouse mandibular cell line ST_{885} (Poronnik *et al*., 1992) as well as in isolated rat renal cortex and liver mitochondria (Uyemura *et al*., 1997; Pigoso *et al*., 1998; Jordani *et al*., 2000), it has been shown that FFA, MFA and salicylates induce a mitochondrial Ca^{2+} efflux, probably through the activation of the permeability transition pore (PTP). To assess a possible mitochondrial origin of the

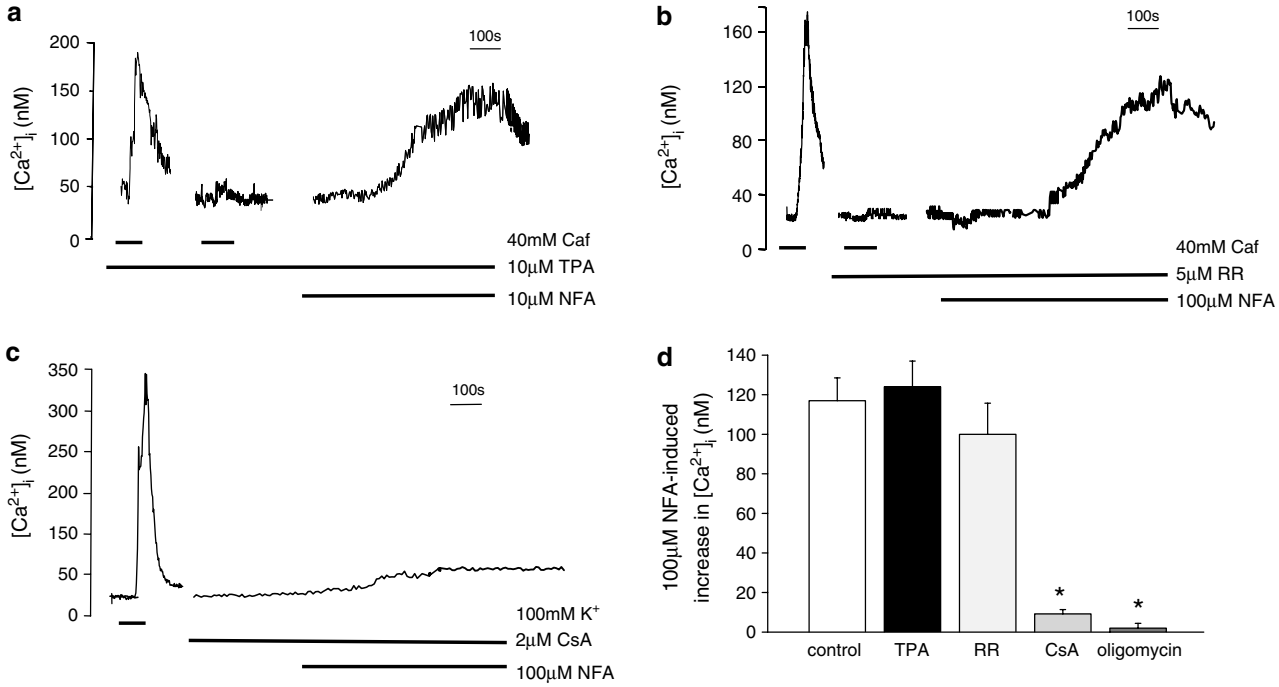

Figure 4 Investigation of the calcium source responsible for the NFA-induced increase in $[Ca^{2+}]_i$. Representative traces showing the effect of 100 μM NFA in the presence of (**a**) the Ca-ATPase inhibitor thapsigargin (TPA); (**b**) the ryanodine receptor antagonist ruthenium red (RR); (**c**) the mitochondrial PTP inhibitor CsA. (**a**) Muscle fibres were incubated with TPA for 15 min before repeated application of caffeine (Caf). Thereafter, NFA was applied. (**b**) After a Caf stimulation in control solution, muscle fibres were incubated with RR for 5 min before Caf re-application. Thereafter, NFA was applied. (**c**) Muscle fibres were incubated with CsA for 10 min before NFA application. (**d**) Summary of 100 μM NFA-induced responses in control conditions ($n=27$) and in the presence of TPA ($n=21$), RR ($n=25$), CsA ($n=21$) and oligomycin ($n=18$). Each bar represents the mean$\pm$s.e.m. *Significantly different from control value with, $P<0.05$.

NFA-induced $[Ca^{2+}]_i$ increase in native muscle fibres, we evaluated the effect of NFA in the presence of CsA, an inhibitor of the mitochondrial PTP (Crompton *et al*., 1999). As shown in Figure 4c, pretreatment with 2 μM CsA indeed markedly inhibited the NFA-induced $[Ca^{2+}]_i$ increase. Furthermore, pretreatment with oligomycin, a mitochondrial inhibitor that inhibits the ATP synthase, also completely prevented the effect of NFA on $[Ca^{2+}]_i$ (Figure 4d), further corroborating the idea that the ability of NFA to increase $[Ca^{2+}]_i$ is dependent on functionally intact mitochondria.

NFA-induced $[Ca^{2+}]_i$ increase is not dependent on cyclooxygenase inhibition. To test the possibility that the NFA-induced increase of $[Ca^{2+}]_i$ is mediated through an inhibition of cyclooxygenases, we used indomethacin and meloxicam, two structurally unrelated inhibitors of cyclooxygenases (Vane and Botting, 1998). At 100 μM, a concentration that is well above that needed to inhibit cyclooxygenases (Turck *et al*.,. 1996; Kalgutkar *et al*., 2000), neither indomethacin nor meloxicam induced a significant increase in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ was 24.9$\pm$1.8 and 26.2$\pm$1.6 nM in the absence and in the presence, respectively, of indomethacin, and 26.9$\pm$2.3 and 27.5$\pm$1.7 nM in the absence and presence, respectively, of meloxicam; $P>0.35$ in both cases).

Is NFA-induced $[Ca^{2+}]_i$ increase dependent on Cl^- channel block? It is possible that the increase of $[Ca^{2+}]_i$ induced by NFA is indirectly caused by an inhibitory effect on the gCl. For example, a block of gCl might depolarize the sarcolemma and this might lead to secondary effects on $[Ca^{2+}]_i$. With this in mind, we evaluated the effect of S(−)−CPP, a well-characterized blocker of CLC-1 and native gCl (Conte Camerino *et al*. 1988; Pusch *et al*. 2000; Liantonio *et al*. 2003). As shown in Figure 5, 100 μm S(−)−CPP, a concentration that blocks more than 80% of gCl (Conte Camerino *et al*., 1988), failed to increase $[Ca^{2+}]_i$. Hence, the NFA-mediated $[Ca^{2+}]_i$ increase is not dependent on CLC-1 channel block.

Molecular prerequisites of NFA for inhibiting CLC-1 channel activity and for increasing $[Ca^{2+}]_i$ in skeletal muscle: a structure-activity study

In order to define the structural determinants of NFA for mediating the two different activities on skeletal muscle fibres, that is, direct block of CLC-1-mediated currents and increase of $[Ca^{2+}]_i$, we performed a structure-activity study evaluating the effect of a series of NFA derivatives on both parameters. In particular, we evaluated the role of: the CF_3 group by substituting or shifting it (compound MT-6, MT-7 and MT-4 in Figure 6a), the carboxylic group by substituting it with an alcoholic function (EB-168 in Figure 6b), the anilinic nitrogen by substituting it with an oxygen atom (Li-derivatives in Figure 7a) and of the pyridinic group by substituting it with a phenyl one (FFA derivatives in Figure 7b).

As the inhibitory IC_{50} of NFA on native gCl is 42 μM, and the $[Ca^{2+}]_i$ increase is half maximal at about 100 μM NFA, we

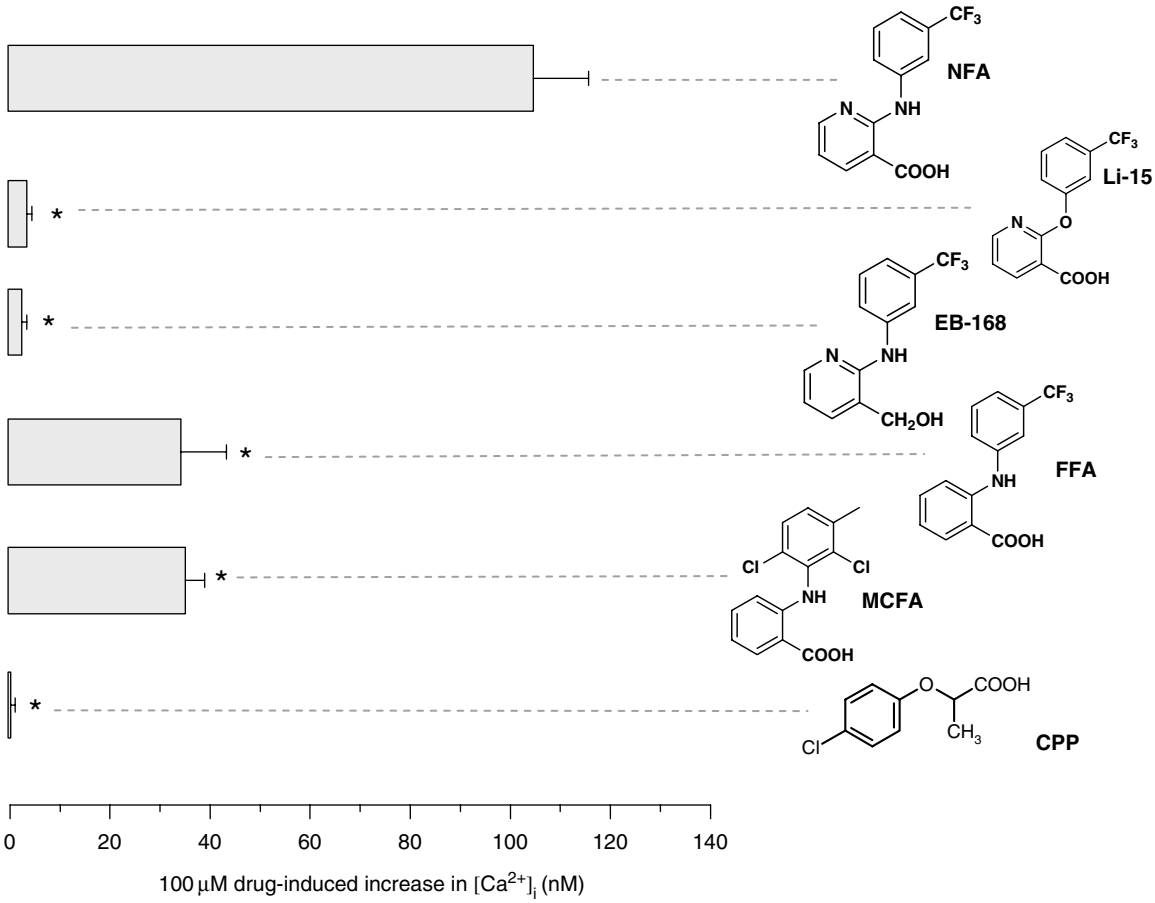

Figure 5 Structure-activity relationship for the increase in $[Ca^{2+}]_i$ in rat EDL muscle fibres induced by NFA derivatives. Each bar represents the enhancement of $[Ca^{2+}]_i$ induced by the compound indicated. For a comparison with other chloride channel blockers, the lack of effect of S(−)−CPP was also presented. Data are expressed as mean ± s.e.m. of 15–20 fibres. *Significantly different from the response to NFA with $P<0.05$.

used the fixed concentration of 100 μM to compare drug activity on gCl and $[Ca^{2+}]_i$ in native skeletal muscle. Furthermore, as the native gCl recordings were performed at the fibre's resting potential (around −80 mV), we used as an index of drug potency on heterologously expressed CLC-1, the apparent K_D value measured at a similarly negative voltage (−100 mV). The results from these experiments are summarized in Figures 5–7. With the exception of FFA derivatives, all chemical modifications compromised CLC-1 blocking activity, leading to less potent derivatives compared to NFA. Interestingly, the affinities of FFA and TFA are about four- to fivefold higher than that of NFA (K_D values of FFA and TFA at −100 mV are 4.5 ± 1.3 and 5.9 ± 0.8 μM, respectively). This increased potency was not observed with the other FFA derivatives that showed a CLC-1 block similar to that of NFA (Figure 7b). The main conclusion from these data is that the increased blocking potency of FFA and TFA is dependent on the presence of two phenyl groups, one of which is substituted with an electron attractive group in the meta position.

In contrast to that observed with CLC-1, none of the NFA derivatives showed an increased potency at enhancing $[Ca^{2+}]_i$ compared to NFA. In fact, FFA and MCFA were less potent compared to NFA, whereas Li-15 and EB-168 were practically ineffective (Figure 5). Accordingly, as shown in Figure 8, the relationship between drug-induced increase in $[Ca^{2+}]_i$ and drug K_D value for blocking CLC-1 was consistent with the conclusion that the molecular requirements for mediating the two effects are quite different.

Mechanism of action of NFA on muscle fibres

Effect of NFA on native gCl in the presence of chelerythrine. CLC-1 activity is modulated by Ca-dependent PKC, as indicated by previous studies conducted on native muscle fibres as well as on heterologously expressed CLC-1 (De Luca *et al.*,. 1998; Rosenbohm *et al.*, 1999). Thus, the NFA-evoked increase of $[Ca^{2+}]_i$ may secondarily contribute to an inhibition of native gCl through PKC stimulation. To test this hypothesis, we evaluated the effect of NFA in the presence of chelerythrine, an inhibitor of PKC. We used NFA at 100 μM, a concentration at which the NFA-induced $[Ca^{2+}]_i$ increase could be consistent with the sustained activity of PKC. No significant change of gCl was observed after application of 1 μM chelerythrine alone (Figure 9). Importantly, however, pretreatment with chelerythrine significantly reduced the NFA-induced inhibition of native gCl from 65 ± 2.2 to 43 ± 4% (Figure 9).

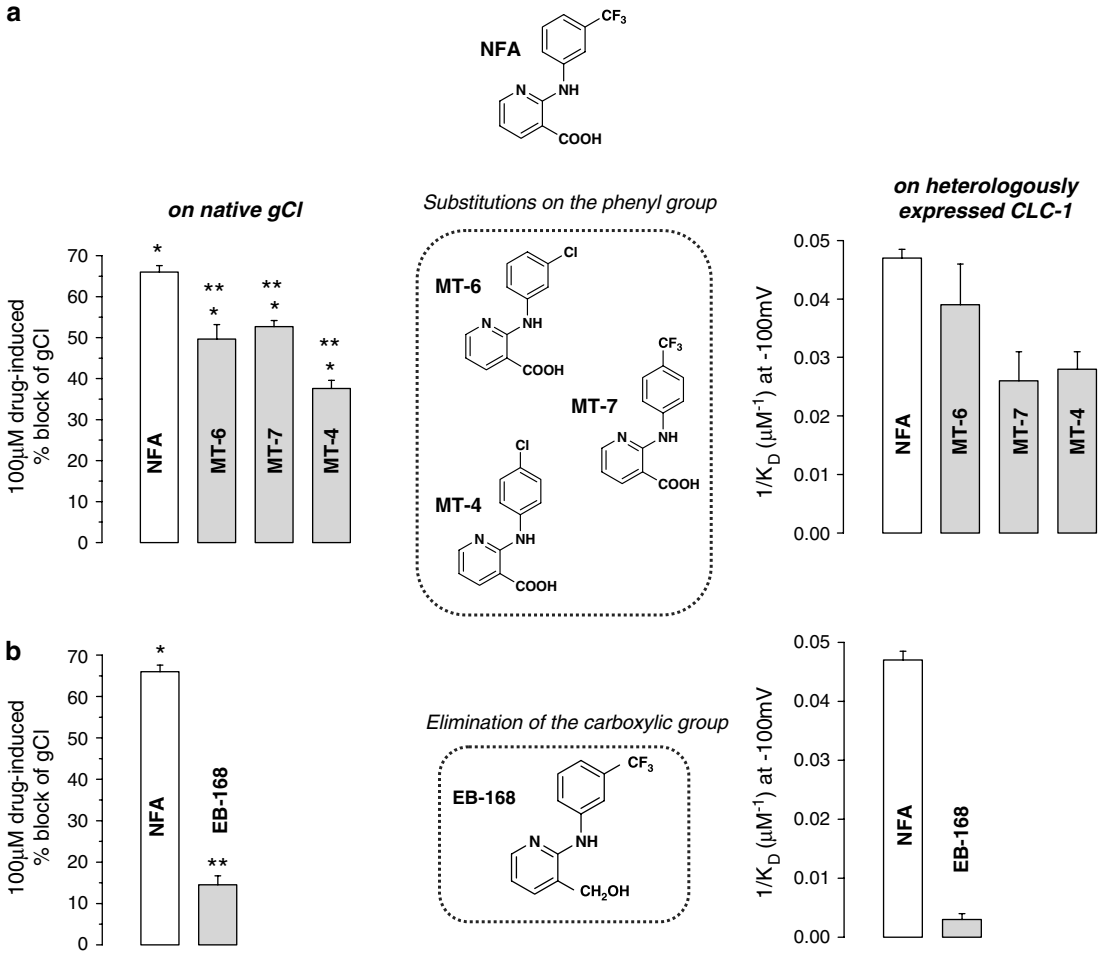

Figure 6 Structure-activity relationships for inhibition of CLC-1 channel activity by NFA derivatives. Effect of compounds with substitutions on the phenyl group (**a**) and of a compound in which the carboxylic group was removed (**b**) on gCl of native rat EDL muscle fibres (on the left, each bar represents the normalized percent block of gCl ± s.e.m. in the presence of each derivative at a concentration of 100 μM; 18–26 fibres from two to three preparations) and on CLC-1 expressed in *Xenopus* oocytes (on the right, each bar represents 1/the apparent K_D calculated at −100 mV for each derivative; at least four patches for each derivative were performed). Note that the different representations of the data (percent block of gCl versus $1/K_D$ for expressed CLC-1) arise from a technical complication: we used a single test drug concentration on gCl, whereas we could reliably estimate the K_D for the inside-out patch experiments. Drug 'potency' parallels both quantities, allowing a direct qualitative comparison. Significantly different from: * the relative control value and ** NFA, with $P<0.05$.

These results indicate that Ca-dependent PKC activation and consequent downregulation of the CLC-1 protein via the NFA-induced $[Ca^{2+}]_i$ increase may add to the direct block of CLC-1 channel activity.

Discussion

Although accumulating data indicate that fenamates are able to modulate a variety of ion channels in several tissues (Gögelein *et al*., 1990; Ottolia and Toro, 1994; Large and Wang, 1996; Lee and Wang, 1999; Peretz *et al*., 2005), very little is known about the effect of these drugs on skeletal muscle ion channels (Astill *et al*., 1996). A characterization of the direct effects of fenamates on the muscle Cl^- channel CLC-1, and of the possible indirect effects in intact skeletal muscle fibres is important to find out possible new ligands acting on CLC-1 and to evaluate possible side effects of molecules belonging to this compound class. In the present study, we showed that the fenamate NFA affects the activity of the CLC-1 channel and calcium homeostasis in native rat skeletal muscle fibres. NFA was found to inhibit in a concentration-dependent manner the CLC-1-mediated current by interacting with a binding site on the channel protein that is directly accessible only from the intracellular side. In fact, the lower potency of NFA on native gCl compared to inside-out recordings on heterologously expressed CLC-1 may be explained by the intracellular location of the binding site. In native skeletal muscle fibres, the drug can only be applied from the extracellular side. Thus, in the fibres, drug potency is strongly dependent on the acidic and lipophilic features as well as on membrane composition, all properties that influence the ability of the drug to cross the plasma membrane. Accordingly, in voltage-clamp recordings, owing to the less permeable oocyte plasma membrane, NFA only blocked heterologous, expressed CLC-1 when

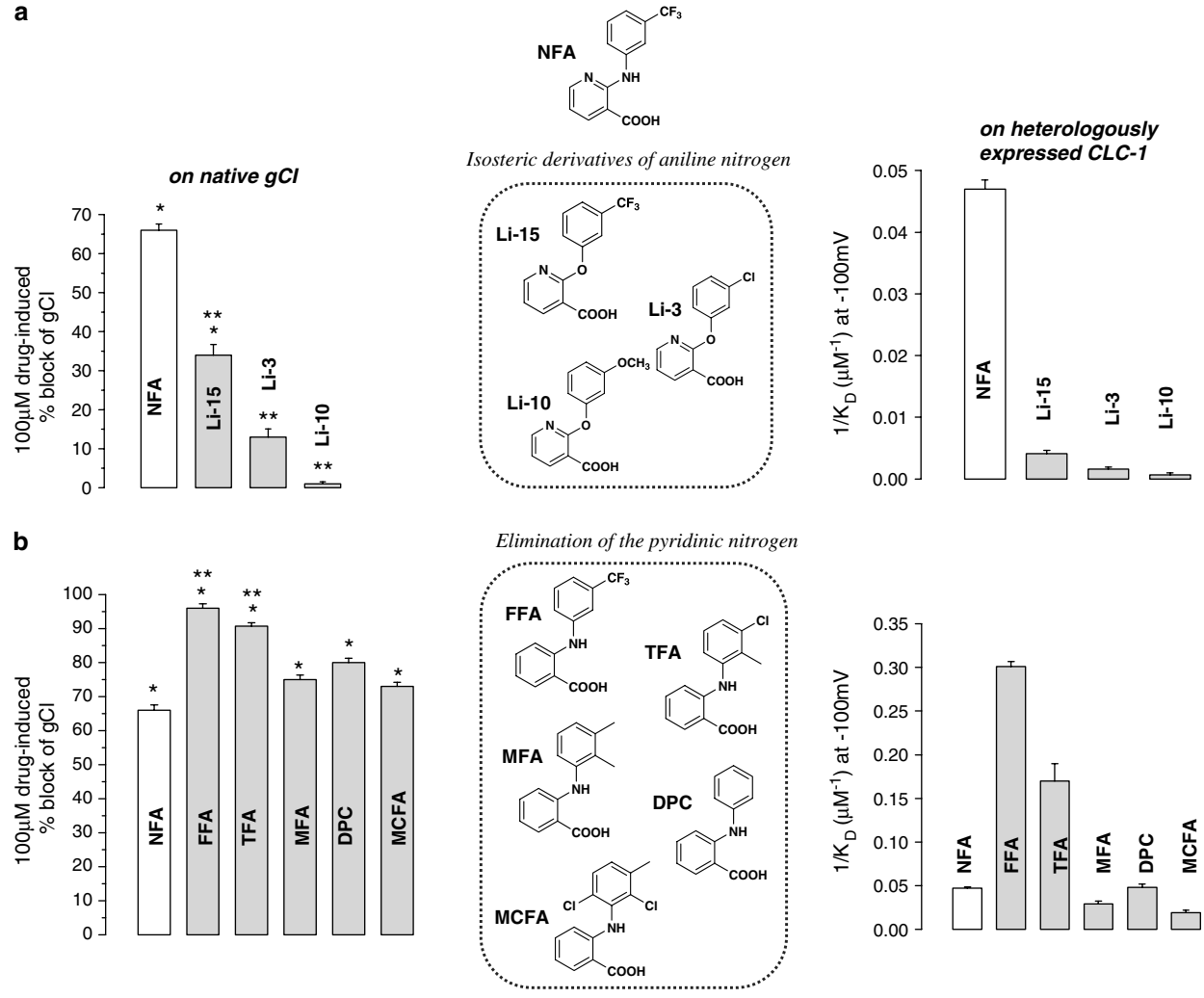

Figure 7 Structure-activity relationships for inhibition of CLC-1 channel activity by NFA derivatives. Effect of compounds with isosteric substitution of the anilinic nitrogen (**a**) and of compounds with elimination of the pyridinic ring (**b**) on gCl of native rat EDL muscle fibres (on the left, each bar represents the normalized percent block of gCl ± s.e.m. in the presence of each derivative; 15–26 fibres from two to three preparations) and on CLC-1 expressed in *Xenopus* oocytes (on the right, each bar represents 1/the apparent K_D calculated at −100 mV for each derivative; at least four patches for each derivative were performed). As in Figure 6, plotting the percent block of gCl and $1/K_D$ for block of CLC-1 allows a straightforward comparison of drug potency in the two systems. Significantly different from: * the relative control value and ** NFA, with $P<0.05$.

applied at a high extracellular concentration ($>500\,\mu$M) for a long incubation period. Actually, this is not surprising, as in a previous study, we observed similar behaviour for CPP, a well-characterized CLC-1 blocker (Conte Camerino *et al*., 1988; Pusch *et al*., 2000).

Our experiments revealed an additional, seemingly independent, effect of NFA in muscle. The compound produced an increase in $[Ca^{2+}]_i$ by releasing Ca^{2+} from an intracellular store. As CsA prevented this effect of NFA but thapsigargin and ruthenium red did not, we deduced that NFA increases $[Ca^{2+}]_i$ mainly by acting on the mitochondrial PTP. Although the ability of fenamates to interfere with the mitochondrial PTP has been demonstrated previously (Uyemura *et al*., 1997; Pigoso *et al*., 1998; Jordani *et al*., 2000), our study is the first to show such activity in native skeletal muscle fibres. Furthermore, *a priori*, the possibility that the effect of NFA on calcium homeostasis in skeletal muscle described here could be a consequence of cyclooxygenase inhibition and, consequently, a reduction of the mediators of the arachidonic acid cascade, needed to be excluded. Indeed fenamates are derivatives of *N*-phenylanthranilic acid and it is well known that the mechanism mediating the anti-inflammatory effect of the latter involves the inhibition of COX-1 and COX-2, enzymes that catalyse the biosynthesis of prostaglandins from arachidonic acid. However, by using indomethacin and meloxicam, two COX-inhibitors with markedly different structures, it was demonstrated that, in skeletal muscle, the effect of NFA on $[Ca^{2+}]_i$ does not involve the cyclooxygenase-prostaglandin pathway.

The structure-activity study involving compounds that varied the NFA molecule at several places, allowed us to conclude that the molecular prerequisites for mediating the two effects, direct CLC-1 block and the increase of $[Ca^{2+}]_i$, are quite different. Regarding the direct interaction with the

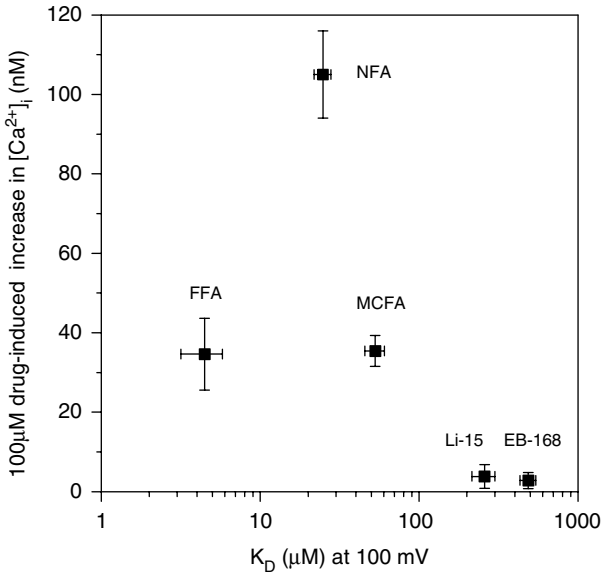

Figure 8 Relationship between the potency of a drug to release Ca^{2+} in native rat skeletal muscle fibres and its ability to block heterologously expressed CLC-1. Each point represents the mean ± s.e.m. of drug-induced $[Ca^{2+}]_i$ increase at 100 μM (15–20 fibres); the K_D value for CLC-1 block was calculated at −100 mV (4–6 patches) for each derivative indicated.

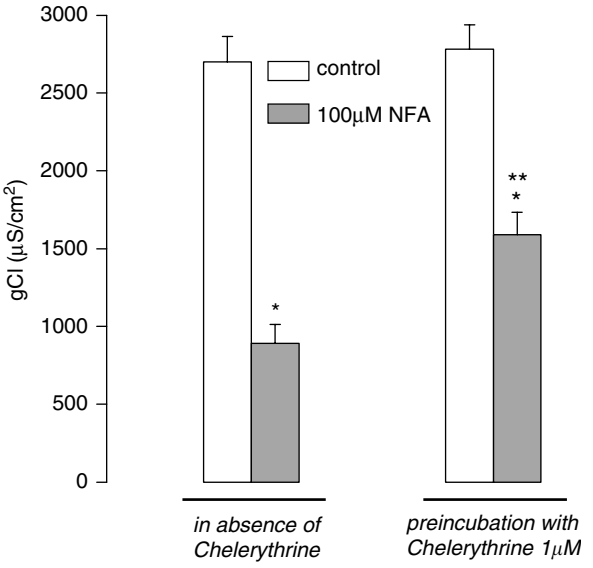

Figure 9 Evaluation of the involvement of PKC in the NFA-mediated inhibition of native gCl. Each bar represents mean gCl ± s.e.m. (from 25 to 30 fibres) obtained in control conditions and after application of 100 μM NFA in the absence or presence of chelerythrine. Significantly different with respect to: * the relative control value and ** in the absence of chelerythrine, with $P<0.05$.

CLC-1 protein, the anilinic nitrogen and the carboxylic group are important determinants, as compounds that lacked the amino bridge (Li-derivatives) or those with decreased acidity (EB-168) were less potent than NFA at inhibiting native gCl and heterologously expressed CLC-1. Interestingly, as observed with the inside-out patch-clamp recordings, the substitution of the nicotinic acid moiety with a benzoic acid ring produced a five- to sixfold increase in drug potency. Also in the case of CPP, the introduction of a second phenyl group, as occurs in bis-phenoxy derivatives (Liantonio *et al.*, 2003), enhanced the inhibitory potency of the drug. In this regard, in relation to the direct inhibition of CLC-1, there are some interesting similarities between the properties exhibited by fenamates and those displayed by CPP derivatives. Firstly, fenamates, just like CPP derivatives, produce a voltage-dependent inhibition by interfering with a binding site that is accessible from the intracellular side of the CLC-1 protein. Secondly, from a structural perspective, despite substantial chemical differences, the moiety of the diphenylamine group of the fenamate compounds exhibits some similarities with the two phenyl rings of the bis-phenoxy derivatives of CPP (Liantonio *et al.*, 2003). In addition to the common carboxylate group, both structures are constructed from two benzene rings, linked to each other through one or more atom bonds. It is not clear, however, whether all these compounds act by the same mechanisms and bind to the same channel site. Mapping of the fenamate binding site on the molecular structure of CLC-1 is needed to elucidate this issue.

Among the tested compounds, NFA was the most potent at increasing $[Ca^{2+}]_i$, indicating that the activity of this drug depends on the presence of the carboxylic group, the anilinic nitrogen and the pyridinic ring. Thus, compared with the molecular prerequisites for binding to CLC-1, it seems that the pyridinic ring is not essential for the CLC-1 inhibitory potency of NFA but is a prerequisite for its ability to increase intracellular calcium levels. In support of this, CPP, a molecule that lacks this particular group, blocks native gCl without effecting calcium homeostasis.

Recently, various NSAIDs have been shown to affect membrane permeability, producing an increase in $[Ca^{2+}]_i$ and this results in NSAID-associated gastrointestinal complications (Tomisato *et al.*, 2004). However, our findings contraindicate such a nonspecific mechanism, but rather support the hypothesis that a direct interaction between the drug and a target protein trigger the NFA-mediated $[Ca^{2+}]_i$ increase. Firstly, the $[Ca^{2+}]_i$ increase is strictly dependent on compound structure. Secondly, it is independent of extracellular calcium, and lastly, it is not prevented by depletion of the SR.

The NFA-induced $[Ca^{2+}]_i$ release most likely interferes indirectly with gCl. In fact, it has been shown that elevating $[Ca^{2+}]_i$ by the ionophore A23187 significantly decreases gCl (De Luca *et al.*, 1994). This reduction in gCl was found to be mediated by a Ca-dependent PKC (Tricarico *et al.*, 1991; De Luca *et al.*, 1994, 1998). In full agreement with the latter finding, we demonstrated that the PKC inhibitor chelerythrine reduced the inhibition of gCl by NFA.

As summarized in Figure 10, we finally conclude that NFA inhibits native gCl in rat skeletal muscle fibres mainly by blocking the CLC-1 channel directly and partly by mobilizing mitochondrial calcium stores, which in turn promote PKC activity and thus CLC-1 current inhibition.

From a toxicological viewpoint our study demonstrates that NFA has the ability to interfere with calcium homeostasis in skeletal muscle. The importance of this effect goes

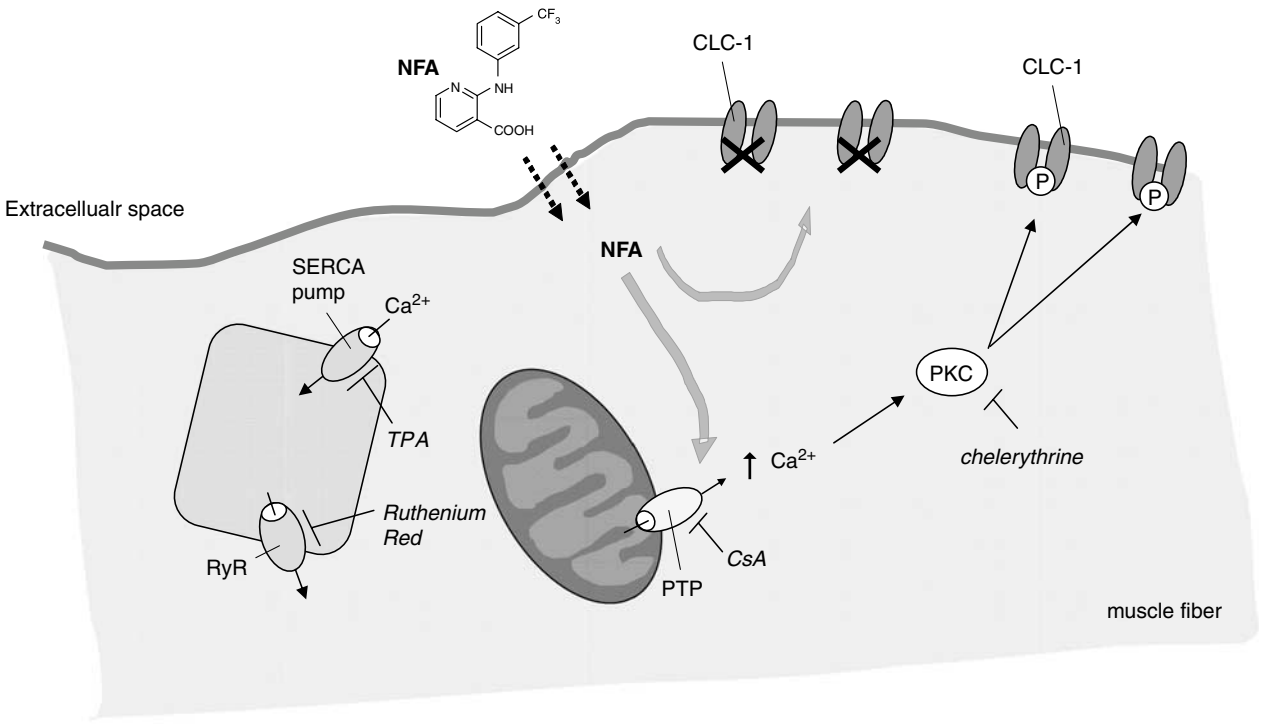

Figure 10 Schematic representation of the effect of NFA on skeletal muscle fibres and the sites of action of the pharmacological tools used in the study. NFA directly blocks CLC-1 channel by interacting with an intracellular binding site. Additionally, through activation of the mitochondrial PTP, NFA promotes mitochondrial calcium efflux, which may result in PKC activation. Thus, the PKC-dependent downregulation of the CLC-1 protein may add to the inhibitory effect mediated by NFA on gCl, which is mainly due to direct channel block by NFA.

beyond the regulation of gCl, because any downstream reaction that depends on $[Ca^{2+}]_i$ will be affected by NFA. In skeletal muscle, an increase in the cytosolic Ca^{2+} level could induce a dysregulation of Ca-activated proteins, promoting apoptosis, protein degradation and muscular remodelling. The toxicological properties of non-steroidal anti-inflammatory agents may be due, at least in part, to their ability to alter the distribution of intracellular calcium. Considering that the NFA-mediated increase of $[Ca^{2+}]_i$ seems to be via a release of calcium from mitochondria and that this is an ubiquitous organelle, our results may be important not only for skeletal muscle but also for other organs and tissues on which the drug could act. Additionally, the importance of gCl for maintenance of excitability and contractile function in working muscle is well known. Thus, the effect of NFA on CLC-1 and $[Ca^{2+}]_i$ could be correlated to toxicity on skeletal muscle in NSAID-treated patients. However, the concentration of NFA needed to inhibit cyclooxygenases is much lower than that required to obtain the effects described above on skeletal muscle. The IC_{50} of some fenamates is lower than 1 μM (Kalgutkar *et al*., 2000) for inhibition of cyclooxygenases and about 25 and 100 μM NFA is required to blocking CLC-1 and increase $[Ca^{2+}]_i$, respectively. Nevertheless, the present findings could help to clarify some of the mechanisms behind the paradox of early functional improvement and late functional impairment observed with the use of NSAIDs in muscle injuries and repair (Prisk and Huard, 2003). The elucidation of the molecular mechanisms of NSAID-induced muscle toxicity and other side effects is pivotal for developing safer NSAIDs.

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Conflict of interest

The authors state no conflict of interest.

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