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Activation and inhibition of kidney CLC-K chloride channels by fenamates

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Running title: Fenamates as CLC-K channels modulators

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The abbreviations used are: CPP, 2-p-(chlorophenoxy)propionic acid; DIDS, 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid; NFA, niflumic acid; FFA, flufenamic acid; MFA, mefenamic acid, MCFA, meclofenamic acid; TFA, tolfenamic acid; DPC, 2-(phenylamino)benzoic acid;

Abstract

CLC-K Cl^- channels are selectively expressed in kidney and ear where they are pivotal for salt homeostasis and loss of function mutations of CLC-Kb produce Bartter's syndrome type III. The only ligand known for CLC-K channels is a derivative of the 2-p-chlorophenoxypropionic acid (CPP), 3-phenyl-CPP, which blocks CLC-Ka, but not CLC-Kb. Here we show that in addition to this blocking site, CLC-K channels bear an activating binding site controlling channel opening. Using voltage-clamp technique on channels expressed in Xenopus oocytes, we find that niflumic acid (NFA) increases CLC-Ka and CLC-Kb currents in the 10-1000 μM range. Flufenamic acid (FFA) derivatives or high doses of NFA produced instead an inhibitory effect on CLC-Ka, but not on CLC-Kb, and on blocker insensitive CLC-Ka mutants, indicating that the activating binding site is distinct from the blocker site. Evaluation of the sensitivity of CLC-Ka to derivatives of NFA and FFA together with a modeling study of these ligands allow us to conclude that one major characteristic of activating compounds is the coplanarity of the two rings of the molecules, while block requires a non-coplanar configuration. These molecules provide a starting point for identification of diuretics or drugs useful in the treatment of Bartter's syndrome.

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Introduction

CLC-Ka and CLC-Kb chloride channels are expressed along the nephron from the thin ascending limb to the collecting duct where they are pivotal for chloride exit across the basolateral membrane (Uchida and Sasaki, 2005; Jentsch et al., 2002). The significance of these channels for salt homeostasis is illustrated by genetic disorders (Jentsch et al., 2005). Defects in the gene encoding CLC-Kb reduce activity producing Bartter's syndrome type III, a disease characterized by severe salt wasting and hypokalemia (Simon et al., 1997). CLC-Kb is implicated in transepithelial NaCl transport in the thick ascending limb and collecting ducts, while CLC-Ka-mediated Cl^- transport in the thin ascending limb constitutes a component of the countercurrent system of the inner medulla. Its loss disrupts the entire system, as suggested by the nephrogenic diabetes insipidus phenotype of CLC-K1 knock-out mice (Akizuki et al., 2001).

CLC-K channels are also expressed in the inner ear where they are involved in endolymph secretion, a mechanism pivotal for sound signal transduction (Estévez et al., 2001). Furthermore, for a correct expression and function, CLC-K channels require the presence of the barttin β subunit (Estévez et al., 2001; Waldegger et al., 2002). Barttin produces an increase of CLC-K channel expression at the plasma membrane (Estévez et al., 2001; Waldegger et al., 2002). Mutations in barttin cause type IV Bartter's syndrome, a disease characterized by renal failure accompanied by sensorineural deafness (Birkenhäger et al., 2001). In support of the hypothesis that defects in barttin impair function of both CLC-K channels, simultaneous CLC-Ka and CLC-Kb mutations result in a phenotype that mimics type IV Bartter's syndrome (Schlingmann et al., 2004).

Interestingly, recently a polymorphism of CLC-Kb has been reported that confers a gain of function of the channel activating CLC-Kb activity (Jeck et al., 2004a) *in vitro* and predisposing to hypertension *in vivo* (Jeck et al., 2004b).

The involvement of these Cl^- channels in various physiophatological processes arouse a considerable interest to identify specific high affinity ligands as tools to explore mechanisms of gating and permeation and as a starting point for drug development.

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In previous studies, we have identified compounds with two chlorophenoxy groups, capable of blocking rat CLC-K1 currents (Liantonio et al., 2002). Successively, we recognized 3-phenyl-CPP as the minimal structure capable of blocking CLC-K1 and CLC-Ka in a reversible and Cl^- ion-dependent manner by interacting with the channel pore from the extracellular side (Liantonio et al., 2004). Surprisingly, the highly homologous CLC-Kb channel was found to be fivefold less sensitive to 3-phenyl-CPP compared to CLC-Ka (Picollo et al., 2004). We identified the amino acid residues that are responsible for the difference in drug sensitivity and thus likely form part of the drug binding site. Particularly, asparagine 68 in CLC-Ka plays a pivotal role for the blocking activity of 3-phenyl-CPP, since exchanging it with a negatively charged aspartate (N68D), as found in CLC-Kb, markedly reduced drug sensitivity. Furthermore, N68 together with glycine 72, is also pivotal for the more pronounced activity of the unrelated stilbene blocker DIDS on CLC-Ka with respect to CLC-Kb (Picollo et al., 2004).

Here we demonstrate that in addition to this blocking binding site, CLC-K channels present also an activating binding site that controls opening of the channels. We show that niflumic acid (NFA), a drug belonging to a class of fenamates usually used as non-steroidal anti-inflammatory drugs, produces an increase of currents carried by CLC-Ka and CLC-Kb. In contrast to this activating effect, application of flufenamic acid (FFA) derivatives or high doses of NFA produced an inhibitory effect on CLC-Ka. The evaluation of the sensitivity to fenamates of CLC-Kb and of CLC-K mutants (Picollo et al., 2004), together with a molecular modeling study of the organic ligands provide insight into the mechanisms of activation and inhibition by fenamates.

This class of molecules represents until now the unique tool able to open and to block renal CLC-K channels and thus provides a starting point for the identification of drugs either with diuretic action or useful in the treatment of type III Bartter's syndrome.

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Materials and Methods

Expression in *Xenopus laevis* oocytes and voltage-clamp analysis

WT CLC-Ka, CLC-Kb and their mutants, obtained as previously described (Picollo et al., 2004), were co-expressed with the activating mutant Y98A of human barttin (Estévez et al., 2001). Expression in oocytes and electrophysiological measurements were performed as previously described (Pusch et al., 2000). Briefly, voltage-clamp data were acquired at room temperature (21-25°C) using the Pulse program (HEKA, Lambrecht, Germany) or a custom acquisition program (Gepulse) and a custom-built amplifier or a TEC03 amplifier (npi electronic, Tamm, Germany). Currents were recorded in the standard solution containing (in mM) 90 NaCl, 10 $CaCl_2$, 1 $MgCl_2$, 10 HEPES at pH 7.3. In experiments with low extracellular [Ca^{2+}], 1.8 mM $CaCl_2$ was used and NaCl was increased to 106 mM to balance osmolarity and salt-strength.

Similar voltage-clamp pulse protocols for CLC-Ka and CLC-Kb, with a longer pulse duration for CLC-Kb, were used: from a holding potential of -30 mV, after a prepulse to 60 mV (or -100 mV) for 100 or 200 ms (for CLC-Ka and CLC-Kb respectively), voltage was stepped from -140 to 80 mV in 20 mV increments for 200 or 500 ms (for CLC-Ka and CLC-Kb respectively), followed by a final tail pulse to -100 mV. To evaluate the on-set and wash-out of drug effects a pulse to 60 mV was applied every 2 s.

As a control, we routinely applied a solution containing 100 mM I^- that blocks currents carried by CLC-K channels but not endogenous currents (Pusch et al., 2000) and used the residual current in 100 mM I^- to estimate the contribution of endogenous currents (Picollo et al., 2004).

Apparent dissociation constants for drugs showing blocking activity, K_D, were determined by calculating the ratio of the steady-state current in the presence and in absence of the drug and fitting the ratios to the equation: $I(c)/I(0) = 1/(1 + c/K_D)$ where c is the concentration (Eq. 1). Errors in figures and in the text are indicated as S.E.M.

Drugs

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NFA derivatives

The following drugs were purchased from Sigma-Aldrich (Milano-Italy): niflumic acid (NFA), flufenamic acid (FFA), mefenamic acid (MFA), meclofenamic acid (MCFA), tolfenamic acid (TFA), 2-(phenylamino)benzoic acid (DPC).

Remaining compounds were synthesized according to previously reported procedures: MT-4, MT-6 and MT-7 were prepared by condensation of 2-chloronicotinic acid with the appropriate substituted amines (Sherlock et al., 1988). The phenoxypyridinecarboxylic acids **(**Li-3, Li-10, Li-6 and Li-11**)** were synthesized condensing 2-chloronicotinic acid with the opportune sodium phenoxide using an excess of the phenol (Villani et al., 1975). For Li15 the condensation was modified using equimolar quantities of the acid and the phenolate in DMF (Fujiwara and Kitagawa, 2000). EB-168 was synthesized by treating commercially available NFA with an excess of boran methylsulfide complex in dry tetrahydrofuran.

Compounds were daily prepared in dimethylsulfoxide stock solutions and the final concentrations were obtained by appropriate dilution with the solution used for the electrophysiological recordings.

Modeling study

We searched the lowest energy conformers for each drug by a systematic MMFF (Merck Molecular Force Field) analysis neglecting solvent presence. A restricted number of conformers (see Table below) were found for the fenamates in the range of 10 kcal/mol. Results were similar to the ones previously reported (Dhanaraj and Vijayan, 1988). 3-phenyl-CPP, a more flexible molecule, showed a larger number of conformers (see Table 1) in the same range of energy. For FFA, MCFA, NFA and 3-phenyl-CPP the same conformational analysis was carried out also in the presence of water and results were close to those without solvent.

In Table 1, we report the number of low energy conformers found after the conformational analysis carried out with and without water.

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When available, as in the case of DPC, FFA, MCFA, MFA, NFA and TFA molecules, the starting geometry parameters were derived from X-ray data from CCDC (Cambridge Crystallographic Data Center) (Cambridge Structural Database V5.26, Nov. 2004, upd. Feb. 2005).

For 3-phenyl-CPP, whose X-ray data were not available, the molecule was first constructed by fragments, the molecular geometry was optimized to DFT B3LYP/6-31G* level theory and then submitted for further calculations (Spartan '04 Wavefunction, Inc. Irvine, CA.). Except for molecular mechanics and semi-empirical models, the calculation methods used in Spartan'04 have been documented in (Kong et al., 2000). We observed that the most populated low energy conformer families are within the 3.0 kcal/mol range; among them the lowest energy conformer was selected and used for the overlay show in Fig. 9B. The conformers were superimposed at the level of the aromatic ring not directly bound to the carboxylic group, and of the heteroatoms, nitrogen or oxygen, bridging the aromatic rings.

All calculations were performed with the SPARTAN ‘04 package (Wavefunction Inc., Irvine, CA). Graphical representations were performed by DS Viewerpro 6.0 trial version (Accelrys Inc., San Diego, CA).

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Results

Effect of fenamates on CLC-Ka

Effect of NFA

In the first series of experiments we characterized the effect of NFA on the CLC-Ka channel. CLC-Ka currents partially deactivate at positive potential and activate at negative potentials (Fig. 1A left).

Application of 200 μM NFA induces an increase of CLC-Ka currents both at negative and positive potentials (Fig. 1A). The on-set of the effect was rapid while the return of the currents to control value upon removal of drug was reached within 7-9 minutes (Fig. 1B). The NFA-mediated current increase was practically voltage independent (I_{drug}/I_0 = 2.38 ± 0.12 at 60 mV and I_{drug}/I_0 = 2.20 ± 0.3 at -140 mV). At all tested potentials, NFA induced an increase of the current amplitude without producing kinetic modifications. While a current increase was seen for concentrations between 50 and 1000 μM, NFA blocked CLC-Ka currents at 2 and 2.5 mM (Fig. 1C). This biphasic dose-response relationship indicates that the mechanism of action of NFA is complex, likely involving two functionally different binding sites with opposite effects.

Dependence of NFA activation on extracellular calcium concentration

It is known that CLC-K channel activity is strongly enhanced by extracellular calcium in the millimolar range (Estévez et al., 2001; Waldegger et al., 2002). We examined whether calcium may interfere with the activating effect of NFA. Lowering $[Ca^{2+}]_{ext}$ from 10 mM to 1.8 mM reduces CLC-Ka currents (data not shown), and, importantly, the relative activating effect of 200 μM NFA was significantly larger in low calcium (Fig. 2). A possible interpretation of increased potency of NFA in low calcium is that in this condition a larger population of channels is available for opening by NFA.

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Structure-activity study

Searching to determine the structural requisites to bind to and to modulate CLC-Ka activity, we evaluated the effect of a series of compounds, modifying in different parts the NFA structure (Fig. 3). We determined the potency of the various derivatives by comparing the effect produced by each drug at 200 μM, a concentration at which the NFA-mediated activating effect was evident.

Substitutions on the phenyl group. Derivative MT-7 in which the CF_3 group on the phenyl ring is shifted from the meta to the para position with respect to the anilinic nitrogen is still able to produce an increase of CLC-Ka currents, albeit with a reduced potency (ratio I_{drug}/I_0 of 1.89 ± 0.04; Fig. 4A). Much more drastic in compromising the activating drug activity resulted the substitution of the CF_3 group with a chlorine atom. Indeed, application of derivatives MT-6 and MT-4 having a chlorine atom in meta or in para position, respectively, only slightly increased or did not produce any effect, respectively (Fig. 4A). These data indicate that an electronegative effect that reduces the density of the electron cloud of this aromatic ring is not sufficient to confer drug activity. Rather, the presence of the threefluoromethyl group seems to confer a specific bulkiness in this part of the molecule suitable for the interaction with the activating binding site.

Elimination of the carboxylic group. The role of the acidic function in the NFA molecule was investigated by substituting the carboxylic group with a hydroxyl methyl one. Application of derivative EB-168 at 200 μM did not produce any modification of CLC-Ka (Fig.4B), suggesting a pivotal role of the carboxylic group in mediating NFA-induced activating effect.

Isosteric substitution of the anilinic nitrogen. Changing the anilinic nitrogen that links the two rings abolished activation of CLC-Ka, independent of other substitutions in the phenyl ring as indicated by the fact that application of each isosteric derivative (substances Li-15, Li-3, Li-10, Li-6, Li-11 in Fig. 3) at 200 μM did not produce any significant change in CLC-Ka currents (Fig. 5A). To exclude

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the formal possibility that the lack of effect of substances Li-15, Li-3, Li-10, Li-6 and Li-11 was due to a compensation of activation and block we evaluated them also at 50 μM. If at 200 μM block would have masked an activating effect, at the lower concentration of 50 μM an increase of currents would be expected. However, also at this concentration, none of these derivatives produced a significant effect (data not shown).

Conversion of the pyridinic ring into a phenyl ring. In FFA, the pyridinic ring is substituted with a phenyl one (Fig. 3). Interestingly, 200 μM FFA blocked CLC-Ka-sustained outward and inward currents in a dose-dependent manner (Fig. 5B, 6A) with a ratio I_{drug}/I_0 of 0.45 ± 0.04 at 60 mV (Fig. 6B). Onset of the effect as well as wash-out were quite rapid (Fig. 6B). The dose response curve was well fitted by a simple titration curve at -140 mV and 60 mV with apparent K_D values reported in Table 2, suggesting 1:1 binding.

While the potentiation of CLC-Ka by NFA was dependent on the presence of a CF_3 group in meta position on the phenyl group, a modification regarding this region of the molecule on FFA led to an increase of the blocking activity. Particularly, the order of potency of FFA derivatives was MCFA>MFA>TFA>FFA (Fig. 5A, Table 2). The elimination of all substituents on this phenyl group (molecule DPC, Fig.3) significantly reduced the drug inhibitory activity (Fig. 5A, Table 2). These results suggest that the substitution of the pyridinic group with a phenyl one completely shifted the affinity of NFA from an activating binding site to an inhibitory binding site with some different structural requirements.

We also evaluated the effect of 3-phenyl-CPP, the CLC-K blocker with highest affinity known up to now (Liantonio et al., 2004; Picollo et al., 2004). In agreement with a previous study (Picollo et al., 2004), 3-phenyl-CPP inhibited CLC-Ka currents with a potency reported in Table 2.

Effect of fenamates on CLC-Kb

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Although highly homologous (Kieferle et al., 1994), the two CLC-K isoforms show a different pharmacological profile (Picollo et al., 2004). We evaluated the sensitivity of CLC-Kb to fenamates using NFA and FFA, the two lead compounds with activating and inhibitory effect on CLC-Ka. At 200 μM, the effect of NFA on CLC-Kb is more pronounced compared to CLC-Ka, with a ratio I_{drug}/I_0 of 3.5 ± 1.0.

Interestingly, in contrast to what observed on CLC-Ka, also application of 200 μM FFA produced an increase of CLC-Kb currents with a ratio I_{drug}/I_0 of 2.09 ± 0.9 at 60 mV (Fig. 7). Furthermore, NFA even at high concentrations (> 1 mM) was incapable of blocking CLC-Kb sustained currents, producing at all tested concentrations a potentiation (data not shown).

Effect of NFA and FFA on CLC-K mutants

We have previously identified N68 on CLC-Ka as a key amino acid of the binding site for 3-phenyl-CPP and DIDS (Picollo et al., 2004), inhibitors of CLC-K1 and CLC-Ka (Liantonio et al., 2004; Picollo et al., 2004). Most features of FFA-mediated inhibition resemble those of 3-phenyl-CPP, suggesting a common inhibitory binding site shared by these two different classes of CLC-Ka inhibitors. To asses this hypothesis we tested FFA and its derivatives on the CLC-Ka mutant N68D, the mutant that was least sensitive to 3-phenyl-CPP block (Picollo et al., 2004). 200 μM FFA did not produce any significant decrease of currents sustained by CLC-Ka N68D, suggesting that this point mutation strongly reduced drug affinity. MCFA, the most potent derivative among the here tested compounds on CLC-Ka, showed a K_D of 632 ± 101 μM, an almost 15-fold decrease in affinity compared to WT. For all other FFA derivatives the apparent K_D value was > 1 mM (Table 2).

We next tested NFA on all mutants previously reported both for CLC-Ka and CLC-Kb (Picollo et al., 2004). Each mutant resulted sensitive to NFA with a potency comparable to that observed in the related wild type (Fig. 8). However, it is interesting to notice that NFA produced a slightly smaller

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effect on CLC-Ka N68D with respect to WT (Fig. 8), indicating that the activating and inhibitory binding site may not be completely independent.

At high concentrations such as 2 mM, NFA is still capable of producing a block of CLC-Ka N68D mutant although less potently with respect to CLC-Ka wild type (I_{drug}/I_0 = 0.75 ± 0.05 and I_{drug}/I_0 = 0.67± 0.04 at 60 mV, respectively).

Modeling study

Conformational search studies show that all examined NFA derivatives exhibit a restricted number of low energy conformers characterized by the presence of an intramolecular hydrogen bond between the amino group and the oxygen atoms of carboxylic moiety. For all examined molecules, the calculated low energy conformations include the ones found in the X-ray crystal structures.

The lowest energy conformer of NFA shows a quite planar structure, differently from the corresponding conformers of the remaining molecules in which the two aromatic rings lie on different planes (Fig. 9A). The NFA planar structure is the result of the intramolecular hydrogen bonding as well as of the conjugation of the amino group lone-pair with the electron withdrawing pyridine ring. This conjugation is less effective in the FFA analogs due to the substitution of the pyridine system with a phenyl; at the same time, this substitution introduces an additional hydrogen atom in ortho position to the amino group causing a steric hindrance in the resulting diphenyl system which forces the non co-planar arrangement of the aromatic rings (Dhanaraj and Vijayan, 1988). This behavior is enhanced in the compounds having bulky substituents in the ortho positions of the aniline moiety such as MCFA, MFA and TFA (Fig. 9A).

Regarding 3-phenyl-CPP all lower energy conformer families exhibit the two aromatic rings lying on different planes due to their molecular flexibility.

An overlay of the lowest energy conformers of the lead molecules, indicates that all blocking compounds such as FFA and 3-phenyl-CPP displayed non-planar geometries in contrast to NFA (Fig.9B).

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Discussion

In the present work we demonstrated that in addition to a blocking site (Picollo et al., 2004), CLC-K chloride channels have also an activating binding site. In dependence on the chemical structure fenamates are capable of blocking or opening CLC-Ka. NFA is able to increase CLC-Ka currents at all tested membrane potentials in the 10-1000 μM range with a rapid on-set and a relative slow, but complete, recovery suggesting binding to a site that is accessible from the extracellular side. The activating effect was markedly increased when the extracellular calcium concentration was lowered. The opposite charge carried by NFA and calcium ions leads us to exclude a possible competition between these CLC-Ka activators for a common binding site. Thus, the increased potency of NFA in low calcium is probably simply due to the fact that in this experimental condition, more channels are available for NFA activity.

Starting from NFA as the lead compound, and considering all double-ring compounds shown in Fig. 3 the following structural requisites for an efficient activation of CLC-Ka emerged: the acidic carboxylic group, two aromatic rings one of which preferentially a pyridinic ring, a CF_3 group in meta position on the phenyl group, an anilinic moiety connecting the two rings. Several of these requisites are shared to enable efficient blocking activity. These include the carboxylic group and the two aromatic moieties linked by an electronegative atom. All FFA derivatives produced a rapid and reversible block with a mechanism of action resembling that of 3-phenyl-CPP. Interestingly, the presence of these groups is pivotal for blocking activity also in the 3-phenyl-CPP structure. Furthermore, as indicated by the different potency shown by the FFA derivatives, in line with what observed with 3-phenyl-CPP structure modifications (Liantonio et al., 2004) the presence of an electronegative group, reducing the charge the electronic cloud of one of the aromatic rings of FFA derivatives, increased the drug affinity toward the binding site.

The fact that NFA at high concentration is able to block CLC-Ka and that FFA is able to open CLC-Kb indicates that both drugs can bind the activating and blocking binding sites but with different affinity. The presence of a pyridinic group favors binding to the activating binding site, although it

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can not be excluded that also at low concentration a certain drug amount binds the inhibitory binding site. In support of this, the activating effect of 200 μM NFA was more evident on CLC-Kb, the CLC-K isoform that is much less sensitive to blockers (Picollo et al., 2004). In parallel, the presence of two phenyl groups, as occurs in FFA derivatives, confers a larger affinity toward the inhibitory binding site on CLC-Ka. However, also in this case it seems that the same molecules are also capable of interacting with the activating binding site as evidenced by the FFA-induced increase of CLC-Kb currents.

In a previous study, we mapped the binding site of 3-phenyl-CPP and DIDS on CLC-Ka, pinpointing the neutral amino acid N68 as crucial for 3-phenyl-CPP-mediated inhibition since mutating it to the negatively charged aspartate (N68D), as occurs in CLC-Kb, markedly reduced drug sensitivity (Picollo et al., 2004). All FFA derivatives resulted much less potent in blocking CLC-Ka N68D mutant with respect to CLC-Ka wild type, indicating that N68 is pivotal for drug blocking activity. Furthermore, we showed that FFA loses its blocking activity on CLC-Kb, producing a potentiation of CLC-Kb. Thus we speculate that, as in the case of 3-phenyl-CPP (Picollo et al., 2004), the electrostatic interaction between amino acids located in the external vestibule of the channel pore and the negatively charged group of FFA derivatives might either permit (CLC-Ka) or impede (CLC-Kb) the interaction of the inhibitors. Surprisingly, at high concentration NFA was able to block CLC-Ka N68D mutant with only slightly less potency with respect to wild type. Apparently this might suggest that the inhibitory effect of NFA and FFA is mediated by different blocking binding sites. Alternatively, considering the possibility of a unique blocking binding site, the presence of the pyridinic ring could account for the different behavior of NFA with respect to FFA on CLC-Ka N68D mutant. In fact, the reduced activity of FFA on mutant N68D is probably due to an electrostatic repulsion (Picollo et al., 2004). The carboxylic group of NFA may have a lower charge density caused by the protonation of the pyridinic ring, an event that can occur at the experimental pH. This could allow NFA to get closer to the blocking binding site than FFA in the mutant.

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One of the most important questions regarding the NFA action is whether the two opposite effects, potentiation and block, are mediated by drug interaction with two different binding sites. In contrast to the FFA blocking effect, the NFA-induced increase of CLC-Ka currents was reproducible on all mutants previously described for the identification of 3-phenyl-CPP binding site (Picollo et al., 2004), although with a slightly smaller potency in the case of CLC-Ka N68D. On the whole, these data indicate the presence of two different binding sites mediating the activating and blocking effect respectively. However, it should be kept in mind that, as we hypothesized for the inhibitory binding site (Picollo et al., 2004), the effective activating binding site could be quite deep within the pore. Thus, it should not be excluded that the two binding site are partially overlapping. The precise identification of the activator site on CLC-K channels will be needed to conclusively resolve this issue.

We attempted to explain the different drug activity by performing modeling investigations that allow us to compare the spatial geometry profiles associated at NFA, FFA and 3-phenyl-CPP structures. The most important conclusion is that NFA and its analogues MT-4 and MT-7 that behave as CLC-Ka openers show nearly planar conformations, while FFA derivatives and 3-phenyl-CPP, exhibiting a CLC-Ka blocking activity, are forced to assume a non co-planar arrangement of the aromatic rings. Overlaying the lowest energy conformers reveals that all blocking compounds display non-planar geometries compared to NFA and that the increasing planarity distortion of the aromatic rings ranging from DPC, FFA, MFA, TFA to MCFA parallels the observed increasing blocking activity in the same order.

The involvement of CLC-Kb in type III Bartter' syndrome and the parallel lack of drug treatment arouse a great interest toward molecules able to open CLC-K channels. The use of a compound similar to NFA for a treatment of Bartter's syndrome could improve diuresis in a direct manner through an increase of CLC-K channel activity and in an indirect manner through cyclooxygenase inhibition. Indeed type III Bartter's syndrome patients show an elevated prostaglandin activity

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(Reinalter et al., 2002) and NFA belongs to the class of non-steroidal anti-inflammatory drugs. However, the activating effect of NFA on mutants of CLC-Kb has not been tested in the current study.

At the same time, considering the involvement of CLC-K channels in the mechanisms of urine concentration, specific inhibitors could represent a new class of drugs with diuretic activity (Fong, 2004). At this regard it is worth to notice that whereas CLC-K2/Kb is clearly restricted to basolateral membranes of renal epithelial cells, the localization of CLC-K1/Ka is still controversial. Both basolateral and apical membranes (Uchida et al., 1995) or only basolateral localization (Vandewalle et al., 1997) have been reported. Thus, CLC-K inhibitors may have to reach their target via the basolateral fluid. This means that effective concentrations have to be reached in the renal interstitium *in vivo* for obtaining the diuretic effect, and the relatively low affinity for the blockers may thus limit the effectiveness. However, this alternative route could ensure drug activity also in some pathological conditions, such as decreased renal blood flow or renal failure, in which the therapeutic effectiveness of furosemide-like diuretics could be seriously compromised.

Besides, recently a polymorphism of CLC-Kb was reported (T481S) that, in contrast to mutations responsible of Bartter's syndrome, confers a gain of function. Indeed, T481S mutant channels led to a dramatic increase in CLC-Kb currents when expressed in oocytes (Jeck et al., 2004a). This polymorphism was associated with hypertension in one study (Jeck et al., 2004b). However, an involvement of this polymorphism was not seen in other studies (Speirs et al., 2005; Kokubo et al., 2005). Thus, a specific inhibition of CLC-K channels as a treatment of such a condition, remains speculative. Although the CLC-K inhibitors identified up to now showed a major affinity toward the CLC-Ka isoform, they represent drugs with good therapeutic potential, permitting a reduction of CLC-Ka activity for counteracting the excessive CLC-Kb activity.

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Footnotes

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D.C.C. and M.P. contributed equally to this work

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Legends for Figures

Figure 1. Effect of NFA on CLC-Ka. (A) Voltage clamp traces of CLC-Ka currents before and during application of 200 μM NFA. The pulse protocol is shown as inset. After a prepulse to -100 mV, voltage was stepped from -140 mV to 80 mV in 20 mV increments for 200 ms. (B) Time course of CLC-Ka current at 60 mV. Lines indicate application of NFA and wash solution. (C) Dose-response relationship of NFA at 60 mV. The ratio of the current in the presence and absence of drug is plotted versus concentration.

Figure 2. Effect of extracellular calcium on the activity of CLC-Ka mediated by NFA. The ratio I_{drug}/I_0 at 60 mV and at –140 mV measured in 10 mM and 1.8 mM extracellular Ca^{2+} are compared.

Figure 3. Chemical structures of niflumic acid (in the inset above on the left) derivatives. (A) Compounds with substitutions on the phenyl group. (B) Compound with elimination of the carboxylic group. (C) Drugs with isosteric substitution of the anilinic nitrogen. (D) Compounds with elimination of the pyridinic ring. For a comparison, in the inset below on the right the molecule of 3-phenyl-CPP is reported.

Figure 4. Substitutions on the phenyl group. In (A) the ratio of the current in the presence and absence of compound is plotted versus time. The arrow indicate application of compounds. (B) Voltage-clamp traces before and during application of 200 μM EB-168.

Figure 5. (A) Structure-activity relationship study: each point represents the average of the ratio I_{drug}/I_0 at 60 mV for each molecule (on the left) obtained from n oocytes (on the right). (B) Current-voltage relationship obtained in control condition, in presence of 200 μM of NFA and 200 μM of FFA. Each point represents the average of determinations obtained from 4-6 oocytes.

Figure 6. Effect of FFA on CLC-Ka. (A) Voltage clamp traces of CLC-Ka currents before and during application of 200 μM FFA. (B) Time course of the CLC-Ka current blocked by 200 μM FFA. Arrow indicates the addition of drug. (C) Dose-response relationship of the block at 60 mV by FFA for CLC-Ka. Line is draw according to equation 1 described in Material and Methods.

Figure 7. Effect of NFA and FFA on CLC-Kb. Voltage-clamp traces of CLC-Kb currents before and during application of 200μM NFA (A) and 200 μM FFA (B) and immediately after wash-out.

Figure 8. Effect of NFA on CLC-Ka, CLC-Kb and their mutants. Each bar represents the ratio I_{drug}/I_0 obtained as the ratio of the current in presence and absence of 200μM NFA for CLC-Ka, CLC-Kb and relative mutants.

Figure 9. Modeling study of the lead compounds. A) Lowest energy conformation of MCFA, 3-phenyl-CPP, FFA and NFA obtained as described in Methods; B) Overlay of 3-phenyl-CPP (blue), FFA (green) and NFA (red) lowest energy conformations. The fitting of the molecules was performed as described in Methods.

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Table 1. Number of low energy conformers obtained from the conformational analysis carried out with and without water (see Materials and Methods).

Molecule	without H_2O	with H_2O
FFA	8	18
MCFA	6	13
NFA	11	12
3-phenyl-CPP	36	50

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Table 2. Isosteric derivatives of the pyridinic nitrogen. The columns from left to right are as follows: compound; CLC-K-type channel; inhibition, K_D value expressed as mean ± S.E.M. calculated for each compound at 60 mV and -140 mV; n, number of oocytes.

Compound	**Channel**	Inhibition		
		K_D (60 mV) (µM)	**K_D (-140 mV) (µM)**	n
FFA	CLC-Ka	121 ± 37	57 ± 8	20
	CLC-Ka N68D	>5000	>1000	5
TFA	CLC-Ka	148 ± 22	195 ± 47	5
	CLC-Ka N68D	>1000	>1000	4
MFA	CLC-Ka	103 ± 24	107 ± 11	4
	CLC-Ka N68D	>1000	>5000	4
MCFA	CLC-Ka	43 ± 6	39 ± 8	5
	CLC-Ka N68D	632 ± 101	172 ± 39	4
DPC	CLC-Ka	502 ± 135	>5000	3
	CLC-Ka N68D	>5000	>3000	3
3-phenyl-CPP	CLC-Ka	79 ± 5	184 ± 18	23
	CLC-Ka N68D	424 ± 38	934 ± 87	10

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Figure 1

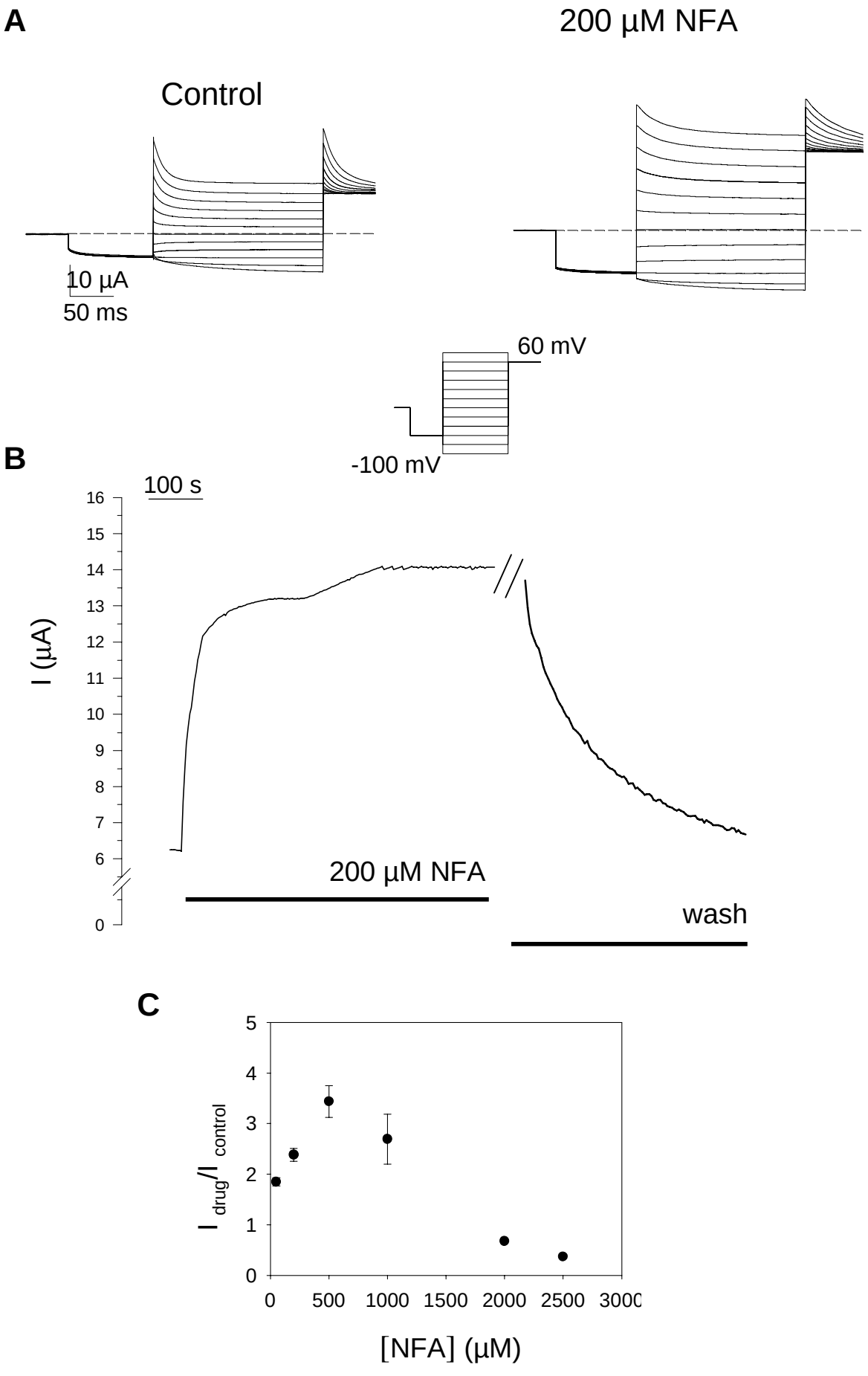

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Figure 2

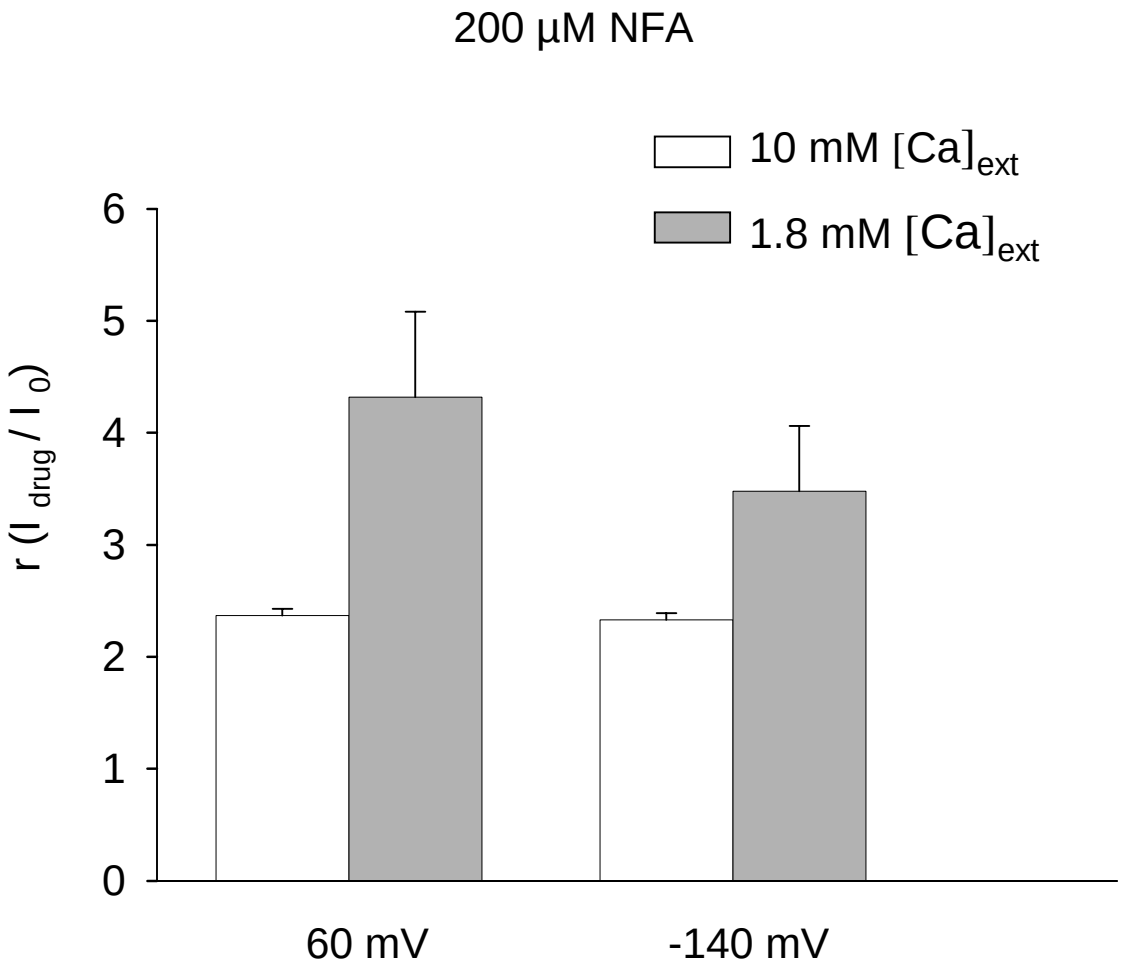

Figure 3

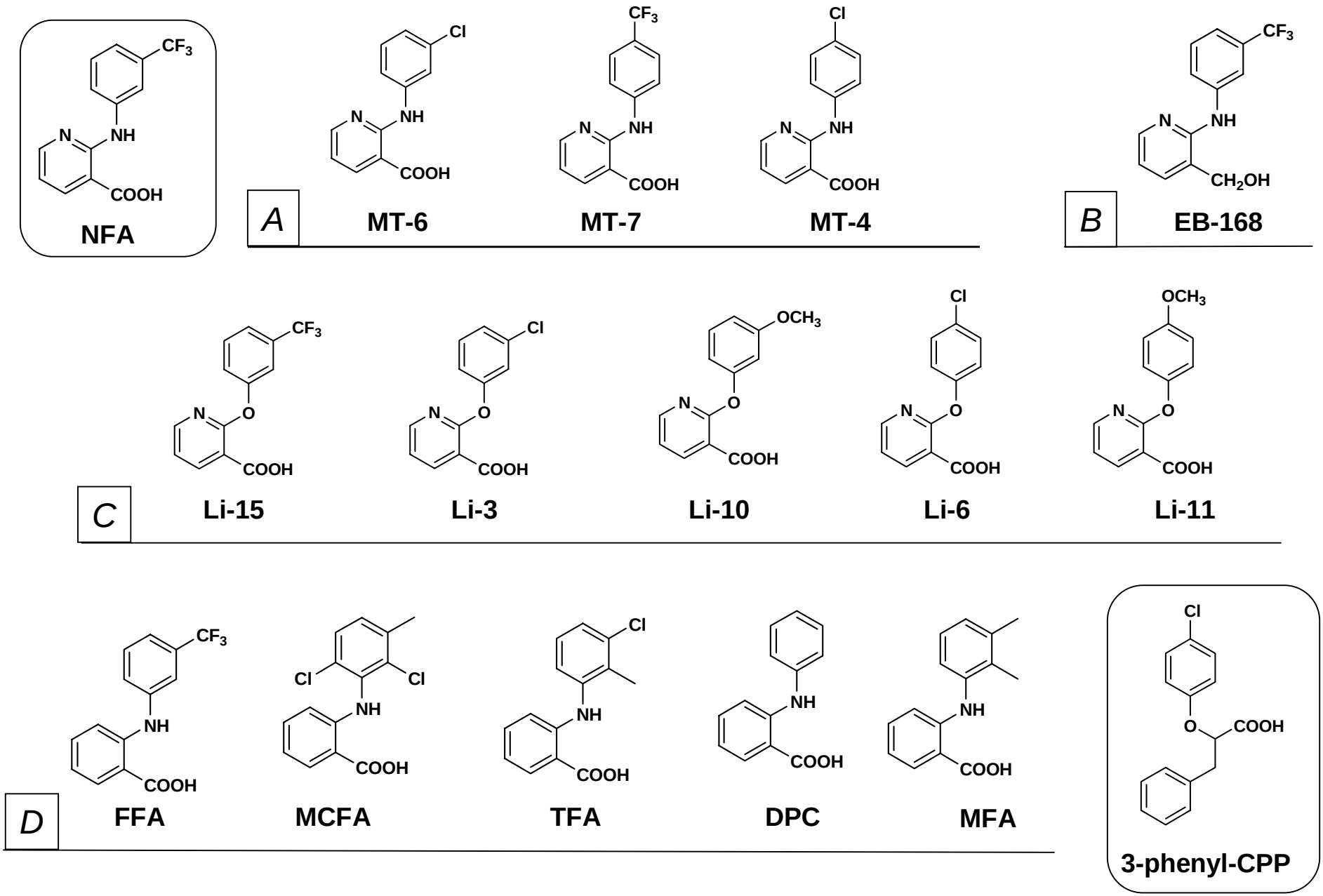

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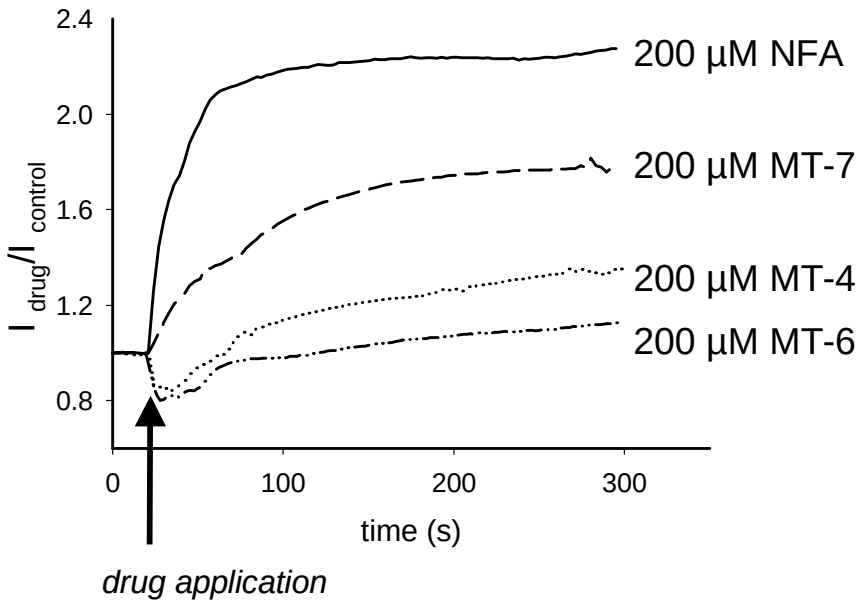
Figure 4

A Substitutions on the phenyl group



B Elimination of the carboxylic group

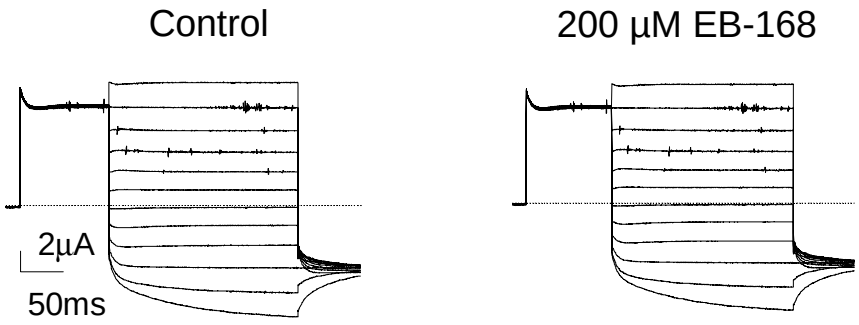


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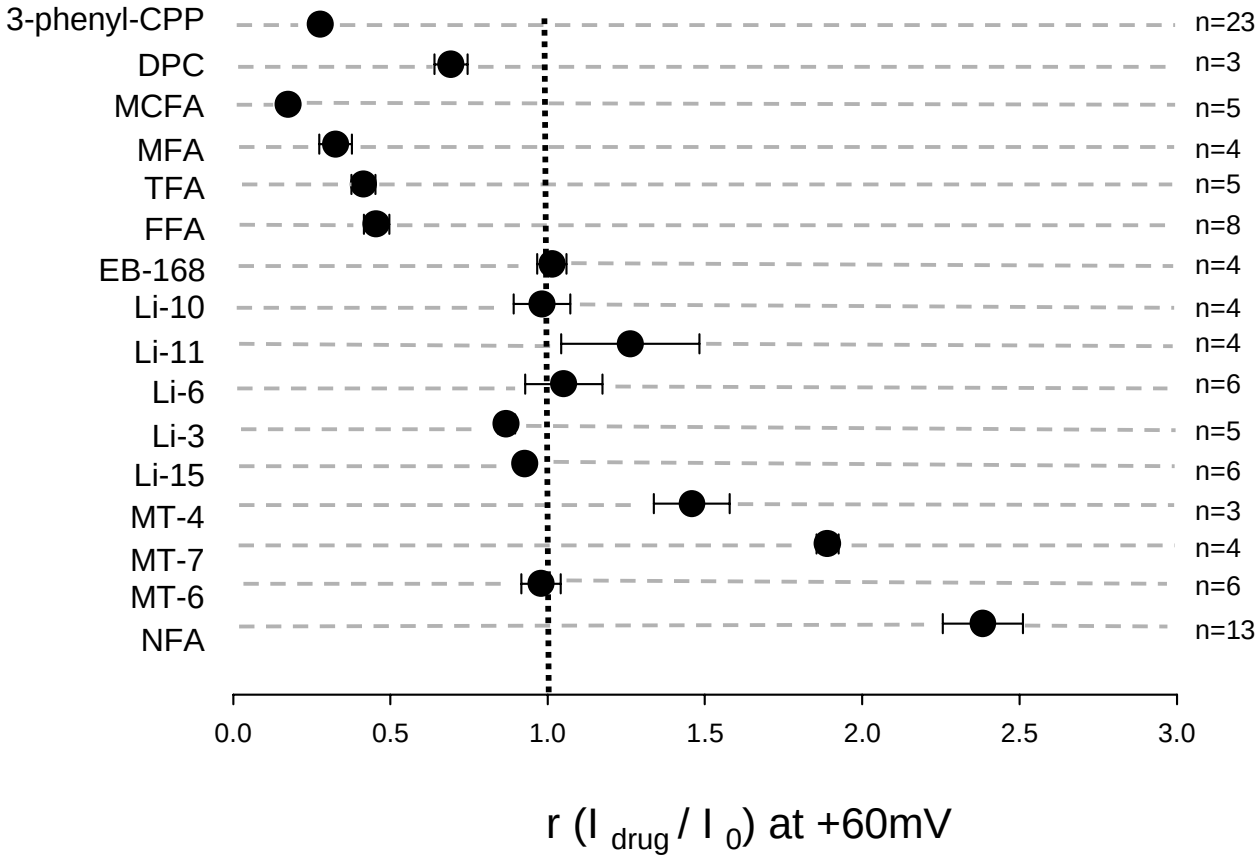
Figure 5

A



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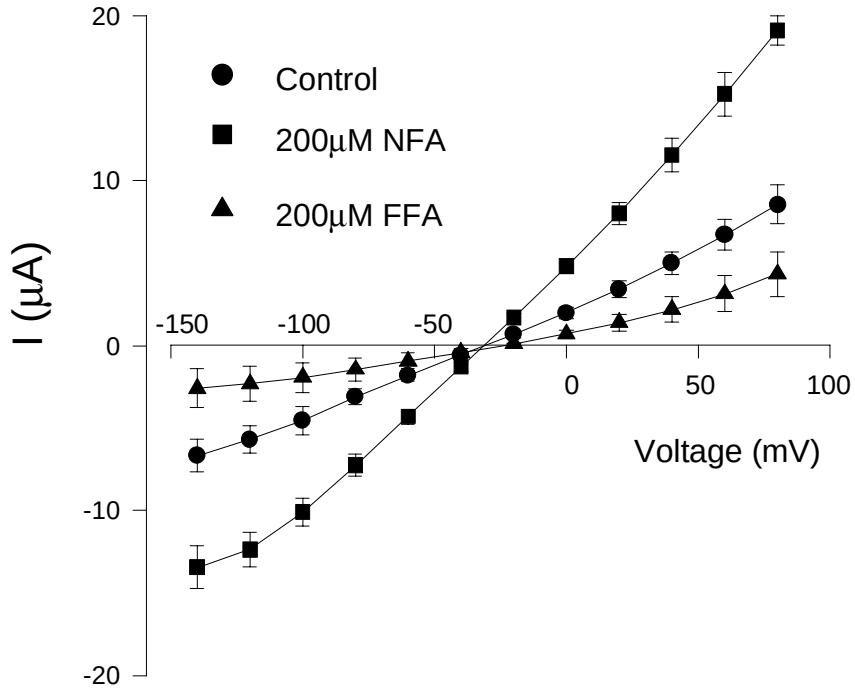

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Figure 6

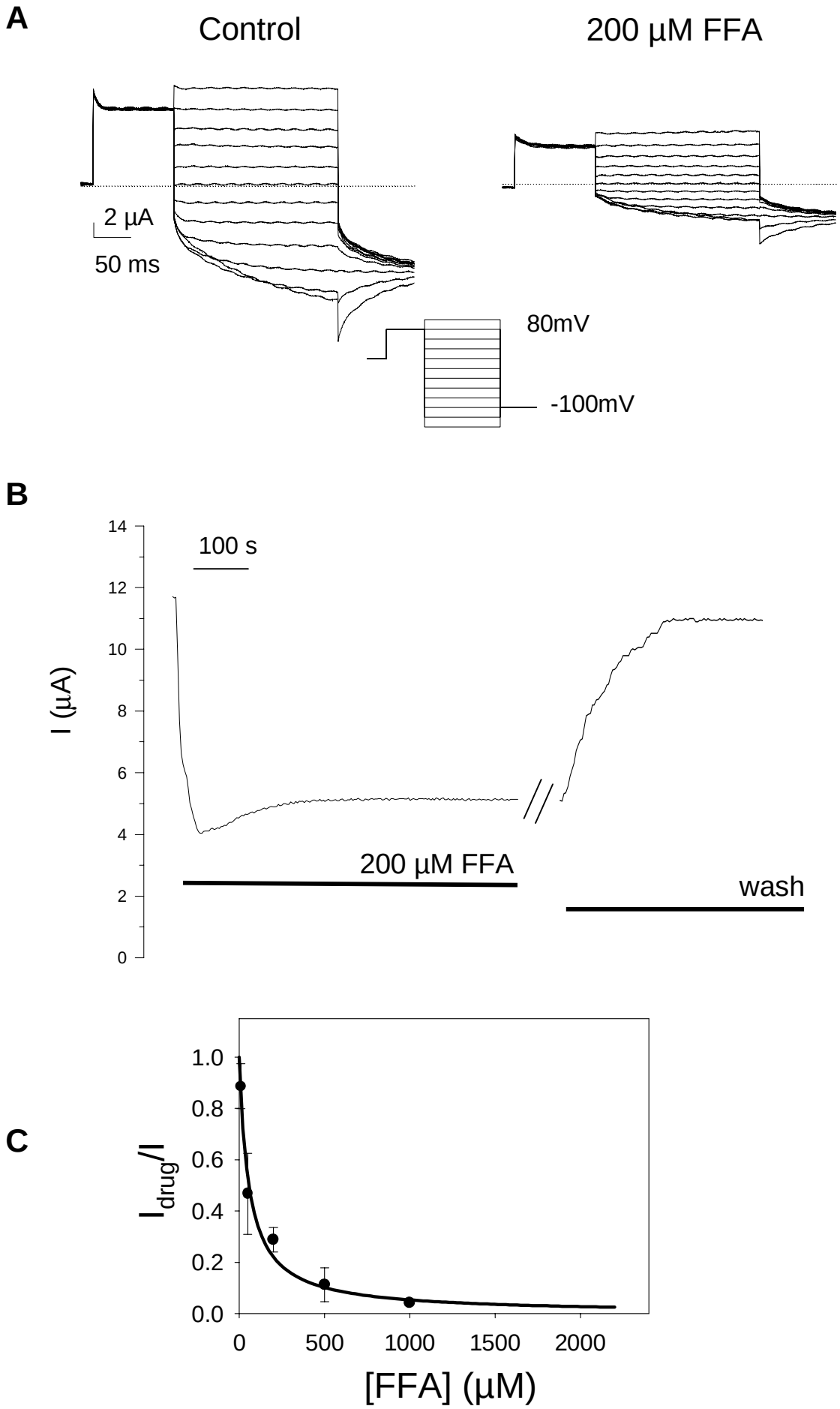

Figure 7

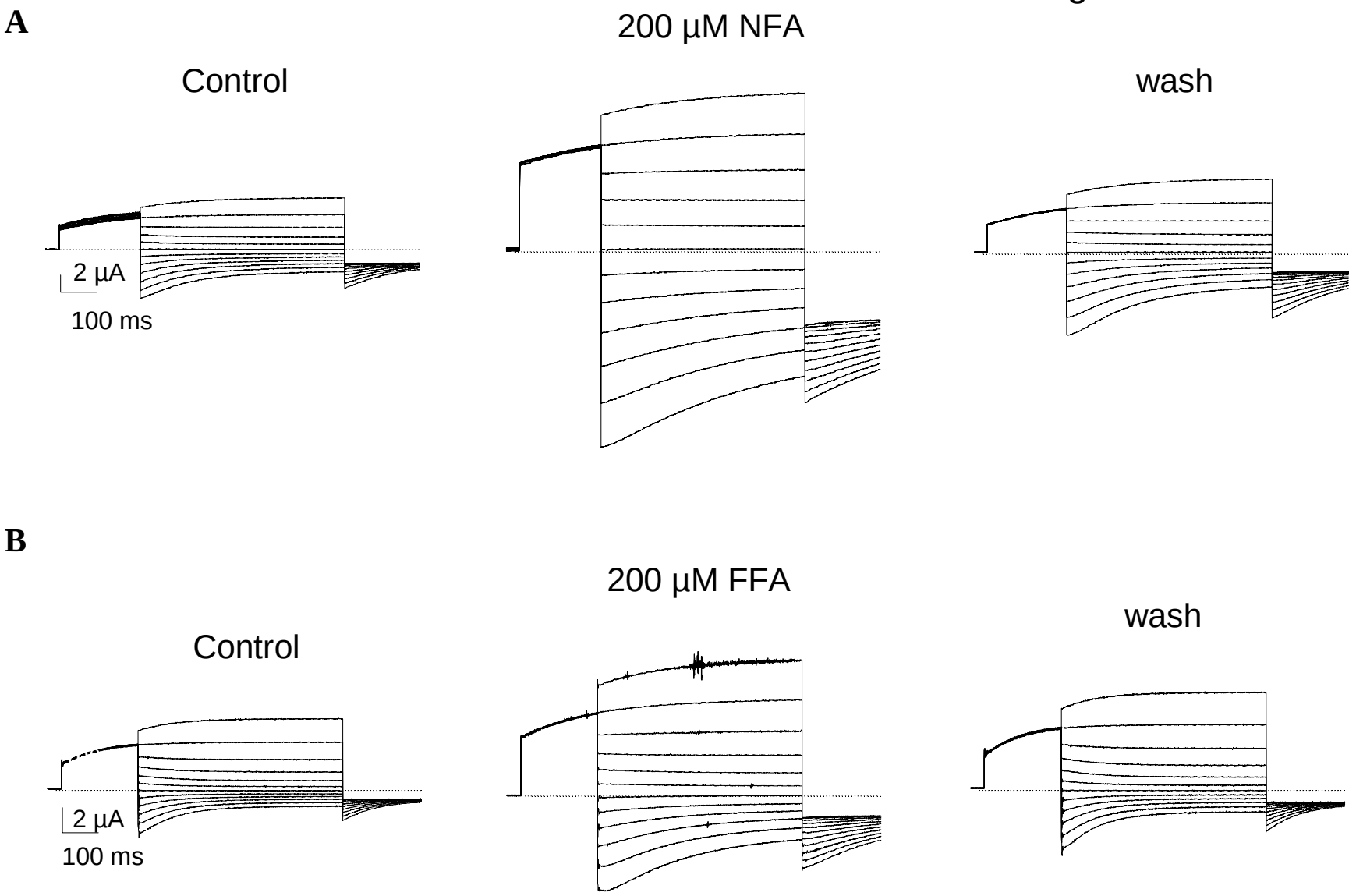

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Figure 8

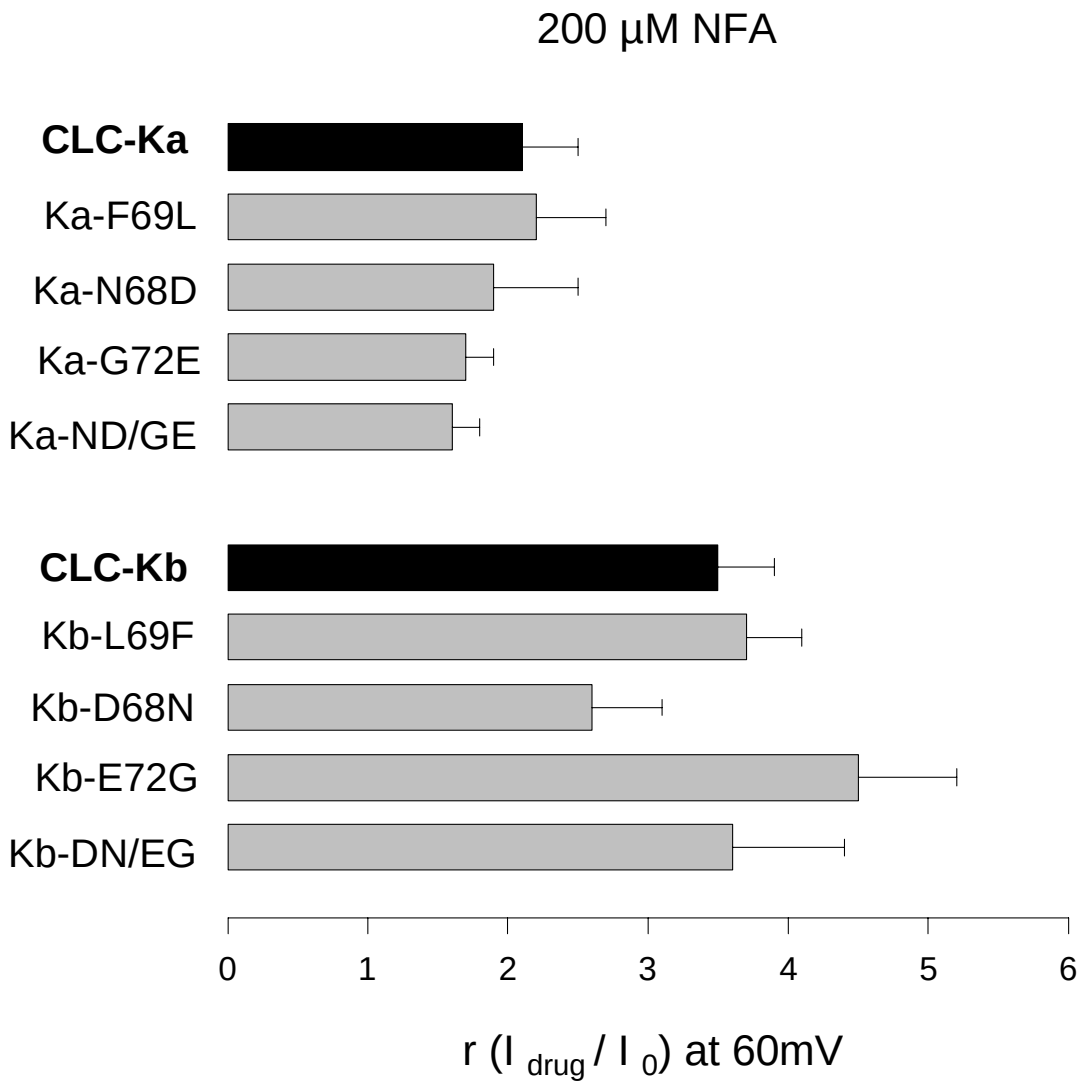

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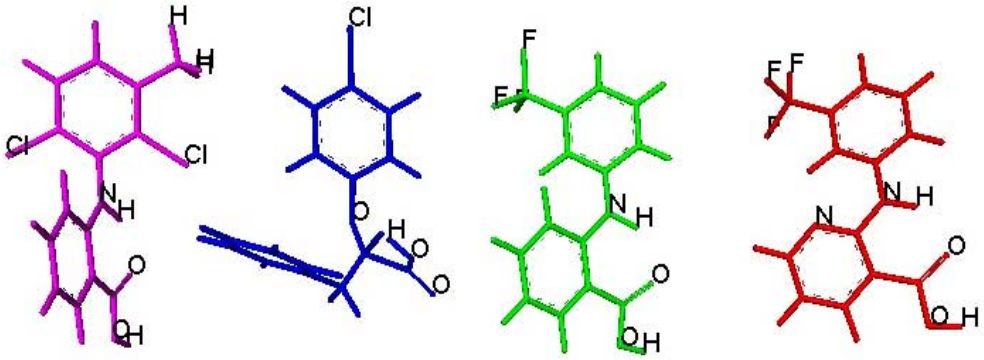
Figure 9

A



MCFA 3-phenyl-CPP FFA NFA

B