

# Activation and Inhibition of Kidney CLC-K Chloride Channels by Fenamates

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## ABSTRACT

CLC-K Cl<sup>-</sup> 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 that controls channel opening. Using the voltage-clamp technique on channels expressed in *Xenopus laevis* oocytes, we found that niflumic acid (NFA) increases CLC-Ka and CLC-Kb currents in the 10 to 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, whereas block requires a noncoplanar configuration. These molecules provide a starting point for identification of diuretics or drugs useful in the treatment of Bartter's syndrome.

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 (Jentsch et al., 2002; Uchida and Sasaki, 2005). The significance of these channels for salt homeostasis is illustrated by genetic disorders (Jentsch, 2005). Defects in the gene encoding CLC-Kb reduce channel 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, whereas CLC-Ka-mediated Cl<sup>-</sup> 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).

It is noteworthy that a polymorphism of CLC-Kb has recently been reported that confers a gain of function of the

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**ABBREVIATIONS:** CLC-K, kidney CLC chloride channels; 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; WT, wild type.

channel-activating CLC-Kb activity (Jeck et al., 2004a) in vitro and predisposes to hypertension in vivo (Jeck et al., 2004b).

The involvement of these Cl<sup>-</sup> channels in various physiological processes arouses 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.

In previous studies, we have identified compounds with two chlorophenoxy groups capable of blocking rat CLC-K1 currents (Liantonio et al., 2002). Subsequently, we recognized 3-phenyl-CPP as the minimal structure capable of blocking CLC-K1 and CLC-Ka in a reversible and Cl<sup>-</sup> ion-dependent manner by interacting with the channel pore from the extracellular side (Liantonio et al., 2004). The highly homologous CLC-Kb channel was found to be 5-fold less sensitive to 3-phenyl-CPP compared with CLC-Ka (Picollo et al., 2004). We identified the amino acid residues that are responsible for the difference in drug sensitivity and thus probably form part of the drug-binding site. In particular, asparagine 68 in CLC-Ka plays a pivotal role for the blocking activity of 3-phenyl-CPP, because exchanging it with a negatively charged aspartate (N68D), as found in CLC-Kb, markedly reduces drug sensitivity. Furthermore, Asn68, together with Gly72, 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).

In this study, we demonstrate that in addition to this blocking binding site, CLC-K channels also present 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 nonsteroidal 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, provides 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.

## Materials and Methods

**Expression in *Xenopus laevis* Oocytes and Voltage-Clamp Analysis.** WT CLC-Ka, CLC-Kb and their mutants, obtained as described previously (Picollo et al., 2004), were coexpressed with the activating mutant Y98A of human barttin (Estévez et al., 2001). Expression in oocytes and electrophysiological measurements were performed as described previously (Pusch et al., 2000). In brief, 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 GmbH, Tamm, Germany). Currents were recorded in the standard solution containing 90 mM NaCl, 10 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES at pH 7.3. In experiments with low extracellular [Ca<sup>2+</sup>], 1.8 mM CaCl<sub>2</sub> 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 washout 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<sup>-</sup> 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<sup>-</sup> 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. Errors in figures and in the text are indicated as S.E.M.

**NFA Derivatives.** The following drugs were purchased from Sigma-Aldrich (Milano-Italy): NFA, FFA, mefenamic acid (MFA), meclofenamic acid (MCFA), tolfenamic acid (TFA), and 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 phenoxy pyridine-carboxylic acids (Li-3, Li-10, Li-6, and Li-11) were synthesized by condensing 2-chloronicotinic acid with the opportune sodium phenoxide using an excess of the phenol (Villani et al., 1975). For Li-15, 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 borane methyl sulfide complex in dry tetrahydrofuran. Compounds were daily prepared in dimethyl sulfoxide stock solutions, and the final concentrations were obtained by appropriate dilution with the solution used for the electrophysiological recordings. (See Fig. 3 for structures of compounds listed in this paragraph.)

**Modeling Study.** We searched the lowest energy conformers for each drug by a systematic Merck Molecular Force Field analysis neglecting solvent presence. A restricted number of conformers (Table 1) were found for the fenamates in the range of 10 kcal/mol. Results were similar to those reported previously (Dhanaraj and Vijayan, 1988). 3-Phenyl-CPP, a more flexible molecule, showed a larger number of conformers (Table 1) in the same range of energy. For FFA, MCFA, NFA, and 3-phenyl-CPP, the same conformational analysis was also carried out 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. 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 Cambridge Crystallographic Data Center (Cambridge Structural Database, ver 5.26).

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

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 <sub>2</sub> O	With H <sub>2</sub> O
FFA	8	18
MCFA	6	13
NFA	11	12
3-Phenyl-CPP	36	50

submitted for further calculations (Spartan '04; Wavefunction, Inc., Irvine, CA). Except for molecular mechanics and semiempirical models, the calculation methods used in Spartan '04 have been documented by 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 shown 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.

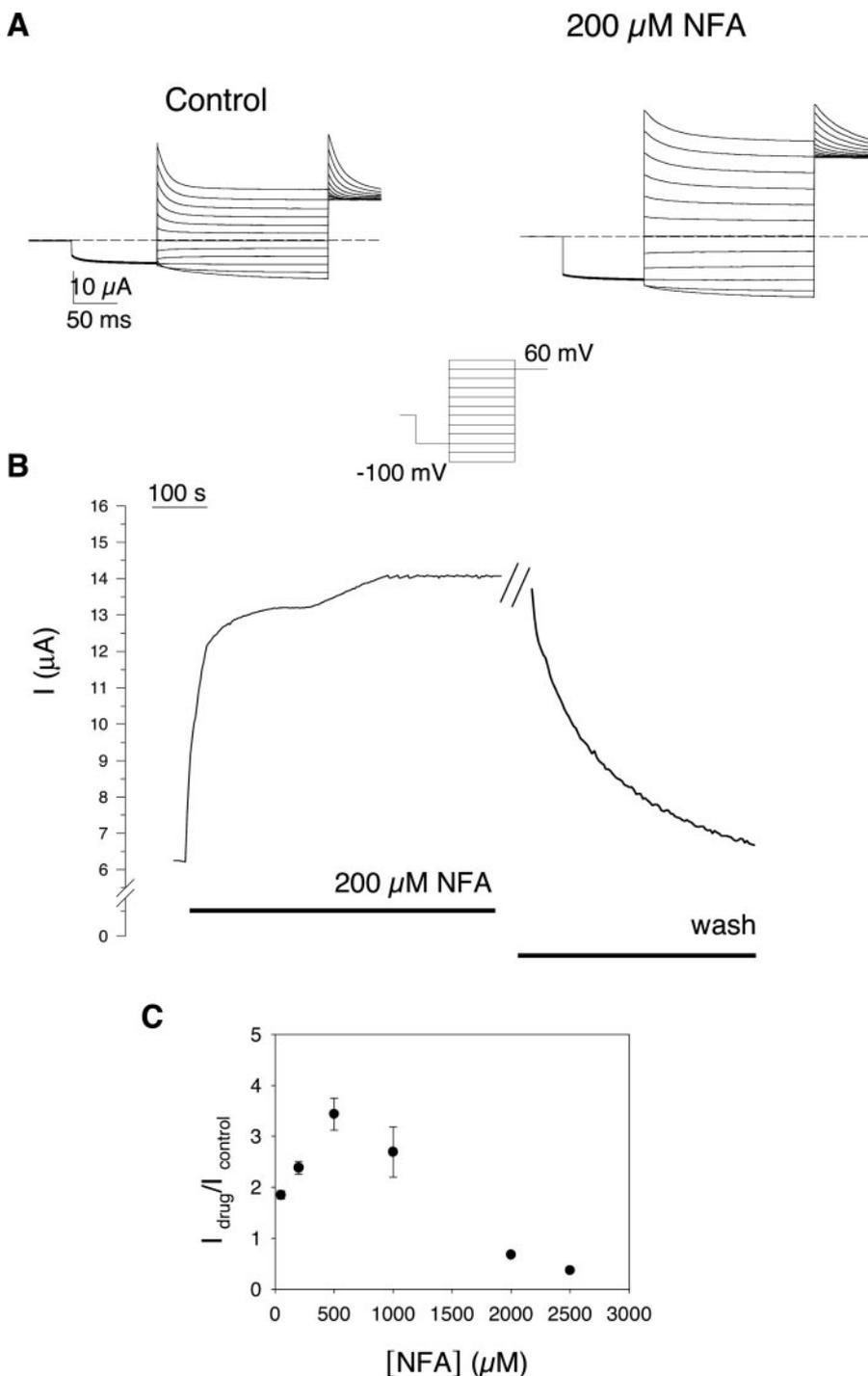
All calculations were performed with the Spartan '04 package (Wavefunction Inc.). Graphical representations were performed by DS Viewerpro 6.0 trial version (Accelrys Inc., San Diego, CA).

## 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 potentials and activate at negative potentials (Fig. 1A, left).

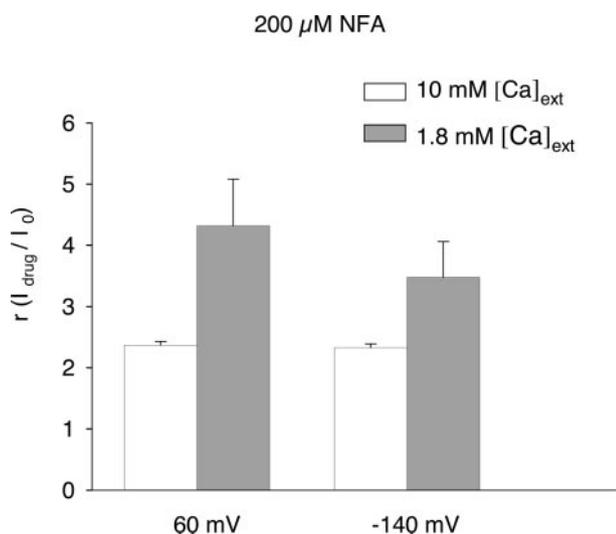
Application of 200  $\mu\text{M}$  NFA induces an increase of CLC-Ka currents both at negative and positive potentials (Fig. 1A). The onset of the effect was rapid, and the return of the currents to control value upon removal of drug was reached within 7 to 9 min (Fig. 1B). The NFA-mediated current in-



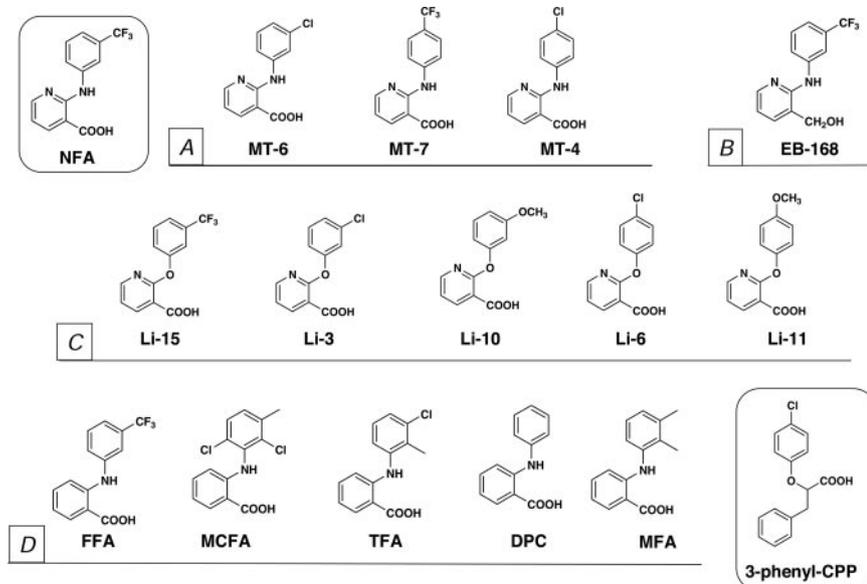
**Fig. 1.** Effect of NFA on CLC-Ka. **A**, voltage-clamp traces of CLC-Ka currents before and during application of 200  $\mu\text{M}$  NFA. The pulse protocol is shown as inset. After a prepulse to -100 mV, voltage was stepped from -140 to 80 mV in 20-mV increments for 200 ms. **B**, time course of 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.

crease was practically voltage-independent ( $I_{\text{drug}}/I_0 = 2.38 \pm 0.12$  at 60 mV and  $I_{\text{drug}}/I_0 = 2.20 \pm 0.3$  at  $-140$  mV). At all tested potentials, NFA induced an increase of the current amplitude without producing kinetic modifications. Although a current increase was seen for concentrations between 50 and 1000  $\mu\text{M}$ , 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 and probably involves 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  $[\text{Ca}^{2+}]_{\text{ext}}$  from 10 to 1.8 mM reduces CLC-Ka currents (data not shown) and, importantly, the relative activating effect of 200  $\mu\text{M}$  NFA was significantly larger in low calcium (Fig. 2). A possible inter-



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



**Fig. 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 comparison, the 3-phenyl-CPP molecule is shown at lower right.

pretation of increased potency of NFA in low calcium is that in this condition, a larger population of channels is available for opening by NFA.

**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  $\mu\text{M}$ , a concentration at which the NFA-mediated activating effect was evident.

**Substitutions on the Phenyl Group.** Derivative MT-7, in which the  $\text{CF}_3$  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 ( $I_{\text{drug}}/I_0$  ratio of  $1.89 \pm 0.04$ ; Fig. 4A). Substitution of the  $\text{CF}_3$  group with a chlorine atom compromised the activating drug activity much more drastically. Indeed, application of derivatives MT-6 and MT-4, which have a chlorine atom in the *meta* or *para* position, respectively, only slightly increased or produced no 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 3-fluoromethyl 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 hydroxymethyl group. Application of derivative EB-168 at 200  $\mu\text{M}$  produced no 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, and Li-11 in Fig. 3) at 200  $\mu\text{M}$  produced no significant change in CLC-Ka currents (Fig. 5A). To exclude

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  $\mu\text{M}$ . If block at 200  $\mu\text{M}$  had masked an activating effect, an increase of currents would be expected at the lower concentration of 50  $\mu\text{M}$ . However, none of these derivatives produced a significant effect at this concentration (data not shown).

**Conversion of the Pyridinic Ring into a Phenyl Ring.** In FFA, the pyridinic ring is substituted with a phenyl ring (Fig. 3). It is noteworthy that 200  $\mu\text{M}$  FFA blocked CLC-Ka-sustained outward and inward currents in a dose-dependent manner (Fig. 5B and 6A) with a  $I_{\text{drug}}/I_0$  ratio of  $0.45 \pm 0.04$  at 60 mV (Fig. 6B). Onset of the effect as well as washout were quite rapid (Fig. 6B). The dose-response curve was well-fitted by a simple titration curve at -140 and 60 mV, with apparent  $K_D$  values reported in Table 2, suggesting 1:1 binding.

Although the potentiation of CLC-Ka by NFA was dependent on the presence of a  $\text{CF}_3$  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. In particular, the order of potency of FFA derivatives was  $\text{MCFA} > \text{MFA} > \text{TFA} > \text{FFA}$  (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 group completely shifted the affinity of NFA from an activating binding site to

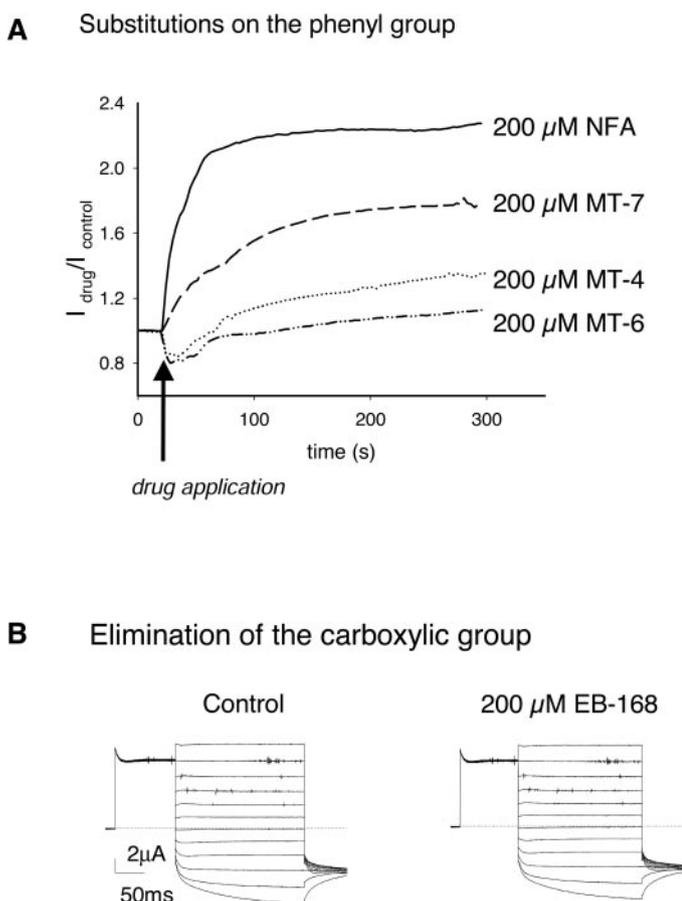
an inhibitory binding site with some different structural requirements.

We also evaluated the effect of 3-phenyl-CPP, the CLC-K blocker with highest known affinity (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.

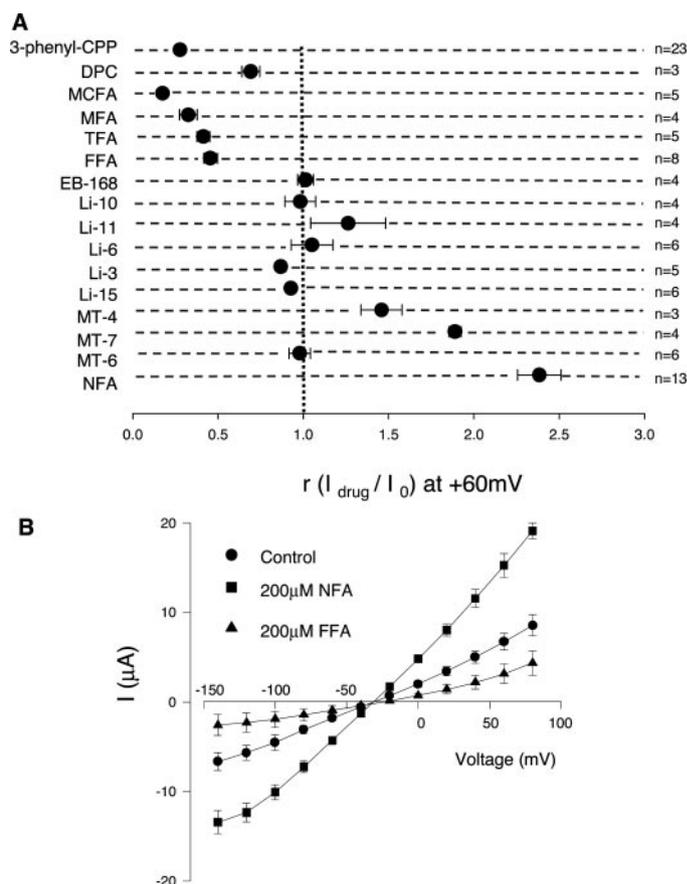
**Effect of Fenamates on CLC-Kb.** Although highly homologous (Kieferle et al., 1994), the two CLC-K isoforms show a different pharmacological profile (Picollo et al., 2004). We evaluated the sensitivity of CLC-Kb to fenamates using NFA and FFA, the two lead compounds with activating and inhibitory effect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

It is noteworthy that in contrast to what we observed with CLC-Ka, application of 200  $\mu\text{M}$  FFA produced an increase of CLC-Kb currents with a  $I_{\text{drug}}/I_0$  ratio of  $2.09 \pm 0.9$  at 60 mV (Fig. 7). Furthermore, even at high concentrations (>1 mM), NFA was incapable of blocking CLC-Kb sustained currents, producing a potentiation at all tested concentrations (data not shown).

**Effect of NFA and FFA on CLC-K Mutants.** We have previously identified Asn68 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.,



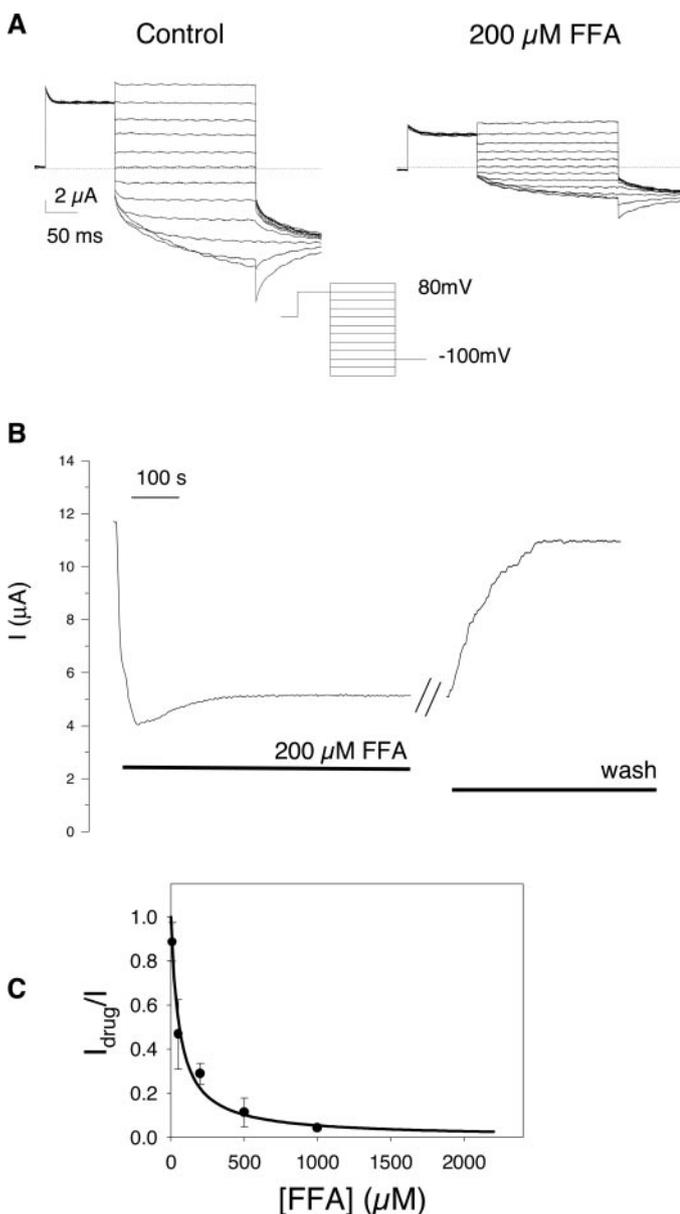
**Fig. 4.** Substitutions on the phenyl group. A, ratio of the current in the presence and absence of compound is plotted versus time. The arrow indicates application of compounds. B, voltage-clamp traces before and during application of 200  $\mu\text{M}$  EB-168.



**Fig. 5.** A, structure-activity relationship study: each point represents the average of the  $I_{\text{drug}}/I_0$  ratio 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  $\mu\text{M}$  NFA and 200  $\mu\text{M}$  FFA. Each point represents the average of determinations obtained from four to six oocytes.

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 assess 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). FFA (200  $\mu\text{M}$ ) produced no 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 compounds tested here on CLC-Ka, showed a  $K_D$  of  $632 \pm 101 \mu\text{M}$ , a decrease in affinity of almost 15-fold compared with WT. For all other FFA derivatives, the apparent  $K_D$  value was  $>1 \text{ mM}$  (Table 2).

We next tested NFA on all mutants previously reported



**Fig. 6.** Effect of FFA on CLC-Ka. A, voltage-clamp traces of CLC-Ka currents before and during application of 200  $\mu\text{M}$  FFA. B, time course of the CLC-Ka current blocked by 200  $\mu\text{M}$  FFA. Arrow indicates the addition of drug. C, dose-response relationship of the block at 60 mV by FFA for CLC-Ka. The line is drawn according to the equation  $I(c)/I(0) = 1/(1 + c/K_D)$ .

both for CLC-Ka and CLC-Kb (Picollo et al., 2004). Each mutant was sensitive to NFA with a potency comparable with that observed in the related wild type (Fig. 8). However, it is interesting that NFA produced a slightly smaller 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 ( $I_{\text{drug}}/I_0 = 0.75 \pm 0.05$  and  $0.67 \pm 0.04$  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 that is different 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 because of the substitution of the pyridine system with a phenyl system; at the same time, this substitution introduces an additional hydrogen atom in the *ortho* position to the amino group, causing a steric hindrance in the resulting diphenyl system that forces the noncoplanar 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 because of their molecular flexibility. An overlay of the lowest energy conformers of the lead molecules indicates that all

TABLE 2

Isosteric derivatives of the pyridinic nitrogen

$K_D$  values are expressed as mean  $\pm$  S.E.M. calculated for each compound.  $n$  indicates number of oocytes.

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

blocking compounds, such as FFA and 3-phenyl-CPP, displayed nonplanar geometries in contrast to NFA (Fig. 9B).

## Discussion

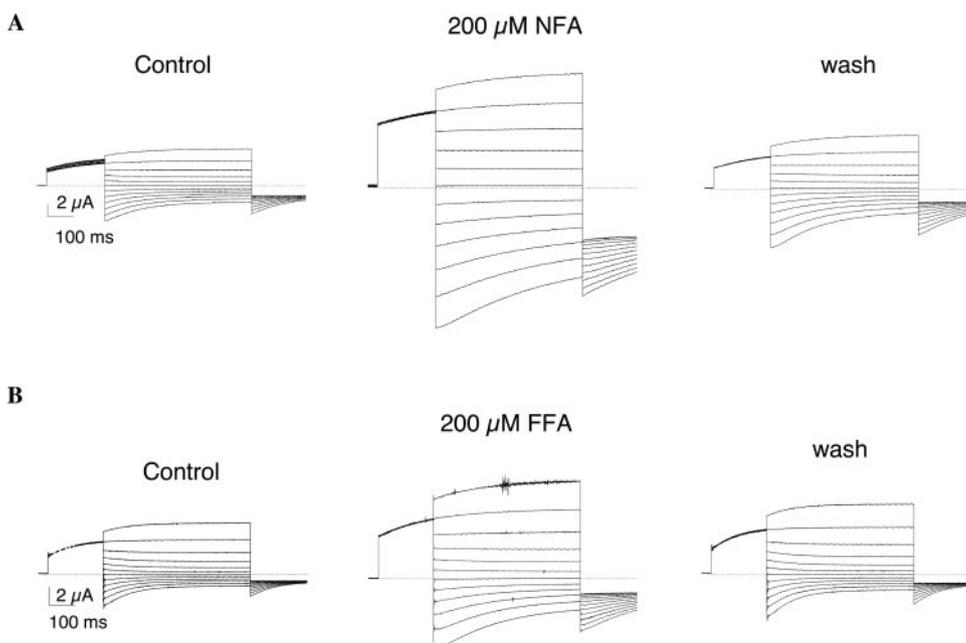
In the present work, we demonstrated that in addition to a blocking site (Picollo et al., 2004), CLC-K chloride channels also have an activating binding site. Depending on the chemical structure, fenamates are capable of blocking or opening CLC-Ka. NFA is able to increase CLC-Ka currents at all tested membrane potentials in the 10 to 1000  $\mu\text{M}$  range with a rapid onset 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 because under these experimental conditions, 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 should be a pyridinic ring), a  $\text{CF}_3$  group in the *meta* position on the phenyl group, and an anilinic moiety connecting the two rings. Combinations of these requisites 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. It is noteworthy that 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 was 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.

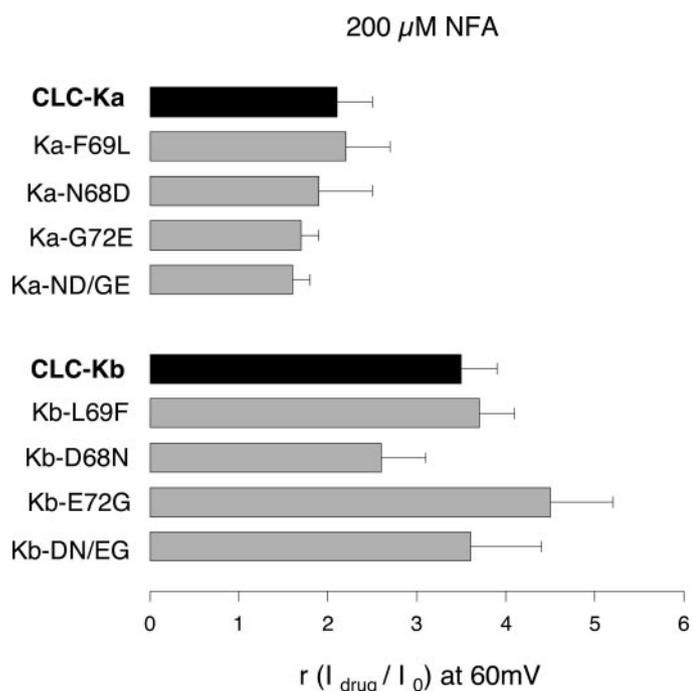
The fact that NFA at high concentration is able to block CLC-Ka and that FFA is able to open CLC-Kb indicates that both drugs can bind the activating and blocking binding sites but with different affinity. The presence of a pyridinic group favors binding to the activating binding site, although it cannot be excluded that a certain drug amount binds the inhibitory binding site at low concentrations. In support of this, the activating effect of 200  $\mu\text{M}$  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, 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 Asn68 as crucial for 3-phenyl-CPP-mediated inhibition, because mutating it to the negatively charged aspartate (N68D), as occurs in CLC-Kb, markedly reduced drug sensitivity (Picollo et al., 2004). All FFA derivatives were much less potent in blocking CLC-Ka N68D mutant with respect to CLC-Ka wild type, indicating that Asn68 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. At high concentrations, NFA was able to block CLC-Ka N68D mutant with only slightly less potency with respect to wild type. This might suggest that the inhibitory effect of NFA and FFA is mediated by different blocking binding sites. On the other hand, considering the possibility of a unique blocking binding site, the presence of the pyridinic ring could account for the dif-

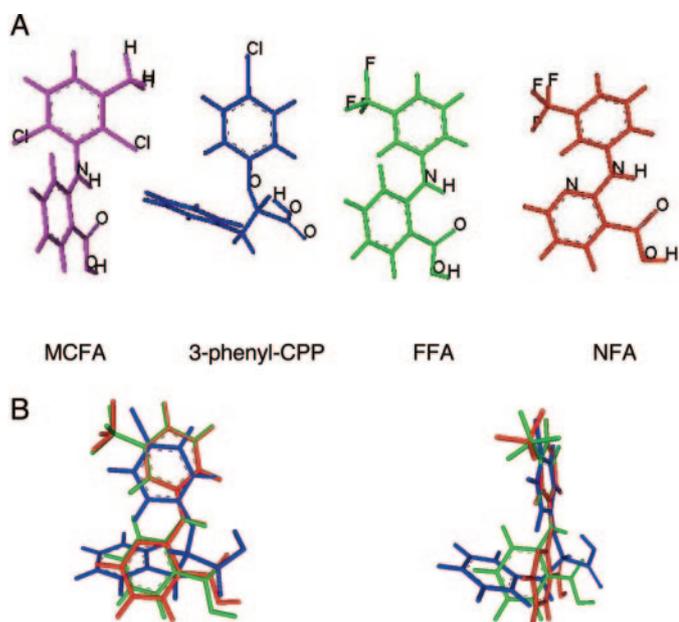


**Fig. 7.** Effect of NFA and FFA on CLC-Kb. Voltage-clamp traces of CLC-Kb currents before and during application of 200  $\mu\text{M}$  NFA (A) and 200  $\mu\text{M}$  FFA (B) and immediately after washout.

ferent behavior of NFA with respect to FFA on CLC-Ka N68D mutant. In fact, the reduced activity of FFA on mutant N68D is probably caused by 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.



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



**Fig. 9.** Modeling study of the lead compounds. A, lowest energy conformation of MCFA, 3-phenyl-CPP, FFA, and NFA obtained as described under *Materials and 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 under *Materials and Methods*.

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 described previously 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. Overall, these data indicate the presence of two different binding sites mediating the activating and blocking effects. 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 sites 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 allowed 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 analogs MT-4 and MT-7 that behave as CLC-Ka openers show nearly planar conformations, whereas FFA derivatives and 3-phenyl-CPP, exhibiting a CLC-Ka blocking activity, are forced to assume a noncoplanar arrangement of the aromatic rings. Overlaying the lowest energy conformers reveals that all blocking compounds display nonplanar geometries compared with NFA and that the increasing planarity distortion of the aromatic rings ranging from DPC, FFA, MFA, and TFA to MCFA parallels the observed increasing blocking activity in the same order.

The involvement of CLC-Kb in type III Bartter's 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 elevated prostaglandin activity (Reinalter et al., 2002), and NFA belongs to the class of nonsteroidal 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). From this regard, it noteworthy 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 targets via the basolateral fluid. This means that effective concentrations must 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, a polymorphism of CLC-Kb was recently 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 (Kokubo et al., 2005; Speirs 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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#### References

- Akizuki N, Uchida S, Sasaki S, and Marumo F (2001) Impaired solute accumulation in inner medulla of CLCNK1  $-/-$  mice kidney. *Am J Physiol* **280**:F79–F87.
- Birkenhäger R, Otto E, Schurmann MJ, Völlmer M, Ruf E-M, Maier-Lutz I, Beekmann F, Fekete A, Omran H, Feldmann D, et al. (2001) Mutation of BSND causes Bartter syndrome with sensorineural deafness and kidney failure. *Nat Genet* **29**:310–314.
- Dhanaraj V and Vijayan M (1988) Structural studies of analgesics and their interactions. XII. Structure and interactions of anti-inflammatory fenamates. A concerted crystallographic and theoretical conformational study. *Acta Crystallogr B* **44**:406–412.
- Estévez R, Boettger T, Stein V, Birkenhäger R, Otto E, Hildebrandt F, and Jentsch TJ (2001) Barttin is a Cl channel beta-subunit crucial for renal Cl reabsorption and inner ear K secretion. *Nature (Lond)* **414**:558–561.
- Fong P (2004) CLC-K channels: if the drug fits, use it. *EMBO (Eur Mol Biol Organ) Rep* **5**:565–566.
- Fujiwara H and Kitagawa K (2000) Synthesis of pyridopyranquinolines by the Skraup reaction of amino-5H-benzopyrano[2,3-b]pyridine-5-ones. *Heterocycles* **53**:409–417.
- Jeck N, Waldegger P, Doroszewicz J, Seyberth H, and Waldegger S (2004a) A common sequence variation of the CLCNKB gene strongly activates ClC-Kb chloride channel activity. *Kidney Int* **65**:190–197.
- Jeck N, Waldegger S, Lampert A, Boehmer C, Waldegger P, Lang PA, Wissinger B, Friedrich B, Risler T, Moehle R, et al. (2004b) Activating mutation of the renal epithelial chloride channel ClC-Kb predisposing to hypertension. *Hypertension* **43**:1175–1181.
- Jentsch TJ (2005) Chloride transport in the kidney: lessons from human disease and knockout mice. *J Am Soc Nephrol* **16**:1549–1561.
- Jentsch TJ, Stein V, Weinreich F, and Zdebik AA (2002) Molecular structure and physiological function of chloride channels. *Physiol Rev* **82**:503–568.
- Kieferle S, Fong P, Bens M, Vandewalle A, and Jentsch TJ (1994) Two highly homologous members of the ClC chloride channel family in both rat and human kidney. *Proc Natl Acad Sci USA* **91**:6943–6947.
- Kokubo Y, Iwai N, Tago N, Inamoto N, Okayama A, Yamawaki H, Naraba H, and Tomoike H (2005) Association analysis between hypertension and CYBA, CLC-NKB and KCNMB1 functional polymorphisms in the Japanese population—the Suita Study. *Circ J* **69**:138–142.
- Kong J, White CA, Krylov AI, Sherrill CD, Adamson RD, Furlani TR, Lee MS, Lee AM, Gwaltney SR, Adams TR, et al. (2000) Q-Chem 2.0: a high-performance *ab initio* electronic structure program package. *J Computational Chem* **21**:1532–1548.
- Liantonio A, Accardi A, Carbonara G, Fracchiolla G, Loidice F, Tortorella P, Traverso S, Guida P, Pierno S, De Luca A, et al. (2002) Molecular requisites for drug binding to muscle CLC-1 and renal CLC-K channel revealed by the use of phenoxyalkyl derivatives of 2-(p-chlorophenoxy)propionic acid. *Mol Pharmacol* **62**:265–271.
- Liantonio A, Pusch M, Piccolo A, Guida P, De Luca A, Pierno S, Fracchiolla G, Loidice F, Tortorella P, and Conte Camerino D (2004) Investigations of pharmacologic properties of the renal CLC-K1 chloride channel co-expressed with barttin by the use of 2-(p-chlorophenoxy)propionic acid derivatives and other structurally unrelated chloride channels blockers. *J Am Soc Nephrol* **15**:13–20.
- Piccolo A, Liantonio A, Didonna MP, Elia L, Camerino DC, and Pusch M (2004) Molecular determinants of differential pore blocking of kidney CLC-K chloride channels. *EMBO (Eur Mol Biol Organ) Rep* **5**:584–589.
- Pusch M, Liantonio A, Bertorello L, Accardi A, De Luca A, Pierno S, Tortorella V, and Conte Camerino D (2000) Pharmacological characterization of the chloride channels belonging to the ClC family by the use of chiral clofibrate acid derivatives. *Mol Pharmacol* **58**:498–507.
- Reinalter SC, Jeck N, Brochhausen C, Watzter B, Nusing RM, Seyberth HW, and Komhoff M (2002) Role of cyclooxygenase-2 in hyperprostaglandin E syndrome/antenatal Bartter syndrome. *Kidney Int* **62**:253–260.
- Schlingmann KP, Konrad M, Jeck N, Waldegger P, Reinalter SC, Holder M, Seyberth HW, and Waldegger S (2004) Salt wasting and deafness resulting from mutations in two chloride channels. *N Engl J Med* **350**:1314–1319.
- Sherlock MH, Kaminski JJ, Tom WC, Lee JF, Wong S-C, Kreutner W, Bryant RW, and McPhail AT (1988) Antiallergy agents. 1. Substituted 1,8-naphthyridin-2(1H)-ones as inhibitors of SRS-A release. *J Med Chem* **31**:2108–2121.
- Simon DB, Bindra RS, Mansfield TA, Nelson-Williams C, Mendonca E, Stone R, Schurman S, Nayir A, Alay H, Bakkaloglu A, et al. (1997) Mutations in the chloride channel gene, CLCNKB, cause Bartter's syndrome type III. *Nat Genet* **17**:171–178.
- Speirs HJ, Wang WY, Benjafield AV, and Morris BJ (2005) No association with hypertension of CLCNKB and TNFRSF1B polymorphisms at a hypertension locus on chromosome 1p36. *J Hyperten* **23**:1491–1496.
- Uchida S and Sasaki S (2005) Function of chloride channels in the kidney. *Annu Rev Physiol* **67**:759–778.
- Uchida S, Sasaki S, Nitta K, Uchida K, Horita S, Nihei H, and Marumo F (1995) Localization and functional characterization of rat kidney-specific chloride channel, ClC-K1. *J Clin Invest* **95**:104–113.
- Vandewalle A, Cluzeaud F, Bens M, Kieferle S, Steinmeyer K, and Jentsch TJ (1997) Localization and induction by dehydration of ClC-K chloride channels in the rat kidney. *Am J Physiol* **272**:F678–F688.
- Villani FJ, Mann TA, Wefer EA, Hannon J, Larca LL, Landon MJ, Spivak W, Vashi D, Tozzi S, Danko G, et al. (1975) Benzopyranopyridine derivatives. 1. Aminoalkyl derivatives of the azaxanthenes as bronchodilating agents. *J Med Chem* **18**:1–8.
- Waldegger S, Jeck N, Barth P, Peters M, Vitzthum H, Wolf K, Kurtz A, Konrad M, and Seyberth HW (2002) Barttin increases surface expression and changes current properties of CLC-K channels. *Pflug Arch Eur J Physiol* **444**:411–418.

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