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RESEARCH PAPER

Identification of sites responsible for the potentiating effect of niflumic acid on ClC-Ka kidney chloride channels

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Background and purpose: ClC-K kidney Cl^- channels are important for renal and inner ear transepithelial Cl^- transport, and are potentially interesting pharmacological targets. They are modulated by niflumic acid (NFA), a non-steroidal anti-inflammatory drug, in a biphasic way: NFA activates ClC-Ka at low concentrations, but blocks the channel above ~1 mM. We attempted to identify the amino acids involved in the activation of ClC-Ka by NFA.
Experimental approach: We used site-directed mutagenesis and two-electrode voltage clamp analysis of wild-type and mutant channels expressed in *Xenopus* oocytes. Guided by the crystal structure of a bacterial CLC homolog, we screened 97 ClC-Ka mutations for alterations of NFA effects.
Key results: Mutations of five residues significantly reduced the potentiating effect of NFA. Two of these (G167A and F213A) drastically altered general gating properties and are unlikely to be involved in NFA binding. The three remaining mutants (L155A, G345S and A349E) severely impaired or abolished NFA potentiation.
Conclusions and implications: The three key residues identified (L155, G345, A349) are localized in two different protein regions that, based on the crystal structure of bacterial CLC homologs, are expected to be exposed to the extracellular side of the channel, relatively close to each other, and are thus good candidates for being part of the potentiating NFA binding site. Alternatively, the protein region identified mediates conformational changes following NFA binding. Our results are an important step towards the development of ClC-Ka activators for treating Bartter syndrome types III and IV with residual channel activity.
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Keywords: CLC-K channels; Bartter syndrome; diuretic; chloride channel; niflumic acid; kidney
Abbreviations: NFA, niflumic acid

Introduction

ClC-K proteins (ClC-Ka and -Kb in humans, and their rodent orthologues ClC-K1 and -K2) (Uchida *et al.*, 1993; Adachi *et al.*, 1994; Kieferle *et al.*, 1994) are Cl^- channels forming a distinct branch of the CLC protein family (Zifarelli and Pusch, 2007) that are expressed in the kidney and the inner ear (Jentsch, 2005; Uchida and Sasaki, 2005). They both co-express with an auxiliary β subunit, barttin (Estévez *et al.*, 2001), which regulates their plasma membrane localization and modulates their functional properties (Estévez *et al.*, 2001; Waldegger *et al.*, 2002; Scholl *et al.*, 2006).

From the insurgence of diabetes insipitus in ClC-K1 knock-out mice (Matsumura *et al.*, 1999; Akizuki *et al.*, 2001), it has been inferred that ClC-Ka is a crucial component in the countercurrent system that preserves a correct urine concentration, even though, to date, no mutation in ClC-Ka has been reported to cause a defect in the ability to concentrate urine in humans. Loss-of-function mutations in ClC-Kb result in Bartter syndrome type III (Simon *et al.*, 1997), characterized by massive salt loss due to impaired NaCl reabsorption in the thick ascending limb. In the inner ear, ClC-K channels are important for K^+ secretion into the scala media (Rickheit *et al.*, 2008). This is the reason why patients affected by Bartter syndrome type IV, caused by loss-of-function mutations in barttin, present both renal dysfunction (more severe than in

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Bartter syndrome type III) and deafness (Birkenhäger *et al*., 2001). This also occurs in patients in which both ClC-K isoforms are non-functional, but this is very rare (Schlingmann *et al*., 2004).

From a pharmacological point of view, ClC-K channels are the only members of the CLC family for which a significant advance has been achieved (Liantonio *et al*., 2002; 2004; 2006; 2008; Picollo *et al*., 2004; Pusch *et al*., 2007; Matulef *et al*., 2008). The binding site for the ClC-Ka blockers DIDS, 3-phenyl-CPP and flufenamic acid (FFA), a fenamate derivative, has been identified as being close to N68 (Picollo *et al*., 2004), located in the channel pore. Recently, niflumic acid (NFA), a drug belonging to a class of fenamates used as non-steroidal anti-inflammatory drugs, has been shown to have a very peculiar effect on ClC-K channels. NFA potentiates ClC-Ka currents at concentrations up to ~1 mM, whereas at higher concentrations it has an inhibitory effect (Liantonio *et al*., 2006). NFA is far from being a specific modulator of ClC-K channels. In fact, NFA or other fenamates are able to interact with both Cl^- channels (Qu and Hartzell, 2001; Coyne *et al*., 2007; Greenwood and Leblanc, 2007; Verkman and Galietta, 2008) and Shaker-like cation channels (Busch *et al*., 1994; Abitbol *et al*., 1999; Malykhina *et al*., 2002; Greenwood and Leblanc, 2007; Fernandez *et al*., 2008; Cheng and Sanguinetti, 2009; Foster *et al*., 2009). Detailed studies on HCN hyperpolarization of activated cation channels suggested an interaction of NFA with positive residues of the S4 segment, which leads to an alteration of voltage sensing (Cheng and Sanguinetti, 2009), whereas an interaction motif in the S3–S4 linker was found in eag K^+ channels (Fernandez *et al*., 2008). $GABA_A$ receptor Cl^- channels are strongly activated by mefenamic acid (Coyne *et al*., 2007). Among Cl^- channels, Ca^{2+}-activated Cl^- channels are probably the most sensitive to extracellular NFA (Qu and Hartzell, 2001; Greenwood and Leblanc, 2007). The muscle CLC Cl^- channel, ClC-1, is blocked by intracellular NFA in a voltage-dependent manner (Liantonio *et al*., 2007). However, of the CLC proteins tested (ClC-1, ClC-5, ClC-K), only ClC-K channels have been shown to be sensitive to extracellularly applied NFA (Liantonio *et al*., 2006). Despite a high sequence conservation, the three ClC-K channels that have been tested for NFA modulation show a different response: rat ClC-K1 is blocked in a dose-dependent manner; human ClC-Kb is activated at concentrations up to 2 mM (Liantonio *et al*., 2006); ClC-Ka is potentiated at low concentrations (<~1 mM), but blocked at higher concentrations (Liantonio *et al*., 2006; Picollo *et al*., 2007). Such a different effect on channels that are highly homologous (homology is 91% between –Ka and –Kb, and 83% between –Ka and –K1) argues for a very specific pharmacological profile. To explain the biphasic dependence of ClC-Ka on NFA, Picollo *et al*. (2007) suggested the presence of two distinct binding sites for NFA. The results from Cl^- competition experiments indicated that neither of the two sites is located in the channel pore and, consistently, none of the binding sites for NFA involve N68, the blocking site for FFA and CPP derivatives.

The different action of NFA and FFA on ClC-Ka is related to the different geometrical arrangement of the two aromatic rings in the two molecules. A planar geometry, as in NFA, favours potentiation, whereas a non-planar geometry, endowed with a greater degree of flexibility (as found in FFA), favours block (Liantonio *et al*., 2008).

In order to identify the NFA binding sites, we performed extensive site-directed mutagenesis on ClC-Ka. Of the 97 mutations screened, only five eliminated the potentiating effect of NFA. Two of these (G167A and F213A) drastically altered the general gating properties, such that no conclusion can be established about their role in NFA potentiation. The three remaining mutations (L155A, G345S and A349E) greatly reduced or abolished the potentiating effect of NFA without affecting its blocking ability. L155 is located on the extracellular side of helix E, whereas G345 and A349 are in the loop connecting helices K and L. Based on the crystal structure of the bacterial CLC homologs, these two regions are expected to be exposed to the extracellular side of the channel, relatively close to each other, and are thus good candidates for being part of the potentiating NFA binding site or responsible for conformational changes that follow NFA binding leading to current potentiation.

Methods

Molecular biology and heterologous expression

Mutations were introduced by recombinant PCR as previously described (Accardi and Pusch, 2003). All constructs were in the pTLN vector (Lorenz *et al*., 1996) and were co-expressed with the barttin mutant Y98A which increases expression (Estévez *et al*., 2001). RNA was prepared using the mMessage mMachine SP6 and T7 kits (Applied Biosystems Italia, Monza, Italy) and injected into *Xenopus* oocytes as previously described (Pusch *et al*., 2000).

The ion channel nomenclature used conforms to BJP's *Guide to Receptors and Channels* (Alexander *et al*., 2008).

Electrophysiology

Voltage clamp data were acquired at 21–25°C using the custom acquisition program Gepulse (available at http://www.ge.cnr.it/ICB/conti_moran_pusch/programs_pusch/software-mik.htm) and a TURBO TEC-03X amplifier (npi electronic, Tamm, Germany). Currents were recorded in a bath solution containing 90 mM NaCl, 1 mM $MgCl_2$, 10 mM $CaCl_2$ and 10 mM HEPES at pH 7.3. Different NFA concentrations were prepared by dissolving NFA in dimethyl sulphoxide (DMSO) and diluting the resultant solution in the bath solution. Final DMSO concentration was ≤0.2%. DMSO alone had no effect on ClC-Ka-mediated currents at this concentration. NFA and all other reagents were purchased from Sigma-Aldrich (Milan, Italy). Pulses were elicited from a holding potential corresponding to the resting membrane potential (–30 to –50 mV). The acute effect of NFA was evaluated by applying repetitive pulses to 60 mV of 100 ms duration, and calculating the ratio between the current measured in the presence of NFA and that in control conditions. ClC-K channels are efficiently blocked by extracellular iodide (Liantonio *et al*., 2002). This was exploited to estimate the contribution of endogenous currents and leak by replacing extracellular chloride by iodide and by removing extracellular calcium at the end of the protocol (this solution contained 100 mM NaI,

5 mM $MgSO_4$, 10 mM HEPES, pH 7.3). Outward currents in the presence of iodide were taken as being purely endogenous. The estimated leak currents were subtracted from the control currents in the absence and presence of NFA to calculate the ratio $I(c)/I(0)$, where c is the concentration of NFA.

Non-stationary noise analysis

For patch clamping, the intracellular solution contained (in mM): 100 *N*-methyl-D-glucamine-Cl (NMDG-Cl), 2 $MgCl_2$, 10 HEPES, 2 EGTA, pH 7.3, whereas the extracellular solution contained 90 NMDG-Cl, 10 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.3. Patch pipettes were pulled from aluminosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) and had resistances of 1–2 $\times$ 10^6 ohm in the recording solutions. Recordings were performed in the inside-out configuration. Currents were recorded at 50 kHz after filtering at 10 kHz with an eight-pole Bessel filter. For noise analysis, 30–100 identical pulses to –100 or –140 mV were applied, and the mean response, I, was calculated. The variance, σ^2, was calculated from the averaged squared difference of consecutive traces. Background variance at 0 mV was subtracted, and the variance–mean plot was fitted by $\sigma^2 = iI - I^2/N$, with the single-channel current, i, and the number of channels, N, as free parameters (Pusch *et al*., 1994). The maximal open probability, P_{max}, was estimated by $P_{max} = I_{max}/(N * i)$, where I_{max} is the maximal current.

Statistical analysis

Each mutant screened was expressed and analysed in at least two batches of oocytes, and measurements were performed for at least three oocytes. For all injections, control measurements were performed for wild-type (WT) ClC-Ka. Statistical analysis was performed using Student's unpaired *t*-test. The five mutants mentioned in the Abstract had a highly significantly different response to NFA at 200 μM compared to WT ($P < 0.01$), whereas none of the other mutants was significantly different ($P > 0.05$).

Results

Application of NFA from the extracellular side of the membrane leads to a rapid potentiation of ClC-Ka (Liantonio *et al*., 2006; Picollo *et al*., 2007) (Figure 1). Therefore, the NFA binding site(s) involved in this potentiating effect are most likely accessible from the outside. Thus, in order to identify the residues involved in NFA binding, we selected several amino acids which are predicted to be accessible from the extracellular side, based on the crystal structure of the bacterial ClC-ec1 (Dutzler *et al*., 2002; 2003). This model has been useful in previous studies of drug-binding sites on human CLC channels (Estévez *et al*., 2003; Picollo *et al*., 2004). We applied several different criteria to select amino acids to mutate. Because ClC-K1 is only blocked by NFA, whereas ClC-Ka and ClC-Kb are both potentiated, we first identified amino acids that are conserved in ClC-Ka and ClC-Kb, but not in ClC-K1. However, because the homology between all ClC-K channels is very high, it is possible that ClC-K1 presents nevertheless a 'vestigial' potentiating site. Therefore, in addition, we selected

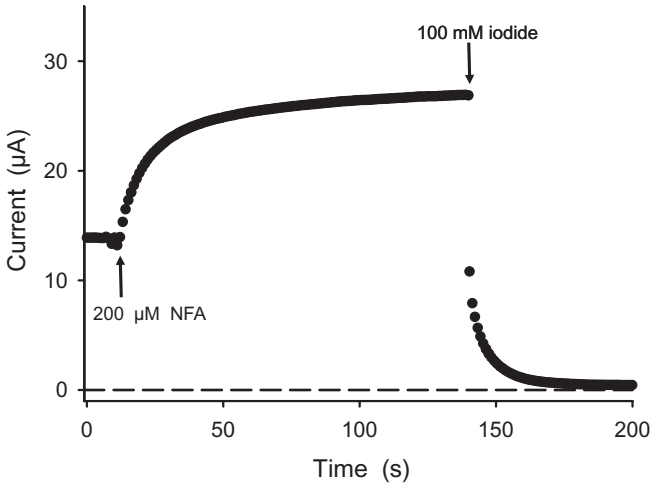

Figure 1 Typical response of WT ClC-Ka to the application of 200 μM NFA. A 60 mV test pulse of 100 ms duration was applied about once per second, and the average current is plotted as a function of time. The current remaining after the application of 100 mM iodide was used as an estimate of endogenous/leak currents.

further residues based on their exposure to the extracellular side. In general, charged residues were neutralized, and if uncharged residues in ClC-Ka corresponded to charged residues in ClC-K1, such a mutation was introduced into ClC-Ka. Furthermore, if uncharged residues conserved in ClC-Ka and -Kb corresponded to a different uncharged residue in ClC-K1, the corresponding mutation was introduced into ClC-Ka. The residues selected are coloured in Figure 2A,B, which shows that we covered a relatively large portion of the surface-accessible part of the protein. Triggered by a recent publication on the effects of NFA on cation channels (Fernandez *et al*., 2008), we also mutated two residues that corresponded to a signature sequence of NFA binding, even though these residues are localized on the intracellular side (R182G and R184G, the latter of which did not yield functional expression).

WT ClC-Ka is robustly activated by more than twofold by 200 μM NFA (Figure 1) (Liantonio *et al*., 2006). Thus, to screen the mutations for a possible alteration of NFA-mediated potentiation, we applied 200 μM NFA to all mutants that could be functionally expressed in *Xenopus* oocytes. Currents were recorded using a test pulse to 60 mV once per second. After a stable baseline had been established in the absence of NFA, 200 μM NFA was continuously perfused, and after a steady state had been reached, we calculated the ratio of the current produced in the presence of NFA and the initial current induced in control conditions (see Figure 1 for a typical recording). Mean values of this ratio are shown in Figure 3. Most mutations only slightly altered the NFA-induced potentiation, the effect being non-significant. However, five mutations abolished the NFA-induced potentiation: L155A, G167A, F213A, G345S and A349E (shown as red bars in Figure 3).

The lack of potentiation by NFA in the presence of these mutations in principle suggests that these residues are contributing to the activating NFA binding site and/or are involved in conformational changes following NFA binding.

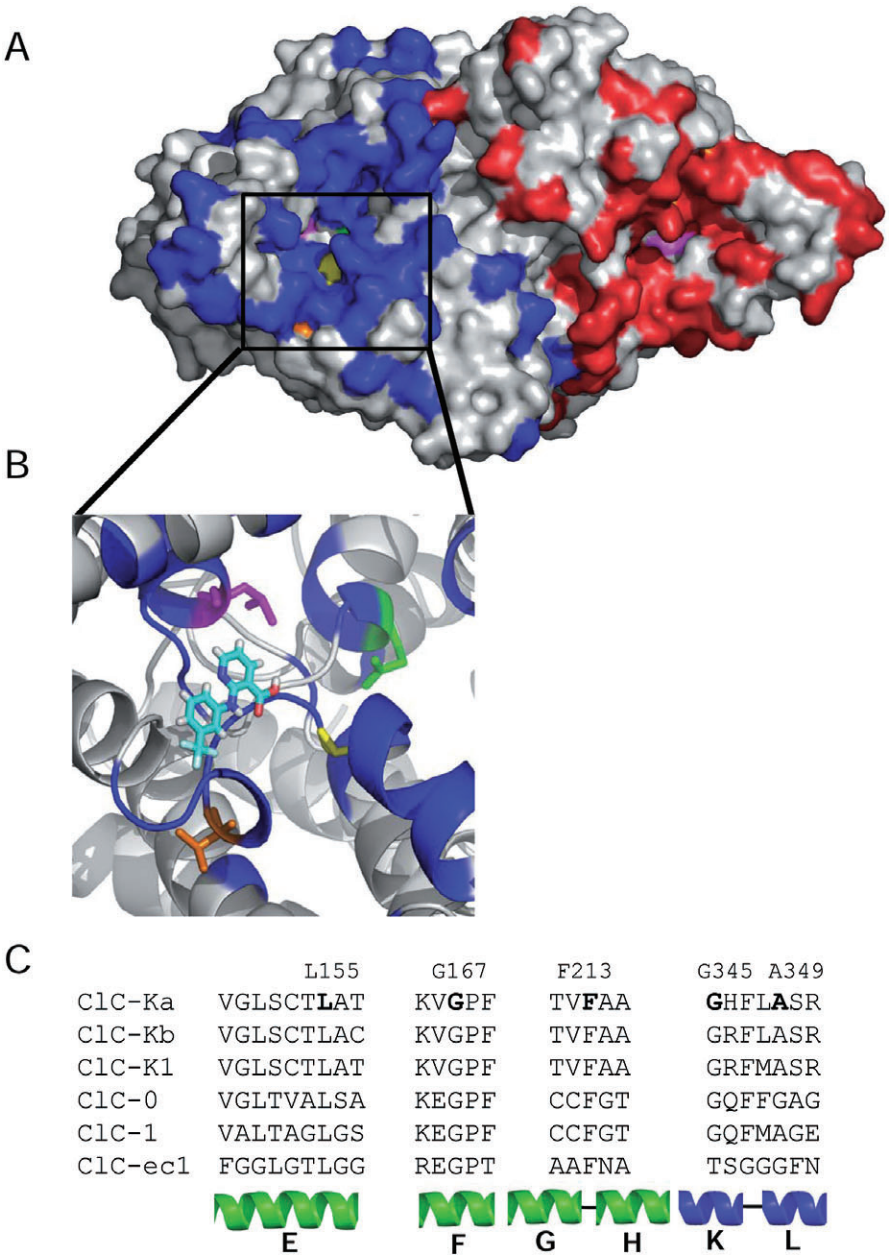

Figure 2 Location of mutants mapped on the structure of ClC-ec1. (A) A surface representation of the bacterial ClC-ec1 (pdb entry 1OTS) viewed from the extracellular side. The central glutamate residue (E148) is coloured in green indicating the pore. The residues corresponding to those selected for mutation are shown in colour. In the left subunit, the residues that emerged as the most interesting ones are coloured in pink (L139 corresponding to L155 of ClC-Ka), orange (T312 corresponding to G345 of ClC-Ka) and yellow (G316 corresponding to A349 of ClC-Ka) respectively. (B) A zoom of the region comprising the extracellular side of helix E and the extracellular loop between helices K and L is shown in cartoon representation with the four residues L139, E148, T312, G316 highlighted as stick with colouring as in (A). An NFA molecule is shown close to the putative binding site. In (C), alignments of sequence stretches comprising the five residues (shown in bold) that affected NFA potentiation are shown.

However, further analysis is needed before such a conclusion can be reached. First of all, it has to be kept in mind that NFA potentiates ClC-Ka by increasing its open probability (Picollo *et al*., 2007). Thus, we first have to exclude that the lack of potentiation is due to an intrinsic modification of the gating properties brought about by the mutation. In fact, two of the positive hit mutants, G167A and F213A, probably affect the NFA potentiation by this mechanism.

G167 is a highly conserved residue adjacent to V166 (Figure 2C), a residue of paramount importance for the gating of all CLC proteins (Zifarelli and Pusch, 2007). The current level of G167A was significantly enhanced compared to WT: whereas WT expressed microampere-sized currents 3 days after injection, G167A-injected oocytes expressed currents after 1 day and the RNA had to be diluted at least fivefold (compared to WT) in order to obtain an expression level below 10 μA (data not shown). Moreover, the currents mediated by G167A lacked the time-dependent relaxations that characterize WT currents, consistent with a drastic effect on channel gating (Figure S1).

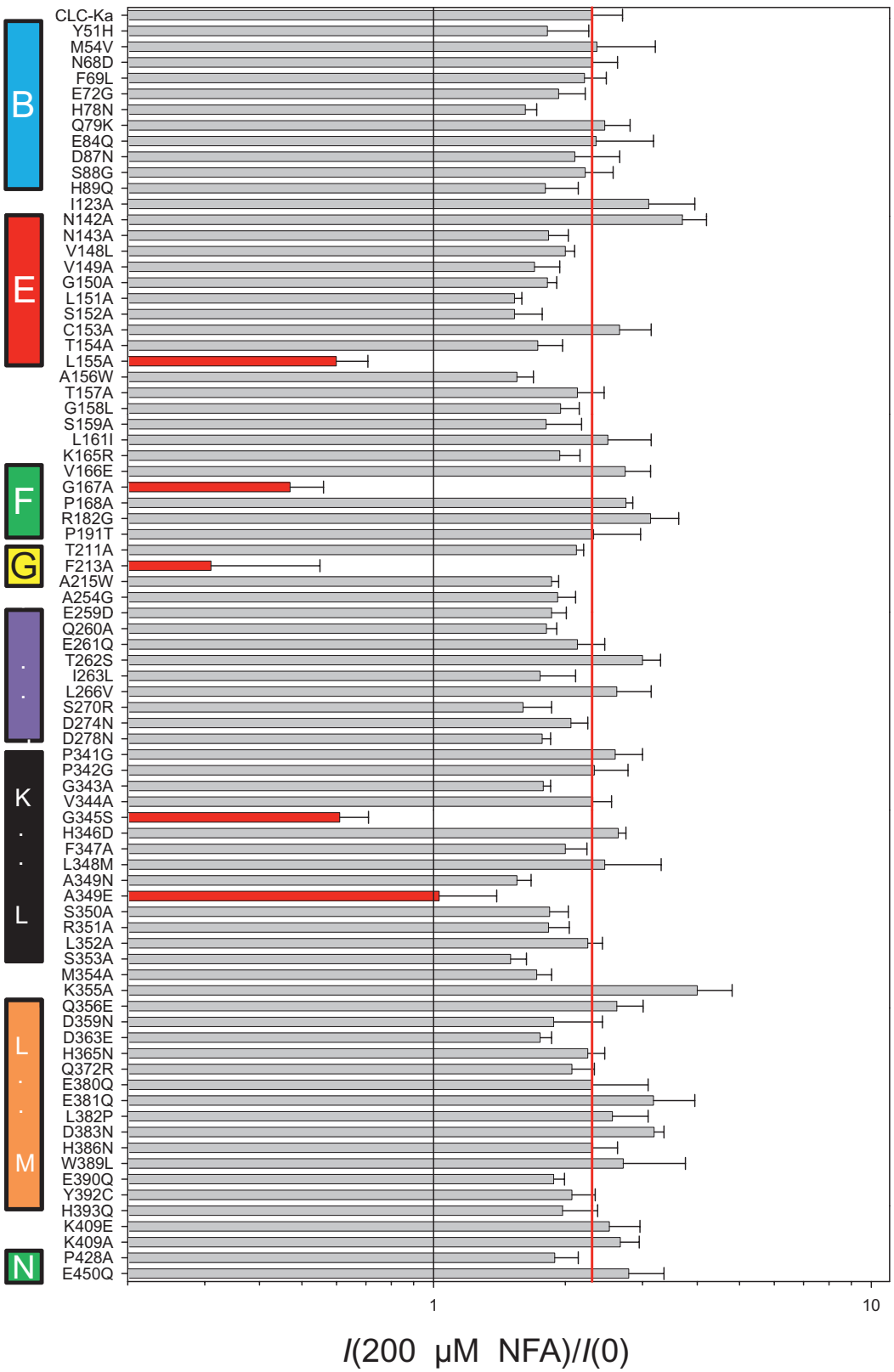

Figure 3 Mutational analysis of NFA-mediated potentiation. The ratio of currents in the presence of 200 µM NFA and in control solution at 60 mV is plotted for the mutations that resulted in significant functional expression ($n \geq 3$; error bars indicate SEM). Mutants L155A, G167A, F213A, G345S and A349E were significantly different from WT ($P < 0.01$), whereas the remaining mutants were not significantly different ($P > 0.05$) (see Methods for statistical analysis). The red line indicates the value for WT; the black line indicates the value '1' (i.e. implying no effect of NFA on current magnitude). The mutants E281D and E442D showed very small expression not allowing a quantitative assessment of NFA effects. The following mutants showed no expression above background: T55A, Y56C, C73R, R184G, V212A, A214W, A214T, E259Q, E281Q, D363N, G424A, M427A, E442Q.

F213 is also a highly conserved residue (Figure 2C). Similar to G167A, the currents mediated by F213A are much larger than those for WT and exhibit much less voltage-dependent gating relaxations (Supporting Information Figure S1).

To assess a possible effect on gating parameters directly, we used non-stationary noise analysis to estimate the single-channel conductance and the absolute open probability (p_{open}) (Sigworth, 1980). However, preliminary single-channel recordings indicated that the single-channel behaviour of ClC-Ka is very flickery (Pusch, unpubl. results). Such flickery behaviour might interfere with the standard noise analysis if the fast transitions are not resolved. We therefore investigated, firstly, the influence of the filter frequency on the results. The power spectrum at –140 mV could be well fitted by the sum of three Lorentzian components (Supporting Information Figure S2A) with corner frequencies of 6.6 Hz, 208 Hz and 2.3 kHz respectively. The latter, high-frequency component, however, had a very small amplitude compared to the other two components (see legend of Supporting Information Figure S2A). Most importantly, the filter frequency did not seem to exert a large effect on the estimate of the single-channel conductance, as the largest difference for this parameter obtained at the most extreme filter frequencies tested (250 and 9000 Hz, respectively) was less than 30% (Supporting Information Figure S2B). Thus, non-stationary noise analysis appears to be a valid method to be applied to the ClC-Ka channel. WT ClC-Ka is characterized by a single-channel conductance of ~18 pS and a p_{open} that is very small under the experimental conditions tested (i.e. 10 mM external calcium, pH 7.3) (Figure 4A; Table 1). In fact, if the p_{open} is less than ~0.1, noise analysis is not suitable for determining the absolute value. Moreover, the application of this technique is somehow problematic for F213A and even more so for G167A, as the lack of current kinetics (Figure 4B,C; left panels) only allows a very limited range of the parabola in the variance–mean plot to be resolved (Figure 4B,C; right panels), rendering the independent estimate of p_{open} and of the single-channel current unreliable. Nevertheless, the noise analysis is compatible with a single-channel conductance similar to that of WT ClC-Ka and a greatly increased $p_{open} \geq 0.5$ (fits in Figure 4B,C; Table 1). Thus, although not conclusive, several pieces of evidence suggest that G167A and F213A have altered gating properties and a drastically increased p_{open} compared to WT. This thwarts any kind of conclusion about the modification of NFA action on these mutants.

Currents elicited by the mutant L155A were found to be similar to WT with respect to both magnitude and kinetics (Figure 5A,B), suggesting that the overall gating characteristics are similar. In fact, non-stationary noise analysis indicated that this mutant is characterized by an open probability that is significantly smaller than 0.1, similar to WT (Figure 4C; Table 1); 200 μM NFA decreased the current in this mutant ($I_{NFA}/I_{control} = 0.43$). L155 is located on the extracellular side of helix E and is very much conserved among CLC proteins (Figure 2C). Interestingly, several other mutations in helix E (L151A, S152A, A156W; see Figure 2) appeared to reduce the NFA-mediated potentiation of the current, but this effect was not significant.

G345 and A349 are located on the extracellular loop connecting helices K and L, and they are also conserved, although

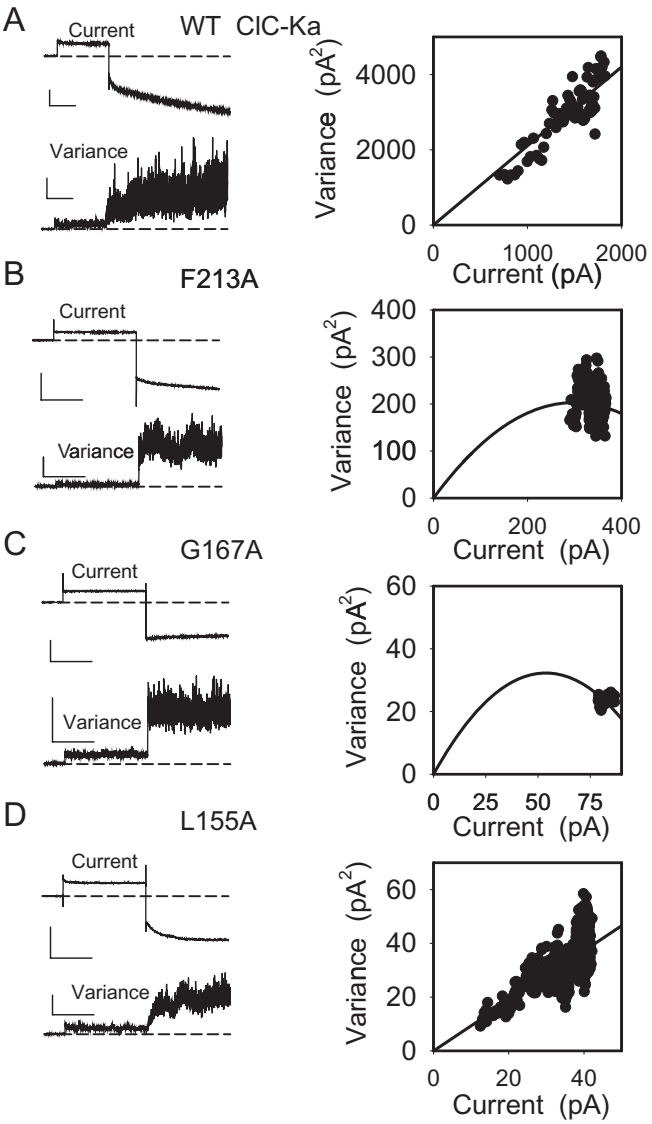

Figure 4 Noise analysis revealed gating alterations. Typical results of non-stationary noise analysis of WT ClC-Ka (A), mutant F213A (B), mutant G167A (C) and mutant L155A (D) are shown. The mean (upper trace) and the variance (lower trace) are shown on the left, while the right shows the variance plotted versus the mean (symbols) and fitted with a parabola (line) as described in Methods. The parameters obtained by the fit are: (A): $i = 2.1$ pA, $P_{max} < 0.1$; (B): $i = 1.4$ pA, $P_{max} = 0.61$; (C): $i = 1.2$, $P_{max} = 0.81$; (D): $i = 0.93$, $P_{max} < 0.1$. Horizontal scale bars indicate 100 ms; vertical scale bars indicate: (A) 500 pA, 2000 pA^2; (B) 200 pA, 100 pA^2; (C) 50 pA, 20 pA^2; (D) 20 pA, 20 pA^2. Mean values of P_{max} are shown in Table 1.

to a lesser extent compared to the other residues analysed (Figure 2C). The currents elicited by G345S were smaller than those in WT, and the current relaxations that characterize the WT were almost completely absent (Figure 5C). Also for this mutant, 200 μM NFA had a blocking rather than a potentiating effect (Figure 5C). The currents mediated by the mutant A349E were very similar to WT (Figure 5D) and were unaffected by 200 μM NFA (Figure 5D). Unfortunately, the levels of the currents of the mutants A349E and G345S were too small to allow noise analysis using the patch clamp technique.

Table 1 Average values for the maximal open probability obtained from non-stationary noise analysis

Construct	P_{max}
WT ClC-Ka	<0.1 (n = 9)
F213A	0.50 ± 0.1 (n = 4)
G167A	0.75 ± 0.06 (n = 4)
L155A	<0.1 (n = 5)

Fit values obtained for WT ClC-Ka and mutant L155A are <0.1 precluding a determination of the true value using this method.

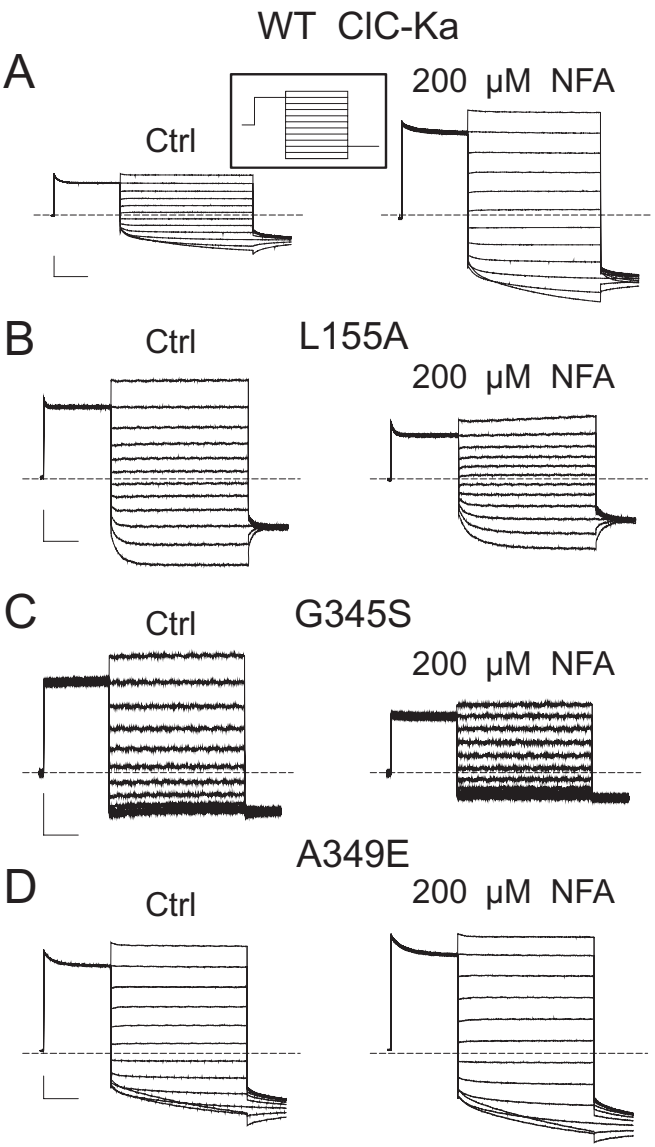

Figure 5 Representative current traces for WT and the indicated mutants in control solution and in 200 µM NFA. Horizontal scale bars correspond to 50 ms; vertical scale bars correspond to 5 µA for WT and 1 µA for the mutants. The pulse protocol comprised a prepulse to 60 mV followed by steps to values from –140 to 80 mV, and a tail pulse to –100 mV and is depicted in (A) as an inset.

Taken together, the lack of a potentiating effect to NFA in the L155A, G345S and A349E mutants indicates that the interaction between these mutants and NFA is drastically altered compared to that in the WT. However, the results

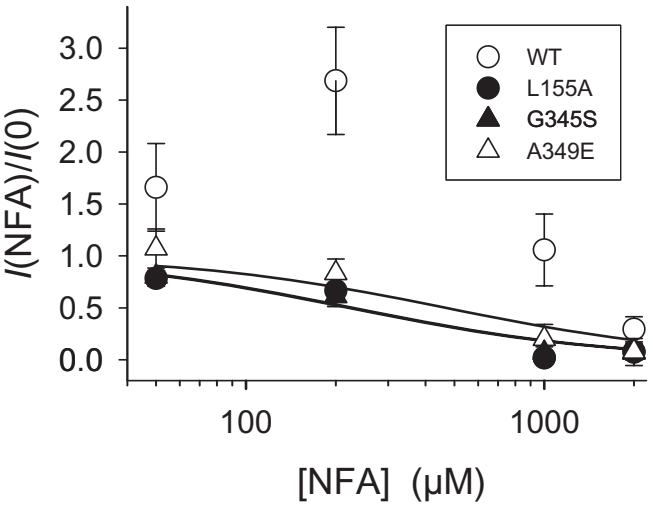

Figure 6 Concentration dependence of the effects of NFA in WT and in the indicated mutants. Lines are fits to the equation $I([NFA])/I(0) = 1/(1 + [NFA]/K)$ with the apparent dissociation constant K as the fitted parameter, resulting in values of 230 µM (L155A), 220 µM (G345S) and 460 µM (A349E) respectively.

could, in principle, be attributed to an enhanced block rather than a reduced potentiation. We therefore tested the response of these mutants to different NFA concentrations (Figure 6). In contrast to the biphasic response of WT ClC-Ka (Liantonio *et al*., 2006), all three mutants show monotonic NFA-induced inhibition with sub-millimolar EC_{50} (see legend of Figure 6). Most importantly, 50 µM NFA, a concentration that leads to a significant potentiation of the WT, had only a small inhibitory effect on the mutants. These results confirm that the mutants specifically ablate the potentiating effect of NFA, but leave the NFA-induced block intact.

Discussion and conclusions

ClC-K channels are essential components in the mechanism of water diuresis and NaCl re-absorption in the kidney and for the hearing process, and are involved in several human genetic diseases (Jentsch, 2005; Uchida and Sasaki, 2005; Zifarelli and Pusch, 2007). ClC-Kb polymorphisms have also been implicated in high blood pressure (Jeck *et al*., 2004), although recent publications contradict these findings (Kokubo *et al*., 2005; Speirs *et al*., 2005). Thus, pharmacological tools able to selectively modulate the activity of ClC-K channels can be of great interest in the treatment of several pathological conditions. Specific modulators of ClC-Ka may regulate water diuresis without affecting osmotic diuresis, mostly due to ClC-Kb (Picollo *et al*., 2004). In particular, increased water diuresis due to ClC-Ka block can be beneficial to reduce cardiac load, for example after heart failure (Fong, 2004). On the other hand, ClC-Ka potentiators might be useful to treat patients with Bartter syndrome type III or type IV, if residual channel activity is present.

NFA is a peculiar ClC-K ligand with unprecedented features; it potentiates ClC-Ka at low concentrations, but blocks the channel at high concentrations. On the basis of a kinetic model, it has been proposed that the two opposite effects of

NFA on ClC-Ka are mediated by two distinct binding sites (Picollo *et al*., 2007). N68, which is part of the blocking site for FFA and CPP derivatives (Picollo *et al*., 2004), does not appear to be part of the NFA binding site(s) (Picollo *et al*., 2007). This suggests that NFA acts on ClC-Ka by a different mechanism compared to FFA and CPP derivatives. The potentiation produced by NFA at low concentrations is caused by an augmentation of the open probability. Thus, NFA can be clearly classified as a gating modifier of ClC-K channels. Similar to its effect on ClC-Ka, NFA and other fenamates potentiate several types of cation channels (Busch *et al*., 1994; Abitbol *et al*., 1999; Malykhina *et al*., 2002; Fernandez *et al*., 2008; Cheng and Sanguinetti, 2009) modulating their open probability. On the other hand, NFA blocks various Cl^- channels by binding to the ion-conducting pore, consistent with its negative charge. This appears to be the case for Ca^{2+}-activated Cl^- channels (Qu and Hartzell, 2001) and for ClC-1, which is blocked from the intracellular side of the membrane (Liantonio *et al*., 2007). For ClC-Ka, it is still unclear whether the NFA-induced block at high concentrations reflects a pore-blocking mechanism.

In the present work, we set out to identify the NFA binding site on ClC-Ka that is responsible for the potentiating effect. The rapid current increase suggests that the binding site for potentiation is exposed to the extracellular side of the membrane. Using the bacterial ClC-ec1 structure as a guide, we screened 97 mutations and found that five mutations abolished the NFA-mediated potentiation. Interestingly, none of these mutations significantly reduced the block induced by NFA at higher concentrations. This finding is consistent with the idea that the potentiating and blocking effects are mediated by different protein regions.

Two of the residues identified (G167 and F213) are unlikely to contribute directly to the activating NFA binding site. Noise analysis of the mutants G167A and F213A, although not conclusive, is at least compatible with an increased open probability compared to WT, consistent with the presence of much larger currents. If this speculative interpretation holds, NFA would not have any effect on these mutants even in the presence of normal binding. However, in the absence of direct binding data, no firm conclusion about NFA binding can be drawn for these residues.

On the other hand, the fact that the mutants L155A, G345S and A349E did not display an increased current expression level, and, with the exception of G345S, exhibited similar current relaxations to those of WT, supports the idea that they specifically alter the NFA/channel interaction. However, an unaltered p_{open} could be directly assessed only for mutant L155A, as this had a sufficient current expression level to allow non-stationary noise analysis measurements. Importantly, from the dose–response analysis, we can safely conclude that these mutations specifically eliminate the potentiating effect without significantly altering the blocking effect of NFA. This is the first direct evidence that the potentiating and blocking effects of NFA are mediated by distinct protein regions. Further studies are required to identify the blocking binding site.

Our results indicate that the extracellular side of helix E and the extracellular loop connecting helices K and L (in particular residues G345 and A349) are critical for the occurrence of the potentiating effect of NFA. In the bacterial ClC-ec1, these regions are exposed to the outside and are relatively close to each other. Given the molecular dimensions of NFA, which is shown in Figure 2 close to the putative binding site, it is possible that these residues contribute to the potentiating binding site for NFA. Because the sequence conservation between ClC-ec1 and ClC-K channels is very low (see e.g. Figure 2), it is impossible to predict the precise spatial orientation of the corresponding amino acids of ClC-Ka, and to draw specific structural conclusions regarding the NFA binding site.

Unfortunately, the NFA affinity associated with the current potentiation is rather low rendering direct ligand binding studies almost impossible. Thus, the possibility that the effect of the mutations could be due to an alteration of conformational changes that follow NFA binding cannot be excluded. Further studies are required to discriminate between these two models. In this respect, it is interesting to note that in addition to L155A, several other mutants located towards the interior helix E, reduced, but not significantly, the NFA-mediated potentiation. It may be speculated that the extracellular end of helix E is involved in direct NFA binding, while a re-arrangement of the helix is involved in the transmission of conformational changes that effectively increase the open probability.

Our results identify a protein region of ClC-Ka specifically responsible for the potentiating effect of NFA, and underscore the physical separation of protein regions responsible for the block and potentiation induced by NFA. They are fundamental for understanding the interaction between ClC-Ka and NFA at a molecular level, and therefore strongly indicate that CLC-K channels are suitable targets for drugs for the treatment of Bartter syndrome.

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Conflict of interest

None.

References

Abitbol I, Peretz A, Lerche C, Busch AE, Attali B (1999). Stilbenes and fenamates rescue the loss of I(KS) channel function induced by an LQT5 mutation and other IsK mutants. *Embo J* **18**: 4137–4448.

Accardi A, Pusch M (2003). Conformational changes in the pore of CLC-0. *J Gen Physiol* **122**: 277–293.

Adachi S, Uchida S, Ito H, Hata M, Hiroe M, Marumo F *et al*. (1994). Two isoforms of a chloride channel predominantly expressed in

thick ascending limb of Henle's loop and collecting ducts of rat kidney. *J Biol Chem* **269**: 17677–17683.

Akizuki N, Uchida S, Sasaki S, Marumo F (2001). Impaired solute accumulation in inner medulla of Clcnk1–/– mice kidney. *Am J Physiol Renal Physiol* **280**: F79–F87.

Alexander SP, Mathie A, Peters JA (2008). *Guide to Receptors and Channels (GRAC)*, 3rd edition. *Br J Pharmacol* **153** (Suppl. 2): S1–209.

Birkenhäger R, Otto E, Schurmann MJ, Vollmer M, Ruf EM, Maier-Lutz I *et al*. (2001). Mutation of BSND causes Bartter syndrome with sensorineural deafness and kidney failure. *Nat Genet* **29**: 310–314.

Busch AE, Herzer T, Wagner CA, Schmidt F, Raber G, Waldegger S *et al*. (1994). Positive regulation by chloride channel blockers of IsK channels expressed in *Xenopus* oocytes. *Mol Pharmacol* **46**: 750–753.

Cheng L, Sanguinetti MC (2009). Niflumic acid alters gating of HCN2 pacemaker channels by interaction with the outer region of S4 voltage sensing domains. *Mol Pharmacol* **75**: 1210–1121.

Coyne L, Su J, Patten D, Halliwell RF (2007). Characterization of the interaction between fenamates and hippocampal neuron GABA(A) receptors. *Neurochem Int* **51**: 440–446.

Dutzler R, Campbell EB, Cadene M, Chait BT, MacKinnon R (2002). X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature* **415**: 287–294.

Dutzler R, Campbell EB, MacKinnon R (2003). Gating the selectivity filter in ClC chloride channels. *Science* **300**: 108–112.

Estévez R, Boettger T, Stein V, Birkenhäger R, Otto E, Hildebrandt F *et al*. (2001). Barttin is a Cl^- channel beta-subunit crucial for renal Cl^- reabsorption and inner ear K^+ secretion. *Nature* **414**: 558–561.

Estévez R, Schroeder BC, Accardi A, Jentsch TJ, Pusch M (2003). Conservation of chloride channel structure revealed by an inhibitor binding site in ClC-1. *Neuron* **38**: 47–59.

Fernandez D, Sargent J, Sachse FB, Sanguinetti MC (2008). Structural basis for ether-a-go-go-related gene K^+ channel subtype-dependent activation by niflumic acid. *Mol Pharmacol* **73**: 1159–1167.

Fong P (2004). CLC-K channels: if the drug fits, use it. *EMBO Rep* **5**: 565–566.

Foster RR, Zadeh MA, Welsh GI, Satchell SC, Ye Y, Mathieson PW *et al*. (2009). Flufenamic acid is a tool for investigating TRPC6-mediated calcium signalling in human conditionally immortalised podocytes and HEK293 cells. *Cell Calcium* **45**: 384–390.

Greenwood IA, Leblanc N (2007). Overlapping pharmacology of Ca^{2+}-activated Cl^- and K^+ channels. *Trends Pharmacol Sci* **28**: 1–5.

Jeck N, Waldegger S, Lampert A, Boehmer C, Waldegger P, Lang PA *et al*. (2004). 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.

Kieferle S, Fong P, Bens M, Vandewalle A, Jentsch TJ (1994). Two highly homologous members of the ClC chloride channel family in both rat and human kidney. *Proc Natl Acad Sci U S A* **91**: 6943–6947.

Kokubo Y, Iwai N, Tago N, Inamoto N, Okayama A, Yamawaki H *et al*. (2005). Association analysis between hypertension and CYBA, CLCNKB, and KCNMB1 functional polymorphisms in the Japanese population – the Suita study. *Circ J* **69**: 138–142.

Liantonio A, Accardi A, Carbonara G, Fracchiolla G, Loiodice F, Tortorella P *et al*. (2002). Molecular requisites for drug binding to muscle CLC-1 and renal CLC-K channel revealed by the use of phenoxy-alkyl derivatives of 2-(*p*-chlorophenoxy)propionic acid. *Mol Pharmacol* **62**: 265–271.

Liantonio A, Pusch M, Picollo A, Guida P, De Luca A, Pierno S *et al*. (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.

Liantonio A, Picollo A, Babini E, Carbonara G, Fracchiolla G, Loiodice F *et al*. (2006). Activation and inhibition of kidney CLC-K chloride channels by fenamates. *Mol Pharmacol* **69**: 165–173.

Liantonio A, Giannuzzi V, Picollo A, Babini E, Pusch M, Conte Camerino D (2007). Niflumic acid inhibits chloride conductance of rat skeletal muscle by directly inhibiting the CLC-1 channel and by increasing intracellular calcium. *Br J Pharmacol* **150**: 235–247.

Liantonio A, Picollo A, Carbonara G, Fracchiolla G, Tortorella P, Loiodice F *et al*. (2008). Molecular switch for CLC-K Cl^- channel block/activation: optimal pharmacophoric requirements towards high-affinity ligands. *Proc Natl Acad Sci U S A* **105**: 1369–1373.

Lorenz C, Pusch M, Jentsch TJ (1996). Heteromultimeric CLC chloride channels with novel properties. *Proc Natl Acad Sci U S A* **93**: 13362–13366.

Malykhina AP, Shoeb F, Akbarali HI (2002). Fenamate-induced enhancement of heterologously expressed HERG currents in *Xenopus* oocytes. *Eur J Pharmacol* **452**: 269–277.

Matsumura Y, Uchida S, Kondo Y, Miyazaki H, Ko SB, Hayama A *et al*. (1999). Overt nephrogenic diabetes insipidus in mice lacking the CLC-K1 chloride channel. *Nat Genet* **21**: 95–98.

Matulef K, Howery AE, Tan L, Kobertz WR, Du Bois J, Maduke M (2008). Discovery of potent CLC chloride channel inhibitors. *ACS Chem Biol* **3**: 419–428.

Picollo A, Liantonio A, Didonna MP, Elia L, Camerino DC, Pusch M (2004). Molecular determinants of differential pore blocking of kidney CLC-K chloride channels. *EMBO Rep* **5**: 584–589.

Picollo A, Liantonio A, Babini E, Camerino DC, Pusch M (2007). Mechanism of interaction of niflumic acid with heterologously expressed kidney CLC-K chloride channels. *J Membr Biol* **216**: 73–82.

Pusch M, Steinmeyer K, Jentsch TJ (1994). Low single channel conductance of the major skeletal muscle chloride channel, ClC-1. *Biophys J* **66**: 149–152.

Pusch M, Liantonio A, Bertorello L, Accardi A, De Luca A, Pierno S *et al*. (2000). Pharmacological characterization of chloride channels belonging to the ClC family by the use of chiral clofibric acid derivatives. *Mol Pharmacol* **58**: 498–507.

Pusch M, Liantonio A, De Luca A, Conte Camerino D (2007). Pharmacology of CLC chloride channels and transporters. In: Pusch M (ed.). Chloride Transport across Biological Membranes*, Vol. 38*. Elsevier: Amsterdam, pp. 83–108.

Qu Z, Hartzell HC (2001). Functional geometry of the permeation pathway of Ca^{2+}-activated Cl^- channels inferred from analysis of voltage-dependent block. *J Biol Chem* **276**: 18423–18429.

Rickheit G, Maier H, Strenzke N, Andreescu CE, De Zeeuw CI, Muenscher A *et al*. (2008). Endocochlear potential depends on Cl^- channels: mechanism underlying deafness in Bartter syndrome IV. *Embo J* **27**: 2907–2917.

Schlingmann KP, Konrad M, Jeck N, Waldegger P, Reinalter SC, Holder M *et al*. (2004). Salt wasting and deafness resulting from mutations in two chloride channels. *N Engl J Med* **350**: 1314–1319.

Scholl U, Hebeisen S, Janssen AG, Müller-Newen G, Alekov A, Fahlke C (2006). Barttin modulates trafficking and function of ClC-K channels. *Proc Natl Acad Sci U S A* **103**: 11411–11416.

Sigworth FJ (1980). The variance of sodium current fluctuations at the node of Ranvier. *J Physiol* **307**: 97–129.

Simon DB, Bindra RS, Mansfield TA, Nelson-Williams C, Mendonca E, Stone R *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, Morris BJ (2005). No association with hypertension of CLCNKB and TNFRSF1B polymorphisms at a hypertension locus on chromosome 1p36. *J Hypertens* **23**: 1491–1496.

Uchida S, Sasaki S (2005). Function of chloride channels in the kidney. *Ann Rev Physiol* **67**: 759–778.

Uchida S, Sasaki S, Furukawa T, Hiraoka M, Imai T, Hirata Y *et al*. (1993). Molecular cloning of a chloride channel that is regulated by dehydration and expressed predominantly in kidney medulla. *J Biol Chem* **268**: 3821–3824.

Verkman AS, Galietta LJ (2008). Chloride channels as drug targets. *Nat Rev Drug Discov* **19**: 19.

Waldegger S, Jeck N, Barth P, Peters M, Vitzthum H, Wolf K *et al*. (2002). Barttin increases surface expression and changes current properties of ClC-K channels. *Pflügers Arch* **444**: 411–418.

Zifarelli G, Pusch M (2007). CLC chloride channels and transporters: a biophysical and physiological perspective. *Rev Physiol Biochem Pharmacol* **158**: 23–76.

Supporting information

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

Figure S1 Representative current traces for the mutants G167A (A) and F213A (B).
Figure S2 Spectral properties of ClC-Ka.

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