Molecular determinants of differential pore blocking of kidney CLC-K chloride channels

Alessandra Picollo^{1*}, Antonella Liantonio^{1,2*}, Maria Paola Didonna^{1,2}, Laura Elia¹, Diana Conte Camerino² & Michael Pusch¹⁺

¹Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Genova, Italy, and ²Sezione di Farmacologia, Dipartimento Farmacobiologico, Università di Bari, Bari, Italy

The highly homologous Cl⁻ channels CLC-Ka and CLC-Kb are important for water and salt conservation in the kidney and for the production of endolymph in the inner ear. Mutations in CLC-Kb lead to Bartter's syndrome and mutations in the small CLC-K subunit barttin lead to Bartter's syndrome and deafness. Here we show that CLC-Ka is blocked by the recently identified blocker 2-(*p*-chlorophenoxy)-3-phenylpropionic acid of the rat channel CLC-K1 with an apparent $K_D \sim 80 \mu\text{M}$. We also found that DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid), a generic Cl⁻ channel blocker, inhibits CLC-Ka ($K_D \sim 90 \mu\text{M}$). Surprisingly, the highly homologous channel CLC-Kb is fivefold less sensitive to both compounds. Guided by the crystal structure of bacterial CLC proteins, we identify two amino acids, N68/D68 and G72/E72, in CLC-Ka and CLC-Kb, respectively, that are responsible for the differential drug sensitivity. Both residues expose their side chains in the extracellular pore mouth, delineating the probable drug binding site. These novel CLC-K channel blockers are promising lead compounds for the development of new diuretic drugs.

Keywords: CLC-Ka; CLC-Kb; DIDS; wild type; block; mutations

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INTRODUCTION

Chloride channels allow the passive flux of anions across biological membranes. CLC Cl⁻ channels are important for several cellular and physiological processes, including transepithelial salt transport, electrical excitability, cell-volume regulation and acidification of internal and external compartments (Jentsch *et al*, 2002). CLC channels have been found in organisms as diverse as mammals, plants and yeast. Mammals have nine different CLC genes. On the basis of sequence homology, they

can be grouped into three branches. The two CLC-K channels (CLC-Ka/CLC-K1 and CLC-Kb/CLC-K2; Uchida *et al*, 1993; Adachi *et al*, 1994; Kieferle *et al*, 1994) are plasma membrane channels that are predominantly expressed in the kidney and the inner ear. They are essential for water and salt conservation in the kidney and for the production of endolymph in the inner ear. Mutations in CLC-Kb lead to Bartter's syndrome (Simon *et al*, 1997) and mutations in the small, essential CLC-K channel subunit barttin lead to Bartter's syndrome and deafness (Birkenhäger *et al*, 2001). Rodent CLC-K1 (the orthologue of CLC-Ka) is important for transepithelial transport in the thin ascending limb, and CLC-K1 knockout mice show nephrogenic diabetes insipidus (Matsumura *et al*, 1999). Recently, CLC-K channels were shown to require the small transmembrane protein barttin as a β -subunit for efficient functional expression (Estévez *et al*, 2001). Coexpression of CLC-K channels with barttin leads to greatly enhanced surface expression (Estévez *et al*, 2001) and maybe also to a change of biophysical properties (Waldegger *et al*, 2002). Thus, only recently has it become possible to study the biophysical and pharmacological properties of CLC-K channels in detail. Small organic ligands that interact with ion channels have been extremely useful tools for understanding the function of these channels (Hille, 2001). Only a few specific blockers exist for CLC channels. Blockage of CLC-0 and CLC-1 by small organic inhibitors that act from the intracellular side has been recently studied in detail (Pusch *et al*, 2000, 2001, 2002; Liantonio *et al*, 2002, 2003; Accardi & Pusch, 2003; Estévez *et al*, 2003; Traverso *et al*, 2003). A different type of inhibition involving a separate binding site has been recently described for CLC-K channels (Liantonio *et al*, 2002). A group of derivatives of 2-(*p*-chlorophenoxy)propionic acid (CPP) containing two chlorophenoxy moieties that inhibit CLC-K1 with micromolar affinity from the extracellular side was identified (Liantonio *et al*, 2002). In a subsequent structure–activity study, the 2-(*p*-chlorophenoxy)-3-phenylpropionic acid (3-phenyl-CPP) (Fig 1C) was identified as the minimal structure required to block CLC-K1 (Liantonio *et al*, 2004). The block was reversible and competitive with extracellular Cl⁻, suggesting a pore location of the binding site. Among 'classical' Cl⁻ channel inhibitors, only DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid; Fig 1C) blocked CLC-K1 with an affinity comparable with that of

¹Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Via de Marini 6, 16149 Genova, Italy

²Sezione di Farmacologia, Dipartimento Farmacobiologico, Università di Bari, 70125 Bari, Italy

*These authors contributed equally to this work

+Corresponding author. Tel: +39 0106475 561/522; Fax +39 0106475 500; E-mail: pusch@ge.ibf.cnr.it

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Fig 1 | Effects of 3-phenyl-CPP and DIDS on CLC-Ka. (A,B) Voltage-clamp traces of CLC-Ka currents before and during application of 200 μM 3-phenyl-CPP (A) and 200 μM DIDS (B), and immediately after wash-out. The pulse protocol is shown in the inset. Horizontal scale bars indicate 50 ms (A,B). Vertical scale bars indicate 5 μA (A,B).

(C) Chemical structures of 3-phenyl-CPP and DIDS. (D) Slow recovery from DIDS block for a representative oocyte. Arrows indicate application of DIDS and wash-out. A pulse to 60 mV was applied every 2 s and the current is shown as a function of the elapsed time.

3-phenyl-CPP, albeit in an apparently irreversible manner (Liantonio *et al*, 2004). We sought to extend our previous studies to the human channels CLC-Ka and CLC-Kb. We found that CLC-Ka, like CLC-K1, is sensitive to 3-phenyl-CPP and DIDS, and that CLC-Kb is much less sensitive to both drugs. This is surprising because the two channels are about 80% identical at the protein sequence level. We then used the crystal structure of bacterial CLC proteins as a guide and identified two amino acids as being responsible for the difference in drug sensitivity.

RESULTS

We examined blockage of the human homologues CLC-Ka and CLC-Kb. CLC-Ka currents activate at negative voltages and partly deactivate at positive voltages (Fig 1A), similarly to CLC-K1 (Liantonio *et al*, 2004). 3-Phenyl-CPP (Fig 1C) inhibited CLC-Ka (Fig 1A, middle) in a quickly reversible manner (Fig 1A, right).

Fig 2 | Effects of 3-phenyl-CPP and DIDS on CLC-Kb. Voltage-clamp traces of CLC-Kb currents before and during application of 200 μM 3-phenyl-CPP (A) and 200 μM DIDS (B), and immediately after wash-out. Horizontal scale bars indicate 100 ms. Vertical scale bars indicate 2 μA (A) and 1 μA (B).

Also DIDS blocked CLC-Ka with a similar affinity; however, the inhibition was not immediately reversible (Fig 1B). Washing for a prolonged time was necessary to recover from DIDS blockage (Fig 1D). This slow recovery rendered difficult a detailed analysis of the blocking mechanism. Surprisingly, CLC-Kb was much less sensitive to both compounds (Fig 2). The voltage dependence and kinetics of CLC-Kb are also inverted with respect to CLC-Ka (Figs 1,2; Estévez *et al*, 2001). Furthermore, the gating kinetics of CLC-Kb are slower than those of CLC-Ka (compare time scales in Figs 1,2). A quantitative dose-response analysis of inhibition by 3-phenyl-CPP and DIDS of CLC-Ka and CLC-Kb revealed that blockage by both substances is practically complete at high concentrations (Fig 3). Inhibition can be described well by a simple 1:1 binding (solid and dashed lines in Fig 3), resulting in the apparent dissociation constants reported in Table 1. As for CLC-K1 (Liantonio *et al*, 2004), blockage by 3-phenyl-CPP and DIDS is slightly voltage dependent, being stronger at positive voltages. We therefore measured inhibition at two voltages: -140 and 60 mV. At 60 mV, 3-phenyl-CPP has a fivefold larger potency on CLC-Ka compared with CLC-Kb, whereas the potency of DIDS is 6.5-fold larger (see Table 1).

The quick onset of blockage indicates that the binding site for 3-phenyl-CPP and DIDS is accessible from the extracellular side. Competition with extracellular chloride suggests that it is located close to the ion-conducting pore (Liantonio *et al*, 2004). To identify the amino acids that are responsible for the different drug sensitivities of CLC-Ka and CLC-Kb, we used the crystal structure of the bacterial StCLC (Dutzler *et al*, 2002) as a guide to identify amino acids that possibly expose their side chains in the extracellular pore entrance (Fig 4A, amino acids shown in dark grey, blue, red, orange and magenta). As both CLC-K1 and CLC-Ka are sensitive to 3-phenyl-CPP and DIDS, whereas CLC-Kb is comparably insensitive, we selected for mutation those residues that were different in CLC-Ka and CLC-Kb and that were identical in CLC-K1 and CLC-Ka. Only four residues fulfilled all criteria. They are all located in helix B (Fig 4). We mutated all four amino acids of CLC-Ka singly to the corresponding residue of CLC-Kb and vice versa. In addition, we combined the two charge-changing mutants (see Table 1 for all mutations). With the exception of CLC-Ka_{Y65C} and CLC-Kb_{D68N/E72G}, all mutants were

Fig 3 | Analysis of inhibition of wild-type channels. Dose–response relationship of inhibition by 3-phenyl-CPP (**A**) and DIDS (**B**) for CLC-Ka and CLC-Kb. The ratio of currents in the presence and absence of the drugs was determined as described in Methods and plotted versus concentration. Lines are drawn according to equation (1), with the apparent K_D values obtained as described in Methods and reported in Table 1.

functional (Fig 5). Most mutants retained the gating properties of their respective parent channel with two notable exceptions: in the double mutant CLC-Ka_{N68D/G72E} gating relaxations were almost completely absent (Fig 5E) and the CLC-Kb mutant D68N showed a gating phenotype similar to that of CLC-Ka (Fig 5H). Table 1 summarizes the K_D values at +60 and –140 mV for all mutations and their respective wild types (WTs). Values that are significantly different from the respective WT value are shown in bold (Table 1). The most marked effect is seen at position 68: mutating the neutral asparagine found in CLC-Ka to the negatively charged aspartate (N68D) markedly reduces the sensitivity to both 3-phenyl-CPP and DIDS, whereas the reverse mutation in CLC-Kb leads to a channel that is almost as sensitive as CLC-Ka to both drugs (Table 1; Fig 5K). Whereas position 68 is important for sensitivity to both 3-phenyl-CPP and DIDS, two other residues specifically alter blockage by DIDS and 3-phenyl-CPP, respectively: CLC-Ka_{G72E} retains WT sensitivity to 3-phenyl-CPP but becomes insensitive to DIDS (Fig 5L). Also this mutation replaces a neutral amino acid with a negatively charged one. The reverse mutation in CLC-Kb, CLC-Kb_{E72G}, renders the channel sensitive to DIDS (Fig 5L) but not to 3-phenyl-CPP. In contrast, mutations at position 69 have no effect on DIDS blockage, but CLC-Kb_{L69F} is nearly as sensitive to 3-phenyl-CPP as is CLC-Ka. The reverse mutation CLC-Ka_{F69L}, however, has no effect on the drug sensitivity of CLC-Ka. No large effects were observed for mutations at position 65. Not surprisingly, the double mutant CLC-Ka_{N68D/G72E} is similar to the single mutant CLC-Ka_{N68D}, being less sensitive to both compounds.

DISCUSSION

Our results clearly identify the outer pore mouth as a crucial part of the binding pocket for the structurally dissimilar molecules 3-phenyl-CPP and DIDS. The most crucial position is 68, with a

Table 1 | Drug sensitivity of wild-type channels and mutant channels

	K_D (μM) 3-phenyl-CPP			K_D (μM) DIDS		
	+60 mV	–140 mV	<i>n</i>	+60 mV	–140 mV	<i>n</i>
CLC-Ka	78.9 ± 5.3	184 ± 18	23	90 ± 7.6	149 ± 19	18
Ka-Y65C ^a	–	–	–	–	–	–
Ka-N68D	424.8 ± 37.7	934.7 ± 86.5	10	1046 ± 422	940 ± 234	12
Ka-F69L	66.8 ± 2.8	134 ± 19	3	105 ± 15	157 ± 30	4
Ka-G72E	78.4 ± 6.7	160 ± 12	15	1298 ± 138	1005 ± 275	6
Ka-ND/GE	405 ± 85	747 ± 82	5	402 ± 31	549 ± 11	7
CLC-Kb	385 ± 47	457 ± 67	13	586 ± 83	981 ± 174	11
Kb-C65Y	322 ± 42	377 ± 68	6	720 ± 174	1734 ± 336	5
Kb-D68N	90.7 ± 5.5	85.2 ± 1.6	9	194 ± 30	227 ± 61	5
Kb-L69F	242 ± 9.5	207 ± 17	11	1013 ± 174	1538 ± 535	3
Kb-E72G	346 ± 36	606 ± 130	13	162 ± 17	248 ± 65	5
Kb-DN/EG ^a	–	–	–	–	–	–

^aNo expression. Apparent K_D values obtained using equation (1) for mutants and respective wild-type (WT) as described in Methods. K_D values were determined under steady-state conditions at +60 and –140 mV. The values that are significantly different from the respective WT value are shown in bold. These are arbitrarily defined as those that differ from the WT value by a factor of at least three. *n* indicates the number of measurements. Errors are obtained as described in Methods.

Fig 4 | Selection of candidate residues involved in drug binding. (A) View of the bacterial channel StCLC (Dutzler *et al*, 2002) from the extracellular side. One subunit of the dimer is shown in wireframe (right-hand side), whereas the other is shown in spacefill (left-hand side). Highlighted in dark grey are all amino acids that possibly expose their side chains in the extracellular pore entrance. Among these, we selected those residues that were different in CLC-Ka and CLC-Kb and that were identical in CLC-K1 and CLC-Ka. Four residues fulfilled this criterion, as shown in (B). The corresponding residues of StCLC are shown in colour in (A) (dark blue: V51, corresponding to CLC-Ka-Y65; red: E54, CLC-Ka-N68; orange: K55, CLC-Ka-F69; magenta: S58, CLC-Ka-G72). All four residues lie in helix B (yellow). E148 (green: V166 in CLC-Ka and CLC-Kb) indicates the location of the selectivity filter (Dutzler *et al*, 2002, 2003). (B) Alignment of the short stretch of helix B. Mutated amino acids are shown in bold. Corresponding residues in CLC-K1 are shown in bold and italic.

neutral asparagine in CLC-Ka and a negatively charged aspartate in CLC-Kb. A negatively charged residue is found in most CLC proteins including the bacterial homologues StCLC and eriC. The side chain of the corresponding E54 in StCLC protrudes into the putative permeation pathway and is close to the positively charged R147 of StCLC (Dutzler *et al*, 2002, 2003). The charge effect on drug affinity is probably due to electrostatic interactions with the negatively charged blockers. N68 of CLC-Ka is important for both 3-phenyl-CPP and DIDS blockage, and CLC-Kb becomes as sensitive as CLC-Ka to both drugs by changing the aspartate to an asparagine. In contrast, a negatively charged residue at position 72 of CLC-Ka or CLC-Kb appears to influence mainly DIDS binding. Position 72 is located more extracellularly than position 68 in the rather wide extracellular vestibule. In this respect, it is important to note that 3-phenyl-CPP carries one, whereas DIDS carries two, negative charges at physiological pH values. Thus, we speculate that the carboxylate group of 3-phenyl-CPP and one of the sulphonate groups of DIDS bind to a deep site, whereas the second sulphonate group of DIDS remains more superficial, where it is able to be influenced electrostatically by a charge at position 72. Inhibition of CLC-K1

and CLC-Ka by DIDS is very slowly reversible. However, an irreversible covalent modification, as observed in the band 3 anion exchanger (Bartel *et al*, 1989), is unlikely because this would predict steady-state inhibition that is almost independent of the applied concentration, in contrast to our measurements.

In addition to the effects on inhibitor binding, mutations at position 68 have a profound effect on the gating of CLC-Kb: gating of mutant D68 strongly resembles gating of CLC-Ka. Thus, a single-point mutation converts the pharmacological as well as the biophysical properties of CLC-Kb to those of CLC-Ka. The inverse mutation of CLC-Ka, however, is not sufficient to change its gating properties markedly. Interestingly, the corresponding CLC-1 mutant (D136G), which is responsible for recessive myotonia, and similar mutations in CLC-0 also markedly alter gating (Fahlke *et al*, 1995; Ludewig *et al*, 1997). It is possible that the charge at position 68 has a marked effect on the local Cl^- concentration, which is known to be important for channel gating (Pusch *et al*, 1995; Chen & Miller, 1996).

The different inhibitor sensitivities of CLC-Ka and CLC-Kb are interesting from a pharmacological viewpoint. In this respect, the pharmacokinetic properties of any drug have to be considered. For example, several commonly used loop diuretics are extensively bound to plasma proteins, reaching their target (the apical Na/K/2Cl co-transporter) by means of the organic-acid transport system of the proximal tubule (Shankar & Brater, 2003). Clofibrate (which is chemically similar to 3-phenyl-CPP) and other drugs used in the treatment of hyperlipoproteinaemias are also probably largely secreted into the tubular lumen, as final excretion of these molecules occurs primarily through the kidneys (Wierzbicki *et al*, 2003). A similar pharmacokinetic profile is predicted for 3-phenyl-CPP. In addition, it must be taken into account that CLC-Ka is localized at both the apical and basolateral levels of the renal epithelium; thus, a CLC-Ka-blocking drug will reach its target by the basolateral fluid, as well as the luminal fluid. CLC-Ka is expressed in the thin ascending limb of Henle's loop such that its inhibition would lead to water diuresis, a situation that might be favourable in certain pathological conditions. Furthermore, application of a CLC-Ka-specific drug would prevent an osmotic diuresis because the function of CLC-Kb in the thick ascending limb of Henle's loop would be preserved. In addition, the important function of CLC-K channels in the inner ear (Birkenhäger *et al*, 2001; Estévez *et al*, 2001) would not be compromised if CLC-Kb is not blocked. Thus, a diuretic drug that specifically inhibits CLC-Ka but not CLC-Kb could have significant advantages compared with a general CLC-K channel blocker.

METHODS

Molecular biology and heterologous expression. Mutations were introduced by recombinant PCR as described previously (Accardi & Pusch, 2003). All constructs were in the PTLN vector (Lorenz *et al*, 1996). Human WT CLC-Ka and CLC-Kb, and their mutants, were coexpressed with mutant Y98A of barttin, leading to an enhancement of expression (Estévez *et al*, 2001).

Electrophysiology. Oocyte expression and electrophysiological measurements were performed as described previously (Pusch *et al*, 2000). Voltage-clamp data were acquired at 21–25 °C using the Pulse program (HEKA, Lambrecht, Germany) and a custom-built amplifier. Currents were recorded in a solution

Fig 5 | Phenotype of mutants. Typical voltage-clamp traces obtained with the pulse protocol described in Methods are shown for the indicated constructs. Horizontal scale bars indicate 50 ms (A–E) and 100 ms (F–J). Vertical scale bars indicate 5 μ A (A,C), 1 μ A (B,I), 3 μ A (D,E), 2 μ A (F), 4 μ A (G), 0.5 μ A (H) and 0.3 μ A (J). Expression levels varied significantly between mutants. In particular, CLC-Kb and its mutants had lower expression levels than those of CLC-Ka. The reason for this difference is unknown. (K,L) Dose–response relationships for 3-phenyl-CPP (K) and DIDS (L) for the indicated constructs. Lines are drawn according to equation (1) with the apparent K_D values reported in Table 1.

containing 90 mM NaCl, 1 mM MgCl₂, 10 mM CaCl₂ and 10 mM HEPES at pH 7.3. For the voltage-clamp measures, we used similar voltage-clamp pulse protocols for CLC-Ka and CLC-Kb with a longer pulse duration for CLC-Kb. Pulses were elicited from a holding potential corresponding to the resting membrane potential (–30 to –50 mV) using a prepulse to +60 mV for 100 or 200 ms (for CLC-Ka and CLC-Kb, respectively). This was followed by steps to various test values (from –140 to +80 mV with 20 mV increments) for 200 or 500 ms (for CLC-Ka and CLC-Kb, respectively) and a final tail pulse to –100 mV. 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).

Because CLC-Kb and several mutants had relatively low expression levels, we used the following strategy to estimate the contribution of endogenous currents. The current amplitude at +60 mV remaining in the presence of 100 mM iodide was taken as being purely endogenous. The leak at –140 mV was estimated by linear extrapolation of this leak conductance. These estimated leak currents were subtracted from the control currents in the absence and presence of an inhibitor to calculate the ratio $I(c)/I(0)$, where c is the concentration of the inhibitor. Apparent dissociation constants, K_D , were determined from the ratio of the steady-

state current in the presence and in the absence of the drug using the equation

$$I(c)/I(0) = 1/(1 + c/K_D). \quad (1)$$

For the determination of K_D , all data points, $I(c)/I(0)$, for a given substance, channel type and voltage were simultaneously fitted by equation (1) without previous averaging of the data points. Thus, all data points count equally in the fit. From this fit (using SigmaPlot, SPSS Inc.), an estimate of the confidence interval of the K_D value was obtained (reported in Table 1). For display, the $I(c)/I(0)$ values at a given concentration were averaged and plotted versus concentration (Fig 3).

DIDS was purchased from Sigma, and racemate 3-phenyl-CPP was synthesized in-house (Liantonio *et al*, 2004).

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