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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

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ABSTRACT

CLC channels are a gene family of Cl^- channels that serve a variety of functions, several of which are involved in genetic diseases. Few specific ligands of CLC channels are known that could be useful as pharmacological tools or potential drugs. We synthesized various derivatives of 2-(*p*-chlorophenoxy)propionic acid, the *S*(−)-enantiomer of which is a specific blocker of the muscle channel CLC-1. In particular, compounds with different alkyl or phenoxy-alkyl groups on the chiral center, isosteres of the oxygen in the aryloxy moiety, or bioisosteres of the carboxy function were prepared. We found that compounds containing a phenoxy and a phenoxy-alkyl group on the chiral center (bis-phenoxy derivatives) specifically inhibited renal CLC-K channels from the extracellular side with an affinity in the 150-μM range and with almost no effect on other CLC channels when applied from the outside. Surprisingly, the same substances inhibited CLC-1 from the intracellular side in a voltage-dependent manner with an apparent K_D of <5 μM at -140 mV, thus being the most potent blockers of a CLC channel known so far. Although the chlorine atom in *para*-position of the second phenoxy group was essential for inhibition of CLC-K channels from the outside, it could be substituted by a methoxy group without changing the potency of block for CLC-1 from the inside. These newly identified substances provide powerful tools for studying the structure-function relationship and the physiological role of CLC channels and may represent a starting point for the development of useful drugs targeting CLC-K channels.

The movement of Cl^- ions through CLC channels is 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., 1999; Maduke et al., 2000; George et al., 2001). The heterogeneity of biological actions in which CLC channels are involved is evidenced by various human genetic diseases that are caused by mutations in CLC genes. It is well known that mutations in *CLCN-1*, the gene encoding the muscle channel CLC-1, lead to myotonia congenita, a disease associated with electrical hyperexcitability of the muscle membrane (Koch et al., 1992). Moreover, CLC-Kb and CLC-5 channels play a pivotal role in renal physiology (Lloyd et al., 1996; Simon et al., 1997; Piwon et al., 2000). In particular, CLC-Kb is predominantly expressed at the basolateral side of the thick ascending limb, where it is involved in NaCl reabsorption. Mutations in the gene coding for CLC-Kb cause Bartter's syndrome, a severe salt-wasting disorder (Simon et al., 1997). Knockout studies have recently shed light on the function of several members of the CLC family, including CLC-2, CLC-3, CLC-5, and CLC-7 in various tissues such as brain, testis, and bone (Piwon et al., 2000; Bösl et al., 2001; Kornak et al., 2001; Stobrawa et al., 2001). In mice, the knockout of the CLC-Ka ortholog CLC-K1 led to diabetes insipidus, further stressing the importance of renal CLC channels (Matsumura et al., 1999).

A limitation to understanding the function of CLC channels is the lack of specific high-affinity ligands and modulators. To date, the only substances available are derivatives of the 2-(*p*-chlorophenoxy)propionic acid (CPP), which are molecules that stereoselectively modulate the macroscopic resting Cl^- conductance (gCl) of skeletal muscle as well as the

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ABBREVIATIONS: CPP, 2-(*p*-chlorophenoxy)propionic acid; gCl, Cl^- conductance of skeletal muscle; WT, wild-type; ΔNCLC-2, deletion mutant of rat CLC-2; hCLC, human CLC; D9, chimera CLC-Kb(D9)K1.

activity of CLC-1, the muscle Cl^- channel underlying gCl (Bettoni et al., 1987; Aromataris et al., 1999). As for native gCl, the *S*(−)-enantiomer is the most potent CPP-enantiomer able to block the currents of heterologously expressed human CLC (hCLC)-1 interfering with channel gating by acting from the intracellular side (Pusch et al., 2000). *S*(−)CPP inhibits individual protopores of the double-barreled structure of CLC-0 and CLC-1 channels. It binds much more strongly to closed channels than to open channels, resulting in a voltage-dependent block that is partially relieved by depolarization that opens the channels (Pusch et al., 2001).

In a previous study, we also demonstrated that substitutions on the chiral carbon atom of CPP changed the potency of the molecule, whereas all derivatives were selective modulators of CLC-1 and were ineffective on an N-terminal deletion mutant of CLC-2 and on CLC-5 (Pusch et al., 2000).

The renal CLC-K channels have so far not been characterized pharmacologically, mainly because of the difficulty in expressing the native channels in heterologous expression systems. Among the renal "CLC-K" channels, so far only the rat homolog CLC-K1 yields functional expression in heterologous expression systems (Waldegger and Jentsch, 2000). However, the macroscopic current amplitude is barely greater than background, rendering pharmacological studies practically impossible. Recently, Waldegger and Jentsch (2000) constructed chimeras of CLC-K1 (rat) and CLC-Kb (human) that yielded much larger expression. In the present study, we initially used these chimeras with the aim of testing first whether the previously studied CPP-like substances are effective on the renal CLC-K channels. We then constructed a whole variety of new derivatives of CPP that we screened as potential ligands of CLC-K and other CLC channels. We identified a particular set of bis-phenoxy derivatives in which a second phenoxy group has been introduced on the chiral center of CPP. These substances specifically inhibit CLC-K channels from the extracellular side. The inhibitory effect was also confirmed on WT rat CLC-K1 coexpressed with the recently identified CLC-K channel β-subunit barttin (Birkenhäger et al., 2001; Estévez et al., 2001). The same class of substances turned out to have a much higher affinity for CLC-1 than the mother compound when applied to the intracellular side.

Materials and Methods

Expression of CLC channels in *Xenopus laevis* Oocytes. The expressed CLC channels were hCLC-1 (Koch et al., 1992), a deletion mutant of rat CLC-2 in which residues 16 to 61 are deleted (Gründer et al., 1992; Pusch et al., 2000), and hCLC-5 (hCLC-5) (Lloyd et al., 1996). As a model for CLC-K channels, two chimeras between hCLC-Kb and rCLC-K1, described by Waldegger and Jentsch (2000), were initially used. The first one, CLC-Kb-D9-K1 (simply termed "D9") is formed by hCLC-Kb up to domain D9 and the rest by rCLC-K1, whereas the second one, CLC-Kb(c), contains an additional hCLC-Kb portion in the C terminus (for details, see Waldegger and Jentsch, 2000). In later experiments, WT rat CLC-K1 was coexpressed with human barttin (Estévez et al., 2001). Oocyte expression and electrophysiological measurements were performed as described previously (Pusch et al., 2000). Briefly, voltage-clamp data were acquired at room temperature (21–25°C) using the Pulse program (HEKA, Lambrecht, Germany) and a custom amplifier. All stereoisomeric substances were applied as racemic mixtures (with the exception of CPP in some experiments). Currents were recorded in a solution containing 100 mM NaCl, 5 mM $MgCl_2$, and 10 mM HEPES at pH 7.3. For the experiments with WT CLC-K channels, the extracellular solution contained 10 mM $CaCl_2$. Voltage-clamp pulses were elicited from a holding potential of −30 mV, using a prepulse to +60 mV for 100 ms followed by steps to various test values (from −140 to +80 mV in 20-mV increments) for 500 ms and a final tail pulse to −100 mV. Patch-clamp measurements were performed at 18 ± 1°C using the inside-out and outside-out configuration with an EPC-7 amplifier (List, Darmstadt, Germany) and the following solutions: intracellular solution, 100 mM *N*-methyl-D-glucamine-chloride, 2 mM $MgCl_2$, 10 mM HEPES, and 2 mM EGTA at pH 7.3; extracellular solution, 100 mM *N*-methyl-D-glucamine-chloride, 5 mM $MgCl_2$, and 10 HEPES at pH 7.3. For patch experiments with CLC-K1, the extracellular solution contained 5 mM $CaCl_2$ instead of 5 mM $MgCl_2$. Pulse protocols similar to those used for the two-electrode voltage clamp were used. Apparent dissociation constants, K_D, were determined by calculating the ratio of the steady-state current in the presence and in the absence of the drug and fitting the ratios at a fixed voltage by use of the equation

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

where *c* is the concentration. Errors in all figures are indicated as S.E.M.

Cl^- Conductance Measurements in Native Rat Skeletal Muscle Fibers. Determination of macroscopic Cl^- conductance (gCl) was made on isolated extensor digitorum longus muscle fibers of adult male Wistar rats by use of the two intracellular-microelectrode technique as detailed elsewhere (De Luca et al., 1992). Briefly, muscles were removed under urethane anesthesia and placed in a temperature-controlled muscle chamber at 30°C and bathed with a physiological solution bubbled with 95% O_2/5% CO_2, pH 7.2, in the absence and presence of the test compound (incubated for 30 min). The normal physiological solution contained 148 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.44 mM NaH_2PO_4, 12 mM $NaHCO_3$, and 5.5 mM glucose. The Cl^--free solution was made by equimolar substitution of methylsulfate salt for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$. Mean gCl was calculated as described previously (De Luca et al., 1992). The number of fibers was ≥19 for each compound.

CPP Derivatives. All derivatives of CPP were synthesized in our laboratory. The derivatives of classes 1, 2, and 4 (see Fig. 1) were synthesized as racemic mixtures by use of the procedures detailed elsewhere (Bettoni et al., 1987; Romstedt et al., 1996; Ferorelli et al., 1997; Carbonara et al., 2001). The derivative N8 of class 3 was synthesized starting from α,4-dichloroanisole and trimethylphosphite; compounds N9 and N10 were obtained by alkaline or acid hydrolysis of compound 8, respectively, according to the procedure described by Cornforth and Wilson (1994) and Bhattacharya and Thyagarajan (1981). The pK_a values of CPP (3.14) and the derivatives of class 4 (N11, 2.50; N12, 2.87; N13, 3.01; N14, 3.07; N15, 3.09; and N16, 3.01) have been calculated using the software Solaris V4.76 (Advanced Chemistry Development, Toronto, Ontario, Canada).

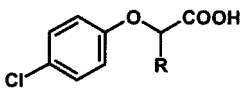
Results

Lack of Specific Block of CLC-K Channels by Extracellular CPP. We first tested the classic CPP and closely related derivatives (compounds N1 to N4 in Fig. 1A) on the chimeric CLC-K construct "D9" (see *Materials and Methods*) expressed in *X. laevis* oocytes using a two-electrode voltage clamp. The currents induced by expression of D9 are quite linear and are almost time-independent (Fig. 2A). Only a slight reduction after prolonged exposure to CPP could be observed (Fig. 2, A and B). As a control that the measured currents were indeed carried by the injected chimera and were not endogenous, we applied to all oocytes a solution in which 90 mM of extracellular Cl^- was replaced with I^-.

Iodide almost completely blocks the currents carried by the chimera (Fig. 2C) (Waldegger and Jentsch, 2000), whereas endogenous currents are not affected or slightly increased in the presence of iodide (data not shown).

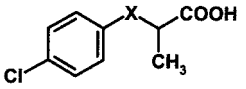
Because CPP inhibits CLC-1 strictly from the intracellular side (Pusch et al., 2001), it would be interesting to test whether CPP could also inhibit CLC-K channels from the inside. Unfortunately, the expression level obtained with the D9 chimera was too low to allow for patch-clamp recordings from oocytes. Also, in HEK-293 cells transfected with D9, the current amplitude was too low to allow the measurement of currents in excised patches that would permit a direct test of a possible intracellular action (data not shown).

A Class 1



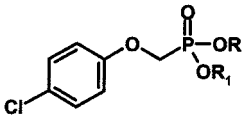
	N	R
CPA	1	H
CPP	2	CH_3
CPV	3	$(CH_2)_2CH_3$
CPPA	4	C_6H_5

B Class 2



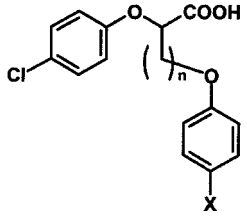
N	X
5	NH
6	CH_2
7	S

C Class 3



N	R	R_1
8	H	H
9	H	CH_3
10	CH_3	CH_3

D Class 4



N	n	X
11	1	Cl
12	2	Cl
13	3	Cl
14	4	Cl
15	5	Cl
16	3	OCH_3

Fig. 1. Chemical structures of different derivatives of CPP. Four different classes (A-D) were synthesized as described under *Materials and Methods*.

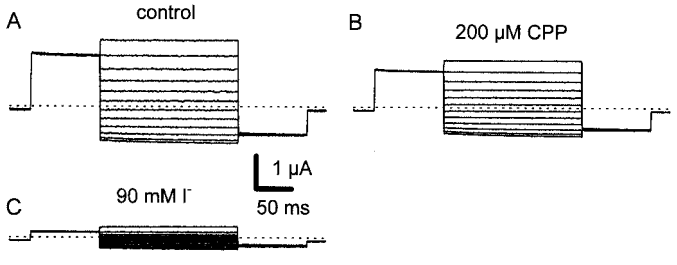


Fig. 2. Slight reduction of CLC-K chimera D9 by CPP. Voltage-clamp traces elicited by a pulse protocol as described under *Materials and Methods* are shown before (A) and after (B) the application of 200 μM *S*(−)-CPP. C, the currents measured after perfusing the oocyte with a solution in which 90 mM Cl^- was exchanged for I^-.

Derivatives of CPP (Classes 2–4). We next synthesized a variety of new derivatives of CPP modified in different parts of the molecular structure to evaluate whether isosteric substitutions of the oxygen of the phenoxy moiety (class 2; Fig. 1B), the substitution of the carboxylic moiety with an isosteric phosphonate group (class 3; Fig. 1C), or the introduction of a second phenoxy moiety at various distances from the chiral center (class 4; Fig. 1D) might increase the affinity for CLC-1 and show an inhibitory activity on the different CLC channels.

Effect of CPP Derivatives (Class 2–4) on Macroscopic gCl of Rat Skeletal Muscle Fibers and on Heterologously Expressed CLC-1. In a first screening, we tested the newly synthesized CPP derivatives for their effect on gCl of rat extensor digitorum longus skeletal muscle fibers. Each compound was tested at 100 μM, a value slightly exceeding the IC_{50} value of racemic CPP in blocking gCl (80 μM) (De Luca et al., 1992). All compounds of classes 2 and 3 were much less potent inhibitors of gCl compared with CPP, with the exception of the isosteric derivative N7 that showed a potency comparable with that of CPP (Table 1), as expected from its similar physicochemical properties. On the contrary, the effects of the compounds of class 4 were dependent on the length of the aliphatic chain, with the compound having three methylenic groups (derivative N13) being the most effective among these derivatives. The substitution of the chlorine atom with a methoxy group on the aromatic ring of the side chain did not affect the blocking activity (compare compounds N16 and N13 in Table 1). In accordance with the effects seen on gCl, none of the newly derived substances showed a larger inhibition of heterologously expressed CLC-1 compared with CPP when applied at a concentration of 200 μM from the extracellular side as measured in two-electrode voltage-clamp experiments (data not shown; see below for the large effect of class 4 substances on CLC-1 applied from the intracellular side).

Inhibition of CLC-K Chimera by Phenoxy-Alkyl Derivatives of CPP (bis-Phenoxy Derivatives). Among all derivatives tested, a significant effect on CLC-K channels was seen only with substances that contained two chlorophenoxy groups (class 4). An example using the phenoxyalkyl derivative N13 is shown in Fig. 3. The substance led to an immediate strong reduction of outward and inward currents at a concentration of 200 μM (Fig. 3A). The effect was almost fully reversible when the substance was washed out immediately (Fig. 3B), whereas no complete washout was obtained after prolonged exposure (data not shown). A similar or even stronger block was seen with the chimera CLC-Kb(c) that contains a larger portion of CLC-Kb (Fig. 3C). However, expression of the chimera CLC-Kb(c) was generally too low to allow a systematic study of its pharmacological properties. The block of the CLC-K chimera by compound N13 was slightly more pronounced at positive voltages than at negative voltages. The dose-response was evaluated separately at +60 mV (Fig. 3D, □) and at −140 mV (Fig. 3D, ●). The concentration-dependence could be well fitted by a simple titration curve (eq. 1) with the apparent inhibition constant, K_D, as a free parameter (solid lines in Fig. 3D), suggesting a simple 1:1 binding. The apparent K_D values were 170 μM at −140 mV and 120 μM at +60 mV. The difference

is small. It was observed, however, in each single oocyte, and a similar voltage-dependence was also seen for WT CLC-K1 channels in two-electrode voltage clamp and in patch-clamp experiments (see below).

Structure Activity Studies of Phenoxy-Alkyl Derivatives of CPP (bis-Phenoxy Derivatives). The finding that the phenylic derivative of CPP (CPPA, compound N4 in Fig. 1A) had no immediate effect on D9 (Table 1) suggests that just a second aromatic group is not sufficient to produce a block of currents of the CLC-K chimera.

We further tested the dependence of the affinity on the distance of the second chloro-phenoxy group from the chiral center. One to five methylenic groups were inserted between the chiral center and the second chloro-phenoxy group (compounds N11 to N15 in Fig. 1). As can be seen in Fig. 4, the largest effect was observed for $n = 3$ methylenic groups, with little difference, however, among the compounds between $n = 1$ to $n = 5$. To determine whether the chlorine atom of the second phenoxy group is essential for activity, we substituted it with a methoxy group in the compound with $n = 3$ (compound N16). This substitution led to almost complete loss of activity with an apparent K_D at +60 mV larger than 1 mM (data not shown).

Effect of Phenoxy-Alkyl Derivatives on WT CLC-K1 Channels. The recent discovery of the CLC-K channel β-subunit barttin allows for the study of WT CLC-K channels in heterologous expression systems (Birkenhäger et al., 2001; Estévez et al., 2001). To confirm that our results obtained with the chimeric channels apply also to WT CLC-K channels, we coexpressed human barttin with rat CLC-K1, the channel that gave the largest expression. Voltage-clamp recordings demonstrated that CLC-K1, similar to the chimera D9, is sensitive to extracellularly applied N13 (Fig. 5), whereas compound N16 was much less effective on CLC-K1 [K_D at 60 mV for N13: 108 ± 62 μM ($n = 4$); K_D at 60 mV for N16: 380 ± 140 μM ($n = 4$)].

Currents obtained by coexpression of CLC-K1 and barttin were large enough to allow for patch-clamp recordings. In Fig. 6A are shown currents from an outside-out patch in control (Fig. 6Aa), after application of 50 μM extracellular N16 (Fig. 6Ab) or 10 μM extracellular N13 (Fig. 6Ac). Extracellular application of N13 clearly leads to a reversible inhibition, whereas N16 is much less effective, in accordance with the voltage-clamp experiments. In contrast, N13 applied to the intracellular side in an inside-out patch experiment (Fig. 6B) was almost without effect.

Strong Inhibition of CLC-1 by Intracellular Phenoxy-Alkyl Derivatives of CPP (bis-Phenoxy Derivatives). Prompted by the effect of the phenoxy-alkyl derivatives on CLC-K channels, we investigated in more detail their effect on CLC-1. As already mentioned briefly above, compound N13 and other bis-phenoxy derivatives had almost no immediate effect on CLC-1 currents when applied from the outside in two-electrode voltage-clamp measurements (Fig. 7, A and B). Interestingly, the derivative N13 turned out to be a quite potent inhibitor of CLC-1 from the intracellular side (Fig. 7, C and D). The block was strongly voltage-dependent, similar to the inhibition by CPP (Fig. 7F). The apparent K_D was lower than 5 μM at −140 mV and increased to >100 μM at positive voltages.

The analog methoxy-derivative N16, which was almost ineffective on CLC-K from the outside (see above), was practically as equipotent as N13 in blocking CLC-1 from the inside (Fig. 7, E and F). A similar degree of inhibition was found also for the derivative N15 (data not shown).

These experiments show that the addition of a second phenoxy group to CPP strongly augments the affinity for CLC-1 by a factor of approximately 10. Furthermore, they show that the phenoxy-alkyl derivatives are not able to diffuse quickly into the oocyte when applied to the outside, because otherwise a strong inhibition of CLC-1 in two-electrode measurements would be observed in the presence of

TABLE 1

Effect of in vitro application of newly synthesized CPP derivatives on gCl and evaluation of their effect on heterologously expressed CLC channels

For the evaluation on heterologously expressed channels currents were measured using two-electrode voltage clamp as described under Materials and Methods; substances were applied at 200 μM. At least three oocytes were measured for each tested substance. For CLC-1 and D9, only the "short-term" effects are evaluated in the Table (i.e. effects after a brief incubation of <30 s). After a longer incubation time, significant effects were observed for CLC-1 for the strict derivatives of CPP (compounds N2 to N4) (Pusch et al., 2000). For CLC-5 and Δ NCLC-2, the effects were evaluated after about 5 to 10 min of incubation.

		Effect on CLC Channels Expressed in Oocytes				
Compound	Effect on Muscle gCl	CLC-1	D9	ΔNCLC-2	CLC-5	CLC-Kb(c)
	% reduction					
CPA (N1)		–	–	–	–	N.D.
CPP (N2)	58 ± 13*	–	N.D.	–	–	N.D.
CPV (N3)		–	N.D.	–	–	N.D.
CPPA (N4)		–	–	–	–	N.D.
N5	16 ± 6*	–	–	–	–	N.D.
N6	4 ± 6	N.D.	N.D.	N.D.	–	N.D.
N7	57 ± 10*	N.D.	N.D.	N.D.	–	N.D.
N8	15 ± 6*	N.D.	N.D.	N.D.	N.D.	N.D.
N9	2 ± 7	N.D.	–	N.D.	N.D.	N.D.
N10	0 ± 7	N.D.	N.D.	N.D.	N.D.	N.D.
N11	13 ± 7*	–	+	–	–	N.D.
N12	25 ± 8*		+	N.D.	N.D.	N.D.
N13	46 ± 10*	–	+	–	–	+
N14	33 ± 8*	–	+	N.D.	–	N.D.
N15	32 ± 8*	–	+	N.D.	–	N.D.
N16	48 ± 9*	–	–	N.D.	N.D.	N.D.

* Significantly different with respect to the control value obtained in the absence of drug ($P < 0.05$).
+, Tested and effective (for details in these cases, see text).
–, Tested and no or only slight effect (less than 30% block).
N.D., not determined.

200 μM, a concentration that is almost 100-fold larger than the apparent K_D at −140 mV.

Effect of Class 1 to 4 Derivatives on ΔN-CLC-2 and CLC-5 in Two-Electrode Voltage-Clamp Measurements. None of the substances tested on CLC-5 or ΔN-CLC-2 had any significant blocking effect at the concentration tested (200 μM), even after prolonged exposure (up to 10 min) (Table 1).

Discussion

Cl^- channels remain the "poor cousins" (Gadsby, 1996) of cation channels, particularly with respect to the availability of specific and high-affinity drugs. In our previous studies, we explored the effect of CPP and close derivatives on some CLC channels (Pusch et al., 2000). As suggested by its inhibitory action on the Cl^- conductance of intact skeletal muscle fibers (Conte Camerino et al., 1988), we have found that the *S*(−)-enantiomer of CPP and of the derivatives belonging to class 1 (Fig. 1A) inhibit CLC-1 in a strongly voltage-dependent manner, acting strictly from the intracellular side (Pusch et al., 2000). Apart from CLC-0, the other two CLC channels tested before (CLC-2 and CLC-5) where much less affected. The sensitivity of the renal "CLC-K" homologs to CPP had not been investigated previously.

In an attempt to find inhibitors for the renal CLC-K channels, we tested in this study a variety of derivatives of CPP initially using chimeras of CLC-K1 and CLC-Kb as a model for CLC-K channels (Waldegger and Jentsch, 2000), and we identified a particular group of substances that are characterized by the presence of two *p*-chloro-phenoxy groups differently joined to the chiral center (Fig. 1D). These bis-phenoxy derivatives were found to inhibit CLC-K chimeras from the extracellular side with an affinity in the 150-μM range, whereas none of the other channels tested exhibited a similar rapid and strong block by this class of substances. These substances are unique in that they seem to be specific for CLC-K channels as blockers from the extracellular side. We initially used chimeras of the rat CLC-K1 and the hCLC-Kb channels that were described by Waldegger and Jentsch (2000) because the respective wild-type channels either do not express functionally or yield only very small current amplitudes, making a systematic testing of inhibitors practically impossible.

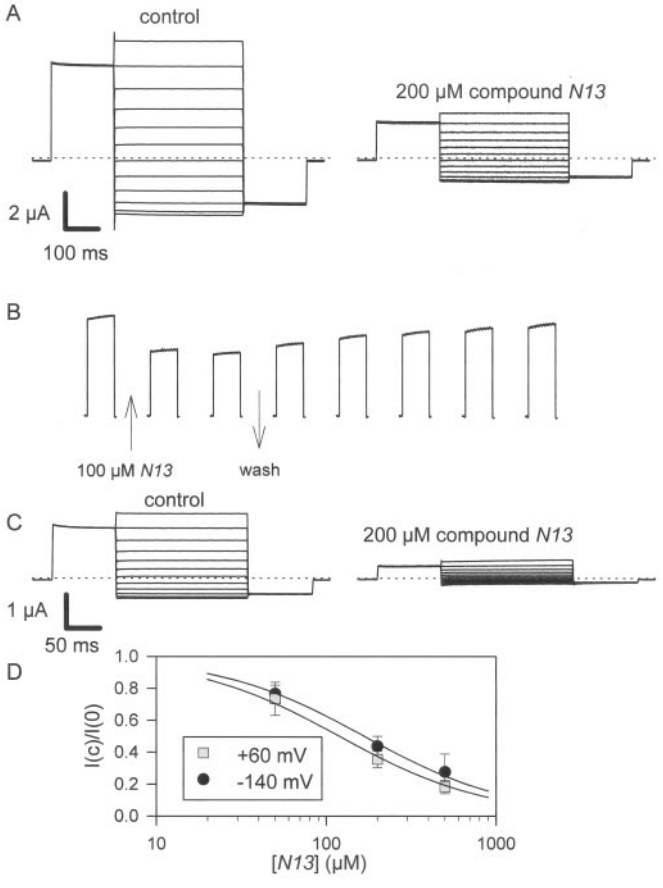


Fig. 3. Inhibition of CLC-K chimeras by phenoxy-alkyl derivatives of CPP. A, effect of compound N13 on chimera D9. B, partial reversibility of the block by phenoxy-alkyl derivatives. Repeated pulses (once every 2 s) were applied up to +60 mV to an oocyte expressing the D9 chimera. After the first pulse shown, 100-μM compound N13 was applied (arrow), leading immediately to a significant reduction that was largely but not fully reversible. C, block of the chimera CLC-Kb(c) by compound N13. D, dose-response relationship of the block of D9 by N13 at −140 mV (●) and +60 mV (□). Plotted are the mean values ($n \geq 4$) of the ratio of the current measured in the presence of the substance and the current in control. The solid lines are fits to eq. 1 with the K_D values reported in the text.

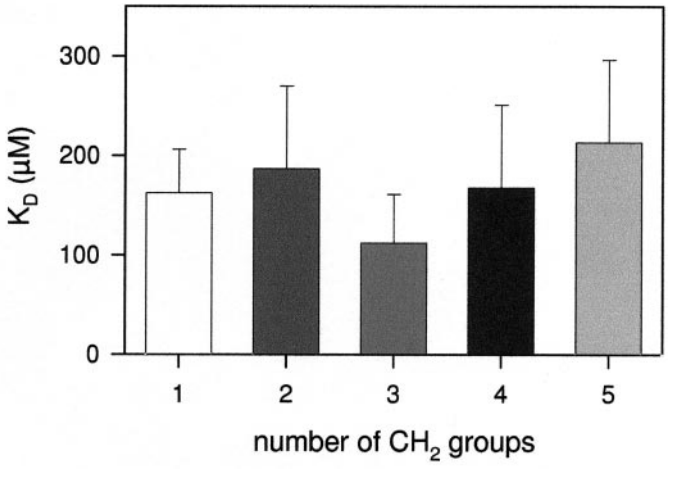


Fig. 4. Structure-activity relationship for the block of CLC-K channels by phenoxy-alkyl derivatives. The apparent K_D at +60 mV is plotted as a function of the number of methylenic groups inserted between the chiral carbon atom and the oxygen atom (compounds N11 to N15). At least four oocytes were tested for each substance.

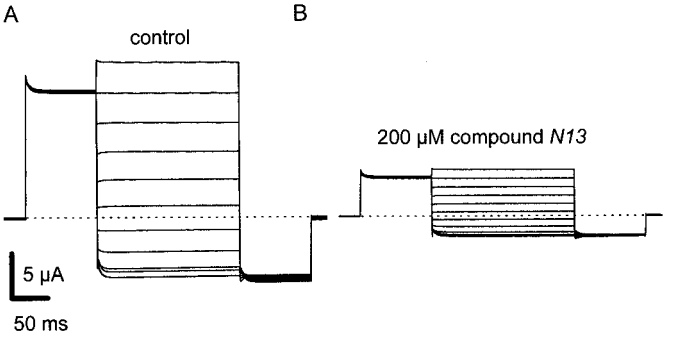


Fig. 5. Block of WT CLC-K1 coexpressed with barttin by extracellular N13. Two-electrode voltage-clamp traces are shown before (A) and after (B) application of 200 μM N13. From similar experiments with 50 μM and 200 μM N13 and N16, mean values of the apparent inhibition constant were determined using eq. 1 at 60 mV as K_D at 60 mV for N13: 108 ± 62 μM ($n = 4$); K_D at 60 mV for N16: 380 ± 140 μM ($n = 4$).

Very recently, a β-subunit of CLC-K channels has been identified whose coexpression with CLC-Ka, CLC-Kb, and CLC-K1 greatly increases the current magnitude in heterologous systems, and that is most probably necessary for a proper function of CLC-K channels in vivo (Estévez et al., 2001). By coexpressing human barttin, we could confirm the inhibitory effect of the bis-phenoxy derivatives on WT CLC-K1 channels. Furthermore, using excised patch-clamp measurements of CLC-K1, we could unequivocally demonstrate that the compounds act from the extracellular side. The affinity of CLC-K1 seemed to be larger in patch-clamp experiments compared with that measured in two-electrode voltage-clamp experiments (compare Figs. 5 and 6). This could be caused by a hindered access from the presence of the vitelline membrane in the whole-oocyte experiments.

Although the bis-phenoxy derivatives are specific inhibitors of CLC-K channels from the outside, they are the most potent inhibitors of CLC-1 when applied from the intracellular side, being until now the ones with the highest affinity for a CLC channel. In fact, measurements of gCl of muscle fibers did not reveal a particularly elevated affinity of CLC-1 for these derivatives, possibly because of a slow diffusion into the muscle fibers. Inside-out patch measurements, however, showed that the affinity of the bis-phenoxy derivatives is increased at least 10-fold compared with the *S*(−)-enantiomer of CPP. The high affinity for CLC-1 leads us to hypothesize the presence of a second hydrophobic pocket where the second phenoxy moiety could be allocated, further stabilizing the interaction with the intracellular binding site. In addition, the interaction with this hypothesized hydrophobic pocket seems not to be affected by the electric cloud of the phenoxy moiety of the side chain because the substitution of the chlorine atom with a methoxy group did not affect its blocking potency for CLC-1. The qualitative properties, including the voltage-dependence and binding/unbinding kinetics of this inhibition, were similar to those described previously for CPP (Pusch et al., 2000) and do not seem to represent a new type of mechanism.

On the contrary, the mechanism of block of the CLC-K chimera by the bis-phenoxy derivatives from the outside seems to be quite different from the internal block produced on CLC-1. In particular, there is only a very small voltage-dependence of block, in contrast to the extreme voltage-dependence of the block of CLC-1, that is probably caused by the different biophysical properties of the channels. Furthermore, our structure-function analysis has revealed very different molecular requisites for the respective block of CLC-K and CLC-1. The substitution of chlorine with a methoxy group on the aromatic ring of the side chain, which did not affect the affinity for CLC-1, led to a drastic reduction of

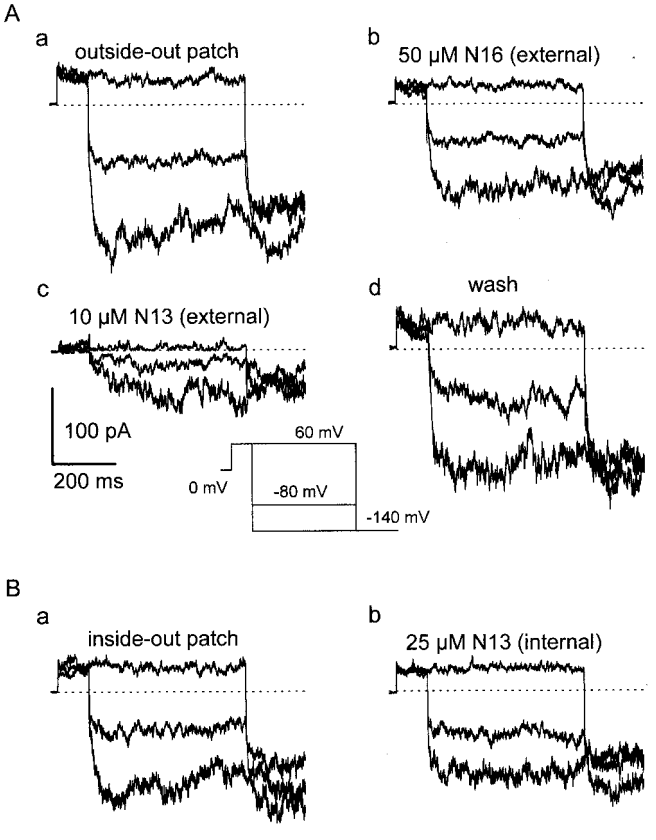


Fig. 6. Effects of externally and internally applied phenoxy-alkyl derivatives on CLC-K1 coexpressed with human barttin measured with patch clamp. A, results from an outside-out patch. Aa, control; Ab, application of 50 μM extracellular N16; Ac, application of 10 μM extracellular N13; Ad, washout. B, results from an inside-out patch before (Ba) and after (Bb) application of 25 μM N13. The voltage-clamp protocol is indicated in the inset. The scale bars apply to the entire figure. Similar results were obtained in three patches for each condition (inside-out and outside-out).

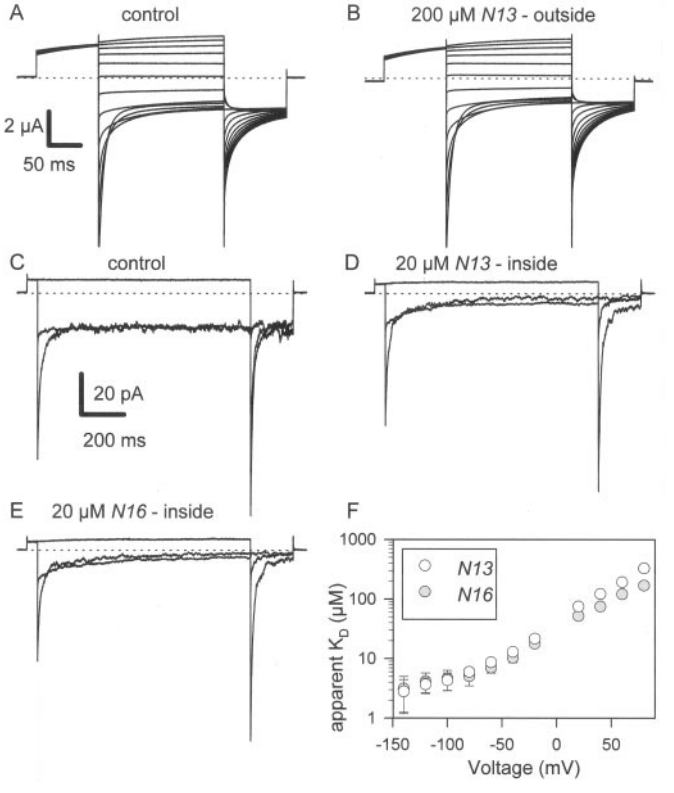


Fig. 7. Effects of externally and internally applied phenoxy-alkyl derivatives on CLC-1. Two-electrode voltage-clamp traces elicited by a pulse-protocol as described under *Materials and Methods* are shown before (A) and after (B) extracellular application of 200 μM N13. C-F, results from inside-out patch clamp recordings in control (C), in the presence of 20 μM compound N13 (D), and in the presence of 20 μM compound N16 (E) (traces are from the same patch). For clarity, only the current traces corresponding to −140, −60, and +60 mV are shown. F, mean values (n = 3) of the apparent inhibition constant obtained by fitting eq. 1 to the inhibition data from the inside obtained at 10, 20, and 50 μM as a function of voltage. Scale bars in A also apply to B, and scale bars in C also apply to D and E.

block of CLC-K channels. Because the chlorine atom is more electronegative than a methoxy group, the aromatic ring of derivative N16 is probably richer in electrons than that of compound N13. This could weaken a hypothetical π- π interaction with an aromatic amino acid present at the binding site. Above all, the presence of two chlorophenoxy groups seems to be a fundamental requirement for drug activity on CLC-K, justifying the lack of effect of extracellularly applied CPP on these renal channels.

It seems to be a coincidence that the very same substance inhibits channels of the same family from opposite sides of the membrane. It will be interesting in future studies to investigate the respective binding sites on the channel proteins using in this way the drugs as a tool to explore the pore structure. Most probably, the negatively charged carboxylic acid group is directly responsible for the pore block, whereas the hydrophobic aromatic rings stabilize the interaction with the channel. Future experiments with chemically altered compounds and mutated channels will allow the establishment of the binding sites of the inhibitors and the mechanisms of inhibition. The recently determined three-dimensional structure of bacterial CLC homologues (Dutzler et al., 2002) will greatly aid such studies.

Because the phenoxyalkyl (bis-phenoxy) derivatives of CPP specifically inhibited CLC-K channels from the outside with practically no effect on CLC-1, CLC-2, and CLC-5, they might be useful for defining the physiological role of these largely unexplored channels and could represent a good starting point for the development of therapeutically useful drugs targeted at CLC-K channels.

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