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Targeting kidney CLC-K channels: Pharmacological profile in a human cell line *versus Xenopus* oocytes

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ABSTRACT

CLC-K chloride channels play a crucial role in kidney physiology and genetic mutations, affecting their function are responsible for severe renal salt loss in humans. Thus, compounds that selectively bind to CLC-Ka and/or CLC-Kb channels and modulate their activity may have a significant therapeutic potential. Here, we compare the biophysical and pharmacological behaviors of human CLC-K channels expressed either in HEK293 cells or in *Xenopus* oocytes and we show that CLC-K channel properties are greatly influenced by the biochemical environment surrounding the channels. Indeed, in HEK293 cells the potentiating effect of niflumic acid (NFA) on CLC-Ka/barttin and CLC-Kb/barttin channels seems to be absent while the blocking efficacy of niflumic acid and benzofuran derivatives observed in oocytes is preserved. The NFA block does not seem to involve the accessory subunit barttin on CLC-K1 channels. In addition, the sensitivity of CLC-Ks to external Ca^{2+} is reduced in HEK293 cells. Based on our findings, we propose that mammalian cell lines are a suitable expression system for the pharmacological profiling of CLC-Ks.

1. Introduction

Human kidney CLC-Ka and CLC-Kb chloride channels (and their rat orthologs CLC-K1 and CLC-K2) are located in Henle's loop, in the distal convoluted tubule and in the cortical collecting duct of the nephron where they govern chloride (Cl^-) absorption and urine concentration [1]. In humans, loss-of-function mutations of the genes encoding CLC-Kb (*CLCNKB*) and the barttin regulatory subunit are responsible for the recessive Bartter syndrome, characterized by urinary salt loss [2,3]. Furthermore, *CLCNKA* and *CLCNKB* gain-of-function polymorphisms have been proposed to be associated with salt sensitive hypertension [4,5]. Thus, the identification of selective CLC-K ligands that can be potentially used in therapy is highly desirable. In parallel, the need of a suitable expression system for a detailed biophysical and pharmacological characterization of human CLC-K channels has proven to be essential to translate *in vitro* pharmacological results to the *in vivo* context.

During the past years, different cellular systems have been tested in order to characterize CLC-K currents. So far, the only data regarding native CLC-K currents have been obtained from rodent renal tubules recordings [6-8] which are highly relevant because preclinical drug screening is mostly performed in these animal models. However, as the precise molecular identity of native rodent channels remains elusive *i.e.* it is unknown if they are mediated by CLC-K1, CLC-K2 or by heteromers of these, a precise comparison with recombinant human channels is still not possible [9]. Furthermore, mouse CLC-K1 and human CLC-Ka might not necessarily behave similarly.

Therefore, most of the information on the pharmacological properties of human CLC-K channels comes from *in vitro* studies performed in *Xenopus* oocytes. For instance, this cellular system allowed to assess that CLC-Ka and CLC-Kb channels require the barttin subunit to become anion conducting [10], whereas rodent CLC-K1 channels show functional expression even in the absence of barttin [11,12]. Moreover, in *Xenopus* oocytes CLC-K/barttin channels are also modulated by multiple signaling cascades, such as the Ser/Thr serum- and glucocorticoid-dependent protein kinases [13]. Remarkably, the expression of human CLC-K channels in *Xenopus* oocytes allowed the rapid screening of novel molecules acting as openers or inhibitors [14,15]. In this framework, we recently discovered that phenyl-benzofuran carboxylic acid derivatives are the most potent CLC-Ka and CLC-Kb inhibitors so far described [15], whereas niflumic acid (NFA) is the most effective activator of human CLC-K channels [14,16]. These data suggested the presence of two distinct binding sites, an activating site and a blocking site [16].

However, when CLC-K channels have been expressed in mammalian cells, several functional differences have been observed. Indeed, while CLC-K channels showed clear voltage-dependent gating in *Xenopus* oocytes, their current kinetics were less pronounced in mammalian cells [9,10,12,15,17–21]. The reasons for this difference are not clear. In

Abbreviations: NFA, niflumic acid; HEK, human embryonic kidney; MDCK, Madin Darby Canine Kidney; ATP, adenosine 5′-triphosphate; DCT, distal convoluted tubule

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addition the pharmacological profile of CLC-K channels expressed in mammalian cell lines is unknown.

Here we provide, for the first time, a pharmacological characterization of CLC-K channels expressed in HEK293 cells. We investigate human CLC-Ka and CLC-Kb as well as rodent CLC-K1 expressed with and without barttin and record their response to blockers and activators, to extracellular pH, Ca^{2+}, kinases and membrane fluidity. By performing a parallel analysis in mammalian cells and *Xenopus* oocytes, we obtained a comprehensive picture of CLC-K channel properties based on the distinct expression system. Our results validate mammalian cell lines as a suitable expression system to test novel chemical compounds targeting human CLC-Ks.

2. Materials and methods

2.1. Electrophysiology

Expression and electrophysiological measurements in oocytes were performed as previously described [14]. WT CLC-Ka, CLC-Kb and CLC-K1 RNAs were co-expressed in *Xenopus* oocytes with the human Y98A barttin in a 1:1 weight ratio (~5 ng each). Currents were recorded in the standard solution containing (in mM) 90 NaCl, 10 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES at pH 7.3. Similar voltage-clamp pulse protocols for CLC-Ka and CLC-Kb, with a longer pulse duration for CLC-Kb, were used: from a holding potential of −30 mV, after a prepulse to 60 mV (or −100 mV) for 100 or 200 ms (for CLC-Ka and CLC-Kb respectively), voltage was stepped from −140 to 80 mV in 20 mV increments for 200 or 500 ms (for CLC-Ka and CLC-Kb respectively), followed by a final tail pulse to −100 mV (or +60 mV). To evaluate the on-set and wash-out of drug effects a pulse to 60 mV was applied every 2 s. The half maximal inhibitory concentrations for drugs showing blocking activity, IC50, were determined by calculating the ratio of the steady-state current with and without the drug and fitting the ratios to the equation: $I(c) / I(0) = 1 / (1 + c / IC50)$ where c is the concentration. For drugs showing an IC50 > 1 mM, we did not determine the accurate IC50 value because it would require large amounts of drug, and it falls outside of the aims of this study. Methyl-beta-cyclodextrin (Sigma Aldrich, St. Louis, MO) and fluvastatin (Sigma Aldrich, St. Louis, MO) were dissolved in the oocyte maintenance solution containing (in mM): 90 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES at pH 7.5. To evaluate the effect of fluvastatin on CLC-Ka in *Xenopus* oocytes, currents were measured for a large number of oocytes (resulting in I_0 for each oocyte), half of which were then exposed to 100 μM fluvastatin for 16 h. After the 16 h period, currents increased for both groups due to increased surface expression of channels resulting in current ratios $r_{Ctrl} = I_{Ctrl} / I_0 = (1.8 \pm 0.1\ SE)$ for the control group and $r_{Fluva} = I_{Fluva} / I_0 = (1.7 \pm 0.3\ SE)$ for the fluvastatin treated group. The current increase was very similar for both groups as shown in Fig. 6 which displays the ratio $r_{Fluva} / r_{Ctrl} = (0.96 \pm 0.2\ SE)$. To evaluate the effect of cyclodextrin, oocytes expressing CLC-Ka currents were measured in standard solution before and after incubation in 1 mM methyl-beta-cyclodextrin (90 min; $r_{cyclodex} / r_{CTRL} = 1.1 \pm 0.05$, $n = 7$).

For mammalian cell expression the constructs were subcloned in the pcDNA3 or pRc/CMV vector. HEK293 cells were transfected with plasmid DNA encoding CLC-Ka and human Y98A barttin in a 1:1 weight ratio using a Ca^{2+} phosphate precipitation method or the Effectene reagent (Qiagen). For the identification of transfected HEK293 cells, a plasmid encoding the CD8 antigen or, alternatively, a plasmid encoding GFP was co-transfected. The transfected cells were identified by microbeads coated with anti-CD8 antibodies (Dynabeads M-450 CD8; Dynal, Great Neck, NY) or by their fluorescence emission. Electrophysiological experiments were performed typically 1–3 days after transient transfection. Whole-cell patch-clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) or an EPC-7 (List, Darmstadt) as previously described [10,12,17]. Pipettes were pulled from borosilicate glass (Harvard Apparatus, Holliston, MA) and had resistances of 2.2 to 3.2 MΩ. The series resistance was compensated 60 to 80% by an analog procedure, such that the estimated voltage error as a result of access resistance was always<2 mV. The extracellular solution contained (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES, whereas the pipette solution contained 120 NaCl, 2 $MgCl_2$, 5 EGTA, and 10 HEPES. Both solutions were adjusted to pH 7.4 with NaOH. For the experiments on MDCK cells and experiments concerning the Ca^{2+} and pH effect, the extracellular standard solution contained (in mM): 145 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES (pH 7.3) (osmolarity, 271 mOsm). To study Ca^{2+} dependence of CLC-Ks the concentration of $CaCl_2$ in the extracellular solution was varied between 0.1 and 30 mM, adjusting the osmolarity by mannitol and keeping the chloride concentration constant. HEPES was replaced by MES buffer in solutions at pH < 7. The intracellular solution contained (in mM): 130 CsCl, 2 EGTA, 2 $MgCl_2$, and 10 HEPES (pH 7.3) (osmolarity 233 mOsm). MDCK cells developed rather large endogenous currents 1 day after splitting. However, at day 1 after transfection and few hours after splitting endogenous currents were negligible and allowed recordings of heterologously expressed CLC-Ka/barttin channels. Patch recordings in HEK293 cells were obtained by voltage pulses from a holding potential of 0 mV to various test potentials, from −120 to +100 mV for 400 ms. Pulses ended with a tail pulse to −80 mV. For MDCK recordings (Fig. 3C), after a prepulse to 60 mV for 100 ms, channels were stimulated with potentials ranging from −140 to 80 mV with 20 mV increments for 1000 ms. Pulses ended with a tail pulse to 80 mV for 200 ms. For CLC-K1 recording (Fig. 5), after a prepulse to 65 mV for 200 ms, channels were stimulated with potentials ranging from −120 to 100 mV with 10 mV increments for 400 ms. Pulses ended with a tail pulse to 65 mV. To evaluate niflumic acid (Fig. 3A), [Ca^{2+}] and kinases effects (Fig. 6A, E), CLC-Ks were stimulated with repetitive 10 ms pulses to 60 mV delivered once per second. Current traces were filtered at 1 kHz with a 4-pole low-pass Bessel filter and acquired at 5 kHz with the Clampex + Clampfit program (Molecular Devices) or with GePulse (custom).

2.2. Chemical synthesis

RT-93 was synthesized in our laboratory. For the electrophysiological recordings, compounds were dissolved in DMSO daily, and diluted to the final concentration in the extracellular solution. DMSO never exceeded 0.2%, a concentration without effect on CLC-K channels.

2.3. Statistical analysis

Data are shown as mean ± SEM, unless specified otherwise; *n* reflects the number of cells. The unpaired *t* test was used to compare the mean values, and $P < 0.05$ or $P < 0.01$ was used to indicate statistical significance.

3. Results

3.1. Whole-cell recordings of CLC-K channels

In agreement with previous studies, when expressed in *Xenopus* oocytes, both CLC-Ka and CLC-Kb channels show time- and voltage-dependent gating (Fig. 1A, B) [10,13,15,19]. Differently, CLC-Ka and CLC-Kb channels expressed in HEK293 give rise to currents with a linear current–voltage relationship (Fig. 1C, D) [17], suggesting that CLC-Ks may behave as constitutively open channels in this cellular system.

3.2. Pharmacological characterization of CLC-K channels

The different biophysical behavior of CLC-Ks in HEK293 cells compared to that in oocytes prompted us to investigate the pharmacological properties of these channels in mammalian cells, using known CLC-K blockers and openers.

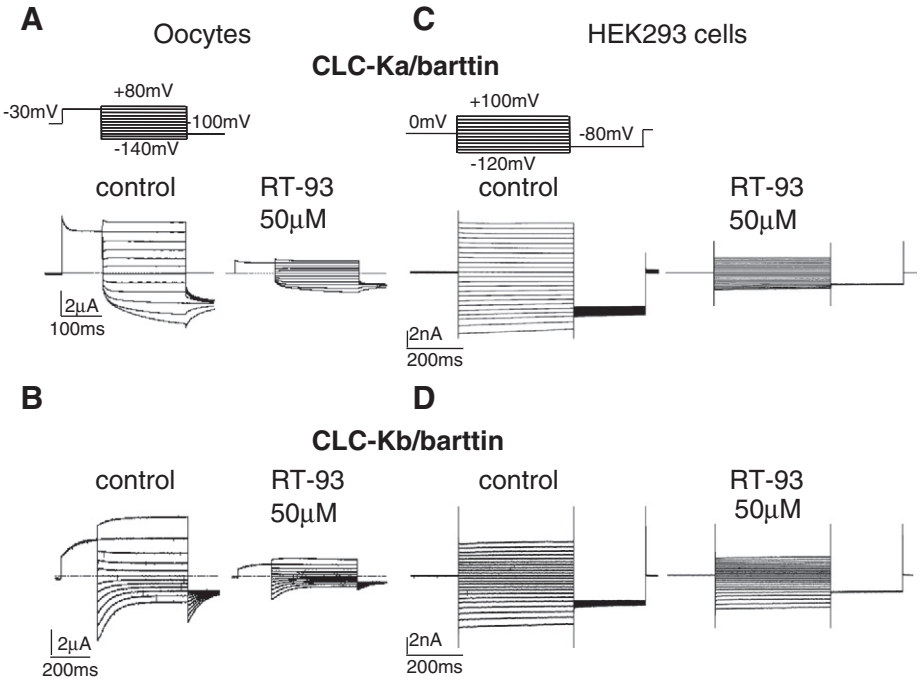

Fig. 1. Benzofuran derivatives inhibit CLC-K channels. Representative current traces of CLC-Ka (*upper panel*) and CLC-Kb (*lower panel*) channels before and after the perfusion of 50 μM RT-93 in *Xenopus* oocytes (A, B) and HEK293 cells (C, D).

3.2.1. Blocking binding site

We have previously demonstrated that the noncoplanar phenylbenzofuran carboxylic acid derivatives are the most potent CLC-Ka inhibitors described so far in *Xenopus* oocytes [15]. Specifically, in a screening of different derivatives, we observed that the compound RT-93 (inset in Fig. 2) showed the highest blocking efficacy, with a concentration of 50 μM able to reduce CLC-Ka and CLC-Kb currents by ~85% in *Xenopus* oocytes (Fig. 1A, B) [15]. When tested in HEK293 cells, the same RT-93 concentration reduces CLC-Ka and CLC-Kb currents by ~80% and ~67%, respectively (Fig. 1C, D). These data indicate that this compound inhibits human CLC-K channels in both cellular systems with a similar affinity (CLC-Ka: IC50 = 7.0 ± 0.9 μM in oocytes and IC50 = 11.3 ± 2.3 μM in HEK293 cells; CLC-Kb: IC50 = 6.0 ± 0.9 μM in oocytes and IC50 = 15.1 ± 3.6 μM in HEK293 cells). The efficacy of benzofuran blockers and the conclusion that a blocking binding site is present in HEK cells are further supported by the observation that other potent benzofuran derivatives, such as MT-189 and JBL-44, are also able to inhibit CLC-Ka and CLC-Kb currents to the same extent as the inhibition observed in oocytes [15] (Fig. 2).

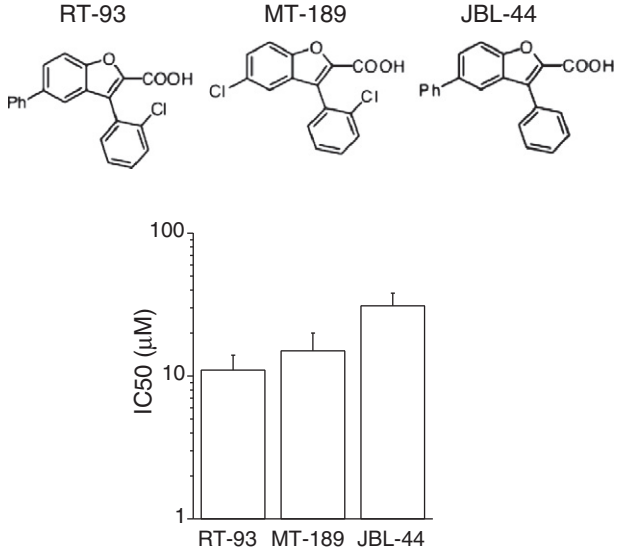

Fig. 2. Effect of benzofuran derivatives on CLC-Ka. The mean values of the IC50 measured at +60 mV for RT-93, MT-189 and JBL-44. The chemical structures of the tested compounds are shown in the upper panel. Data are mean ± SE of $n = 6–12$ cells.

3.2.2. Activating binding site

CLC-Ka in *Xenopus* oocytes is greatly potentiated by [NFA] <1 mM but it is blocked by [NFA] ≥1 mM [14,15], thus displaying a biphasic pharmacological response. This suggests that the mechanism of action of NFA is complex [16] and probably involves the binding to two different binding sites, with opposite effcects [19]. Surprisingly, in HEK293 cells, the application of 300 μM NFA blocks CLC-Ka by ~70% (Fig. 3A). Current inhibition is observed at all tested concentrations in the range between 0.3 μM and 1 mM with an estimated IC50 of ~200 μM (Fig. 3B). In Fig. 3B we illustrate the biphasic response to NFA of CLC-Ka in *Xenopus* oocytes, superimposed with the pure blocking effect in HEK293 cells. We next tested if we could recover NFA-mediated activation of CLC-Ka in a renal epithelial cell line, MDCK cells. However, as in HEK293, also in MDCK cells, CLC-Ka currents exhibit a linear current–voltage relationship (Fig. 3C) and are blocked by NFA with an apparent IC50 of ~200 μM (Fig. 3C, D).

In oocytes, the activating effect of NFA was even more pronounced for CLC-Kb. Indeed, NFA potentiated CLC-Kb even at concentrations higher than 1 mM [14]. In contrast, in HEK293 cells, CLC-Kb is always inhibited with an apparent IC50 of ~300 μM (Fig. 4).

3.3. Insight into the expression system-dependent activity and pharmacology of CLC-K channels

The distinct biophysical and pharmacological profile of human CLC-Ks expressed in HEK293 cells and *Xenopus* oocytes might be influenced by a different channel modulation by extracellular and intracellular

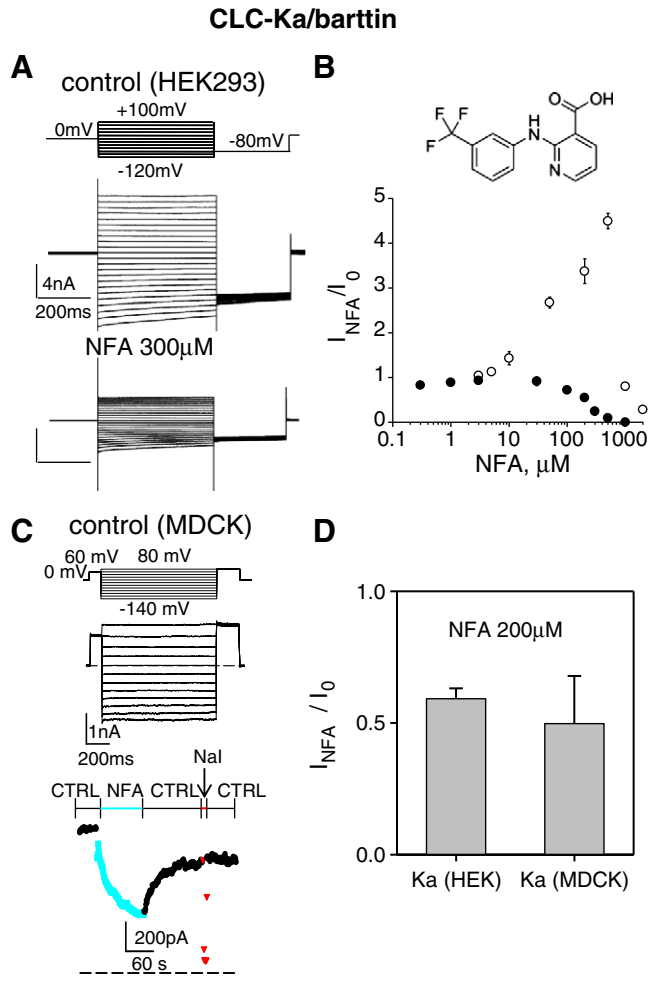

Fig. 3. NFA does not activate CLC-Ka expressed in HEK293 and MDCK cells. (A) CLC-Ka whole-cell patch-clamp traces before and after the perfusion of 300 µM NFA in HEK293 cells. (B) Comparison of the dose–response relationship of NFA (in the inset) for CLC-Ka channels expressed in oocytes (empty circles) and HEK293 cells (filled circles), measured at +60 mV. Data are mean ± SE of $n = 10$–15 cells. (C) Typical current traces of CLC-Ka expressed in MDCK cells in control solution (upper panel) and effect of 200 µM NFA on CLC-Ka in MDCK cells (bottom panel). The mean current was plotted as function of time. Different colors and symbols correspond to the solutions applied during the experiment (CTRL: control; NFA: niflumic acid; NaI: blocking solution) as indicated by the bar on top of the figure. (D) Comparison of the effect of 200 µM NFA on CLC-Ka expressed in HEK293 ($n = 5$) and MDCK cells ($n = 4$). Steady-state currents measured in NFA were normalized to those measured in the absence of NFA. Error bars indicate SD.

factors such as the accessory subunit barttin, Ca^{2+} and H^+ ions, kinases and by the lipid membrane composition.

3.3.1. Role of barttin in NFA-mediated effect

Therefore, we first tested whether the distinct pharmacological properties of CLC-K channels expressed in HEK293 cells may be due to a different interaction with the accessory barttin subunit, whose role in modulating channel gating is not yet well understood [10,21]. Interestingly, CLC-K1 is the only CLC-K channel giving rise to detectable currents even in the absence of barttin [10,21], and, differently from CLC-Ka and CLC-Kb, CLC-K1 is always inhibited by NFA in *Xenopus* oocytes [22]. Therefore, we expressed CLC-K1 channels in HEK293 cells alone or co-transfected with barttin. In agreement with previous studies [22], NFA inhibits CLC-K1/barttin channels at all tested concentrations (Fig. 5A). The application of 300 µM NFA to cells expressing CLC-K1 without barttin still induces a block with a similar potency (Fig. 5B, C). These results suggest that barttin does not modulate NFA block at least on CLC-K1.

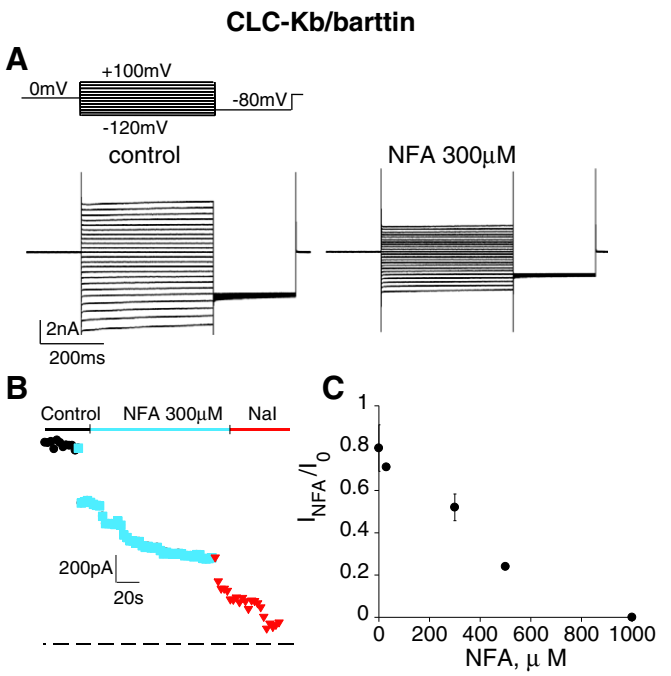

Fig. 4. NFA does not activate CLC-Kb currents in HEK293 cells. (A) CLC-Kb whole-cell patch-clamp traces before and after 300 µM NFA in HEK293 cells. (B) Time-course of NFA inhibition of CLC-Kb channels expressed in HEK293 cells, monitored at +60 mV. Different colors correspond to the different solutions applied during the experiment as specifically indicated by the bars on top of the figure. (C) Dose–response curve of NFA inhibition of CLC-Kb channels expressed in HEK293 cells. Data are mean ± SE of $n = 10$ cells.

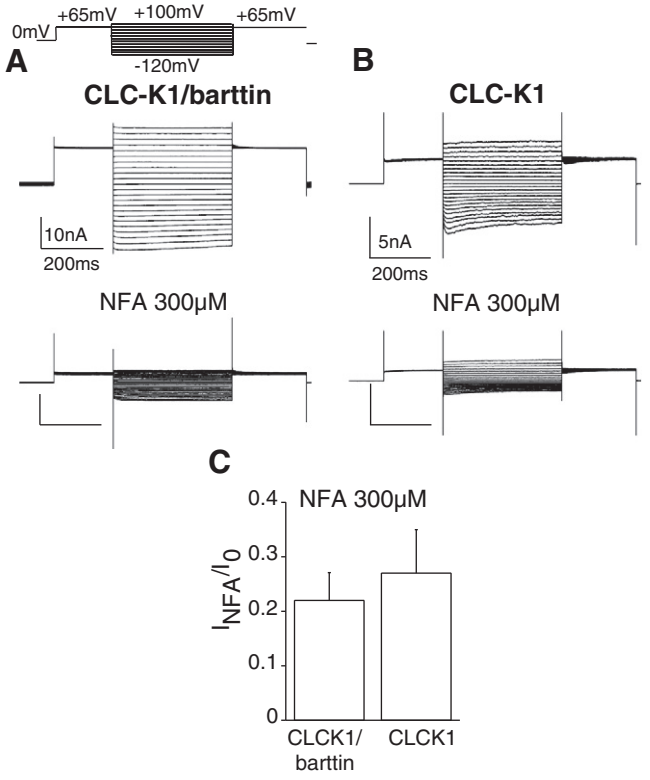

Fig. 5. Barttin does not affect CLC-K1 inhibition by NFA. Representative whole-cell patch-clamp traces of CLC-K1 channels expressed with (A) and without (B) barttin in HEK293 cells, before and after the application of 300 µM NFA. (C) Bar graph of 300 µM NFA inhibition of CLC-K1 channels expressed in HEK293 cells, with and without barttin. Data are mean ± SE of $n = 6$ cells.

3.3.2. Ca^{2+}, pH and protein kinase modulation

We next explored whether a different modulation by extracellular Ca^{2+}, pH and protein kinases could contribute to the different behavior of CLC-K channels in oocytes and mammalian cell lines. Human, rat, and mouse CLC-K channels are activated by extracellular Ca^{2+} when expressed in *Xenopus* oocytes [10,11,23]. Recently, the Ca^{2+} binding site was identified through an extensive mutagenic screen and homology modeling of CLC-Ka, [23,24]. Surprisingly, CLC-Ka currents in HEK293 cells show only a slight (although significant) Ca^{2+}-dependence. At 0.5 mM Ca^{2+}, CLC-Ka currents are ~80% of the currents recorded at 30 mM Ca^{2+} (Fig. 6A, C). Interestingly, CLC-Kb currents exhibit a more marked Ca^{2+} sensitivity resembling that found in oocytes. Indeed, at 0.5 mM Ca^{2+}, CLC-Kb currents are ~50% of the maximum current level at 30 mM (Fig. 6B, C). Therefore we can conclude that CLC-K channels are Ca^{2+} sensitive regardless of the expression system, even though this sensitivity is reduced in HEK293 cells compared to oocytes.

CLC-K channels have a biphasic extracellular pH dependence being strongly inhibited by acidic as well as by alkaline pH in *Xenopus* oocytes [23,25]. Here we establish that inhibition by acidic pH is a feature retained by CLC-K channels expressed in HEK293 cells, even if slightly less evident compared to *Xenopus* oocytes. Indeed, CLC-Ka currents at pH 6.2 are ~56% of those recorded at pH 7.3 and further decrease at pH 5.5 (~11%) (Fig. 6D–F).

CLC-Ks are modulated by protein kinases [7,8,13]. Paulais et al. reported a cAMP-dependent modulation of CLC-K1 in the mouse cortical thick ascending limb [7]. In order to test whether such a regulation is retained in heterologous expression systems, we tested the effect of cAMP on CLC-Ka channels expressed in HEK293 cells using two different approaches. First, we recorded CLC-Ka currents with and without 500 μM cAMP added to the pipette solution. Differently from data on endogenous mCLC-K1 [7], we do not observe any current activation by cAMP in this experimental condition ($n = 10$ cells each; Fig. 7A). In a second approach, we acutely perfused CLC-Ka expressing cells with the membrane permeable cAMP analog DB-cAMP. In this case, we observe no statistically significant current potentiation ($n = 6$ cells, Fig. 7B).

Based on the results by Lourdel et al. on mCLC-K2 in mouse distal convoluted tubule [8], we next tested the potential role of protein kinase C (PKC) on CLC-Kb currents in HEK293 cells. The perfusion of whole-cell CLC-Kb patches with the PKC activator 4β-PDB (500 nM) induces an ~35% inhibition of the currents ($n = 4$ cells, Fig.7C,D). The current reduction induced by 4β-PDB is not observed when 1 μM chelerythrine, a PKC inhibitor, is included in the patch pipette ($n = 4$ cells, Fig.7C,D). Thus, CLC-Kb in HEK293 cells responds to PKC activation similarly to CLC-K2 in mouse kidney tubules.

3.3.3. Role of membrane cholesterol

The different gating and pharmacological properties shown by CLC-Ks when expressed in oocytes and HEK293 cells may arise from different protein–lipid hydrophobic interaction in the membrane of HEK293 cells compared with that in oocytes, as shown for other channels [26]. Therefore, we examined the effects of the reduction in membrane cholesterol on CLC-K channel activity, by treating both oocytes

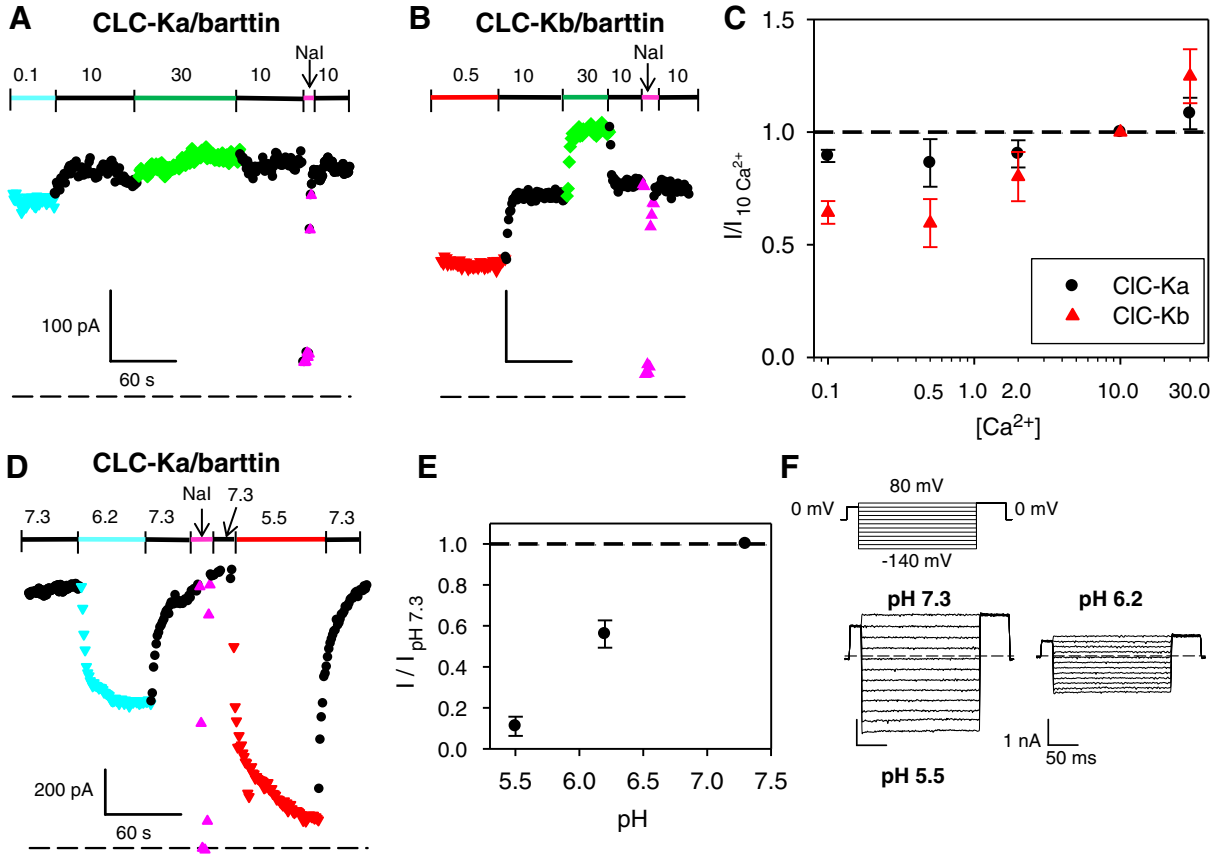

Fig. 6. $[Ca^{2+}]_{ext}$ and pH_{ext} modulation of human CLC-Ks expressed in HEK293 cells. (A–C) Effect of $[Ca^{2+}]_{ext}$ on human CLC-Ks. The mean current of CLC-Ka (A) and CLC-Kb (B) was plotted as a function of time. Different colors and symbols correspond to the $(Ca^{2+})_{ext}$ applied during the experiment (0.1, 0.5, 2, 10, and 30 mM) as indicated by the bars on top of the figure. (C) Ca^{2+} dependence of CLC-Ka (red triangles) and CLC-Kb (black circles). Currents were normalized to values measured at 10 mM Ca^{2+} and plotted versus $[Ca^{2+}]_{ext}$. Data are mean ± SD of $n = 3$–10 cells. (D–E) Effect of external pH on CLC-Ka. (D) The mean current of CLC-Ka was plotted as a function of time. Different colors and symbols correspond to the different pH_{ext} applied during the experiment (7.3, 6.2, and 5.5) as indicated by the bars on top of the figure. (E) CLC-Ka currents normalized to values measured at pH 7.3 were plotted versus pH. Data are mean ± SD of $n = 6$–7 cells. (F) Typical CLC-Ka currents recorded from the same cell at different pH values.

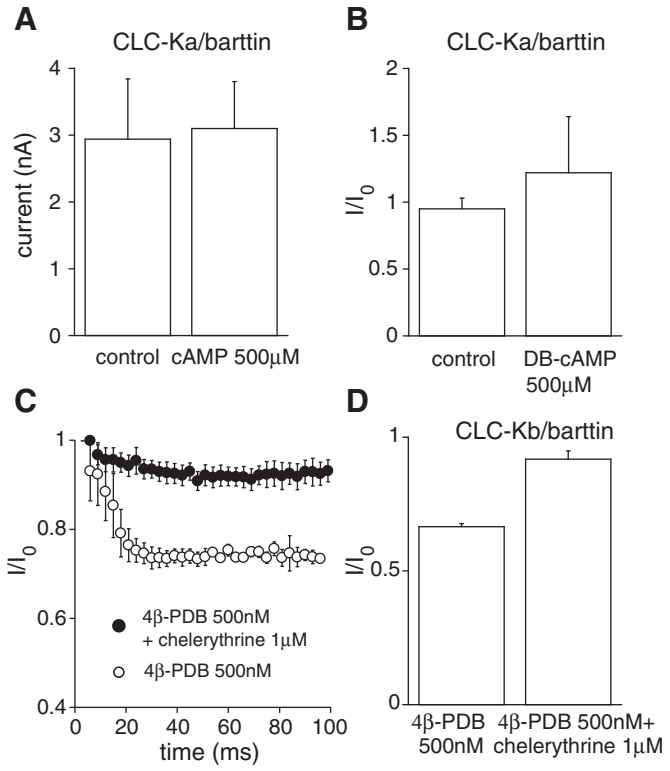

Fig. 7. Protein kinase modulation of human CLC-Ks expressed in HEK293 cells. (A) Bar graph showing average CLC-Ka currents measured at +60 mV with and without cAMP (500 μM) in the pipette solution. (B) Bar graph showing the effect of 5 min perfusion of DB-cAMP on whole-cell CLC-Ka currents. For comparison, whole-cell recordings have been performed on cells perfused with control solution for the same time ($n = 6$, $P > 0.05$). CLC-Ka currents were normalized to those measured before perfusion at +60 mV (I/I_0). (C, D) CLC-Kb current inhibition by 500 nM 4β-PDB alone or with 1 μM chelerythrine added to the patch pipette. CLC-Kb currents were normalized to those measured before perfusion at +60 mV (I/I_0). In the presence of the PKC inhibitor, chelerythrine, in the patch pipette, 4β-PDB induces almost no inhibition ($n = 4$ each, $P < 0.05$). Data are mean ± SE.

and HEK293 cells with methyl-beta-cyclodextrin or fluvastatin. Cyclodextrin lodges the hydrophobic cholesterol molecule inside its ring, thus reducing its concentration and increasing membrane fluidity. Incubation of either oocytes or HEK293 cells expressing CLC-Ka in 1 mM methyl-beta-cyclodextrin does not alter significantly the functional expression level of the channel (Fig. 8A, B). Fluvastatin, instead, lowers

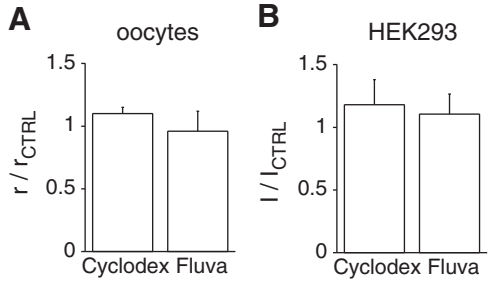

Fig. 8. Reducing membrane cholesterol content does not alter CLC-K functional expression. (A) Bar graph showing CLC-Ka current variation at +60 mV in *Xenopus* oocytes after 90 min incubation with methyl-beta-cyclodextrin 1 mM or after incubation with 100 μM fluvastatin. Data are mean ± SE. (B) Bar graph showing lack of effect of 1 mM methyl-beta-cyclodextrin and 100 μM fluvastatin on CLC-K currents expressed in HEK293 cells. CLC-K currents recorded after incubation with 1 mM methyl-beta-cyclodextrin ($I_{cyclodex} / I_{CTRL} = 1.18 \pm 0.2$, $n = 11$) or after 12 h incubation with 100 μM fluvastatin ($I_{FLUVA} / I_{CTRL} = 1.1 \pm 0.2$, $n = 8$–15) were normalized to those acquired in control solution at +60 mV, in HEK293 cells. Data are mean ± SE.

cholesterol synthesis by inhibiting the HMG-CoA reductase [27]. We observe that incubation with fluvastatin (100 μM) for 16 h does not affect CLC-Ka current magnitude in *Xenopus* oocytes (Fig. 8A). Similarly, the incubation with 100 μM fluvastatin at the time of CLC-Kb transfection or 12 h after transfection does not significantly affect CLC-Kb current amplitude in HEK293 cells (Fig. 8B). These results rule out the possibility that an increase in membrane fluidity, due to a reduction of membrane cholesterol, affects CLC-K channel expression and folding.

4. Discussion

Here we show that CLC-Ka and CLC-Kb channels exhibit different pharmacological properties, in addition to a different biophysical behavior [10,13,15,17,19], when they are expressed in frog cells or in human cell lines. Indeed, in *Xenopus* oocytes, both CLC-Ka and CLC-Kb display time and voltage dependent gating. Conversely, in HEK293 cells, CLC-K currents lack the characteristic time and voltage dependent components. In addition, in *Xenopus* oocytes, a blocking site and an activating binding site have been identified [14,15,19]. In contrast, in HEK293 cells we could only detect the inhibitory activity.

In fact, the analysis of known CLC-K blockers, such as the benzofuran derivatives RT-93 and MT-189, reveals that these compounds are potent inhibitors of human CLC-Ks also in mammalian cell lines, and their channel affinity is similar even though slightly lower than that measured in *Xenopus* oocytes. This result confirms that the blocking binding site is active and it is exposed to high affinity ligands that act from the extracellular side of the membrane in mammalian cells as well as in oocytes. On the other hand, the different efficacy suggests that the activity of CLC-K channels is different in the two biological systems.

Surprisingly, this study further reveals that the NFA-mediated current potentiation found in CLC-Ks expressed in oocytes [14] is not seen in HEK293 and MDCK cells, where NFA blocked CLC-Ka and CLC-Kb at all tested concentrations. One hypothesis to explain the different response to NFA may be that the activating binding site is masked and less easily accessible when human CLC-Ks are expressed in HEK293 cells. Alternatively, and more likely, we could envisage that the open probability of CLC-K channels in HEK293 cells is near maximal [21], such that, even when NFA binds to the activating site, it cannot further activate the channel. Conversely, in oocytes, the low probability of CLC-K channels ($\ll 0.5$) [19,23,25] would allow a dramatic current increase.

Actually, CLC-K openers as NFA would be highly desirable to treat conditions where a reduced channel function is observed, as in recessive Bartter syndrome. In this scenario, we cannot rule out that NFA would still be capable of activating CLC-Kb mutant channels with a reduced open probability, thus restoring the large open probability displayed by wild-type channels when expressed in mammalian cell lines. Further studies with site-directed mutants and/or other compounds are required to unmask the presence of the activating site.

The different channel folding and/or modulation by cellular factors may at least in part account for the different biophysical and pharmacological properties of CLC-Ks in mammalian cell lines and *Xenopus* oocytes. First we investigated whether the regulation by extracellular factors such as Ca^{2+} and pH could be altered and contribute to the reported differences. Indeed, CLC-K channels expressed in *Xenopus* oocytes and endogenous CLC-K channels are highly sensitive to extracellular Ca^{2+} [8–10,23,25]. Interestingly, Ca^{2+} regulation is found only in CLC-Ks among CLC proteins suggesting that this is an important feature of these channels. Furthermore, some CLC-Kb mutants associated to Bartter syndrome present altered Ca^{2+} and pH sensitivity when expressed in *Xenopus* oocytes [28]. In our experiments, CLC-Ka channels expressed in HEK293 cells are much less sensitive to extracellular Ca^{2+}. Interestingly, the reduced Ca^{2+} sensitivity displayed by CLC-Ka in HEK293 cells parallels the lack of NFA potentiation, thus supporting the reciprocal modulation of CLC-Ka observed in oocytes for Ca^{2+} and NFA [14]. The differences in Ca^{2+} sensitivity might again be related to

the different open probability of the channels in *Xenopus* oocytes and HEK293 cells. CLC-Kb channels instead retain a significant Ca^{2+} sensitivity. Although the physiological importance of the Ca^{2+}-dependence of renal chloride channels is unclear, these results support the idea that an increased external [Ca^{2+}] could stimulate NaCl absorption by the thick ascending limb, where CLC-Kb is located, and thus Ca^{2+} reabsorption. The analysis of the pH sensitivity confirms that CLC-Ka channels expressed in HEK293 cells are modulated by a change in extracellular pH, even if slightly less pronounced than those in oocytes.

In addition, specific post-translational modifications, like phosphorylation [7,8,13,29], glycosylation [17], cysteine oxidation [30], or nucleotide binding [30], that are cell-type dependent and likely differ in *Xenopus* oocytes and mammalian cell lines, may account for the observed differences in CLC-K channels. Our results on CLC-Ka and CLC-Kb channel phosphorylation show that CLC-Kb currents in HEK293 cells respond to PKC activation similarly to CLC-K2 in mouse kidney tubules [8] while CLC-Ka seems not to be activated by cAMP as CLC-K1 in mouse kidney tubules [7]. This suggests that CLC-K regulation is complex and may differ in different species. These initial experiments provide a promising starting point for a deeper investigation of the regulation of CLC-K channels by intracellular pathways.

Accessory subunits are known to influence the biophysics and pharmacology of ion channels. Therefore we also attempted to determine whether the regulatory barttin subunit could affect the pharmacology of CLC-Ks [10,21]. As CLC-K1 is the only CLC-K channel that mediates currents also in the absence of barttin we chose this channel isoform to test the contribution of barttin to the effect of NFA. Our results show that barttin does not affect neither CLC-K1 gating nor inhibition by NFA. Nevertheless, as the structural requirements for CLC-K/barttin interaction are still unclear [31,32], we can still expect that different CLC-K channel-barttin contacts occur in HEK293 cells and *Xenopus* oocytes, thus affecting CLC-Ka and CLC-Kb gating, NFA, and Ca^{2+} modulation. Another possibility explaining the different functional properties is that CLC-Ka and CLC-Kb channels assemble with unidentified endogenous accessory subunits in HEK293 cells, as reported for other channels [33,34].

Furthermore, lipid–protein interactions that strictly depend on the specific membrane environment are known to impact channel functions [26]. Our investigation of the role of membrane cholesterol rules out its involvement in CLC-Ks expression and gating in oocytes and mammalian cells.

In conclusion, our results emphasize the importance of the cellular expression environment in the efforts to correlate the biophysical and pharmacological profile of heterologously expressed CLC-K channels with that of endogenous human Cl^- currents. In this context, we conclude that HEK293 cells represent a valid biological system to screen CLC-K high affinity blockers and to drive rational drug development for a translational medicine. Future experiments are required to ascertain the possibility to activate CLC-K channels in mammalian cells and the structural requirements for activating compounds.

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