A common sequence variation of the *CLCNKB* gene strongly activates ClC-Kb chloride channel activity

NIKOLA JECK, PETRA WALDEGGER, JOLANTA DOROSZEWICZ, HANNSJÖRG SEYBERTH, and SIEGFRIED WALDEGGER

Department of Pediatrics, Philipps University of Marburg, Germany

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Background. Tubular transepithelial reabsorption of chloride along the nephron is a major determinant of body salt and water homeostasis and blood pressure regulation. About 40% of the glomerulary filtered sodium chloride are reabsorbed in the distal nephrons. Vectorial transepithelial sodium chloride transport is critically dependent on the function of basolateral ClC-K type chloride channels there. Modulation of ClC-Kb chloride channel activity by polymorphic variations of the *CLCNKB* gene, thus, could form a molecular basis for salt sensitivity of blood pressure regulation. In this study we tested the effect of several polymorphic variants on ClC-Kb chloride channel activity.

Methods. After heterologous expression in *Xenopus* oocytes, ClC-Kb channel activity and surface expression in presence of the ClC-K beta subunit barttin were determined by two-electrode voltage-clamp analysis, immunofluorescence, and ClC-Kb surface enzyme-linked immunosorbent assay (ELISA).

Results. Chloride currents induced by the ClC-Kb variants L27R, G214A, I419V, T562M, and E578K were not significantly different from wild-type currents. The ClC-KbT481S variation, however, which showed a frequency of 20% in our control population, dramatically activated chloride conductance by a factor of 20. Activation of chloride currents was also observed after introducing homologous mutations in ClC-Ka and ClC-K1, but not in ClC-2 and ClC-5 chloride channels. ClC-Kb activation by the T481S mutation did not change intrinsic ion channel pore properties and did not require increased surface expression of ClC-KbT481S.

Conclusion. Genetic heterogeneity of ClC-Kb chloride channels correlates with functional heterogeneity, which assigns ClC-Kb to a set of genes potentially relevant for polygenic salt-sensitivity of blood pressure regulation.

ClC-K channels belong to the CLC family of chloride channels and are nearly exclusively expressed in the kidney and inner ear [1]. Within the kidney, the two highly related isoforms ClC-Ka and ClC-Kb differ somewhat as to their expression pattern along the nephron [2]. ClC-Ka predominates in the thin ascending limb of the inner medulla, whereas ClC-Kb is expressed in the thick ascending limb of Henle's loop and more distal nephron segments [3]. Both isoforms associate with the beta subunit barttin to form functional, constitutively open chloride channels [4, 5]. Genetic evidence strongly suggests the involvement of ClC-K channels in transepithelial transport of chloride along the loop of Henle and distal tubule. Deletion of ClC-K1, the mouse ortholog of ClC-Ka, induces nephrogenic diabetes insipidus due to a urinary concentration defect caused by a decrease in inner medullary interstitial sodium chloride concentration [6, 7]. Mutations in the human ClC-Kb gene (*CLCNKB*) cause classical Bartter syndrome, an autosomal-recessive disorder characterized by urinary salt-wasting [8]. A combined impairment of ClC-Ka and ClC-Kb channel activity by mutations in their common subunit barttin has recently been identified as the molecular defect underlying Bartter syndrome with sensorineural deafness (BSND) [9], a salt losing Bartter syndrome-like disease associated with inner ear deafness. The disturbance of kidney function in all three disease phenotypes is plausibly explained by a derogation of tubular transepithelial chloride reabsorption caused by a loss of function of ClC-K chloride channels. As evident from these diseases, ClC-K chloride channel function is intimately correlated with tubular chloride reabsorbing capability. Variations in ClC-K channel activity, hence, could influence urinary fractional chloride excretion, and by this indirectly affect extracellular fluid volume homeostasis and blood pressure regulation. Variability of ClC-K channel activity could arise from amino acid relevant polymorphisms of the respective ClC-K encoding genes. Indeed, in the course of molecular genetic analyses of patients suffering from salt-losing tubular disorders, several molecular variants of the *CLCNKB* gene have been identified [10]. Until now, however, it was not clear whether this genetic polymorphism also correlated with differences in ClC-Kb channel function. We therefore performed the present study to

elucidate the consequences of *CLCNKB* genetic variants on functional properties of ClC-Kb chloride channels. To this end we expressed several single amino acid variants of ClC-Kb together with barttin in *Xenopus* oocytes and showed that the ClC-KbT481S variation, which was found at a frequency of about 20% in our control population, dramatically activated chloride currents by a factor of about 20 as compared to the wild-type channel. Genetic heterogeneity of ClC-Kb chloride channels, thus, correlated with functional heterogeneity, which assigned ClC-Kb to a set of genes potentially relevant for salt-sensitivity of blood pressure regulation.

METHODS

Molecular genetics

A control population of 200 healthy individuals was screened for polymorphic variations in the *CLCNKB* gene as described previously [10]. In brief, single strand conformational polymorphism (SSCP) analysis was performed on PCR-amplified exonic and adjacent intronic *CLCNKB* sequences. Amplicons with aberrant SSCP patterns were directly sequenced on both strands.

Expression in *Xenopus laevis* oocytes and voltage clamp analysis

Ten ng of in vitro transcribed cRNA (mMessage mMachine kit; Ambion, Austin, TX, USA) for the ClC-K-, ClC-2-, and ClC-5 constructs and 5 ng of barttin cRNA was injected in defolliculated *Xenopus* oocytes, which were kept at 16°C in ND96 storage solution containing 96 mmol/L NaCl, 2 mmol/L KCl, 1.8 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 5 mmol/L HEPES (pH 7.4), 2.5 mmol/L sodium pyruvate, 0.5 mmol/L theophylline, and 20 µg/mL gentamicin. Two to five days after injection, two-electrode voltage clamp measurements were performed at room temperature with a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA, USA). Currents were recorded in ND96 solution without sodium pyruvate, theophylline, and gentamicin. For pH experiments, the indicated proton concentrations were adjusted by titration with HCl or NaOH. Percentage of current inhibition after extracellular acidification was calculated by correlation with current amplitudes at pH 8.5, and the data points were fitted with the Hill equation: pI(H$^+$) = [m1 $\times$ (H$^+$)^{m2}]/[m3^{m2} + (H$^+$)^{m2}]. pI(H$^+$) stands for percentage of current inhibition in dependence of extracellular pH, m1 for maximal current inhibition, m2 for the Hill coefficient, and m3 for the proton concentration required for half-maximal inhibition of currents. For anion replacement experiments, 80 mmol/L chloride was replaced by equivalent amounts of the indicated anions. Data from at least two different batches of oocytes are shown. Statistical analysis was performed on n oocytes derived from one preparation. The error bars in the di-

agrams were calculated from the standard error of the mean (SEM). Site directed mutagenesis was performed with the QuickChange system (Stratagene, La Jolla, CA, USA), and the complete constructs were sequenced to prove the desired nucleotide exchange and to exclude any additional mutations.

Immunostaining of *Xenopus* oocytes

Prior to embedding (Tissue-Tek, Miles, Inc., Torrance, CA, USA) and cutting, *Xenopus* oocytes expressing the indicated epitope-tagged constructs (N-terminal HA epitope for ClC-Ka and C-terminal V5-epitope for barttin) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight. Five µm cryosections were incubated for 30 minutes in PBS containing 10% (v/v) normal goat serum (Sigma Chemical Co., St. Louis, MO, USA), 0.2% (w/v) bovine serum albumin (Sigma), and 0.3% (v/v) Triton X-100 (blocking solution). The primary antibodies (rat monoclonal anti-HA, Roche, Mannheim, Germany; mouse monoclonal anti-V5, Invitrogen, Karlsruhe, Germany) were diluted in the blocking solution (anti-HA 1/400, anti-V5 1/2000) and added to the slices for two hours at room temperature. After the slices were washed with PBS, secondary Cy-2- or Cy-3-coupled antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK) were added at the recommended dilutions for two hours at room temperature. Afterward, the slices were again washed with PBS and mounted with glycerol-gelatin (Sigma). Epifluorescence microscopy was used to detect antibody localization.

ClC-Kb surface ELISA

For quantification of ClC-Kb wild-type and T481S surface expression, a hemagglutinin (HA) epitope was inserted into the extracellular loop between the transmembrane domains L and M (according to the crystal structure published in [11]). As determined by electrophysiologic analysis, the extracellular HA epitope did not interfere with current amplitude or chloride-permeating properties of the ClC-KbHA$_e$ (extracellular) construct (data not shown). Surface quantification of ClC-KbHA$_e$ constructs after expression in *Xenopus* oocytes was performed as described previously [12]. In brief, viable ClC-KbHA$_e$ expressing oocytes were labeled with rat anti-HA monoclonal antibodies (3F10, 1 µg/mL). After rigorous washing in cold ND96-solution, bound antibodies on individual oocytes were detected with horseradish peroxidase-coupled goat antirat antibodies (fab fragments, 1/500 dilution; Jackson Immunoresearch, West Grove, PA, USA) in a peroxidase-catalyzed luminescence reaction using SuperSignal ELISA Femto Maximum Sensitivity chemiluminescent substrate (Pierce, Rockford, IL, USA). The luminescence signal as determined by a luminometer (Berthold Lumat LB9507, Berthold, Technologies, Bad Wildbad, Germany) is given
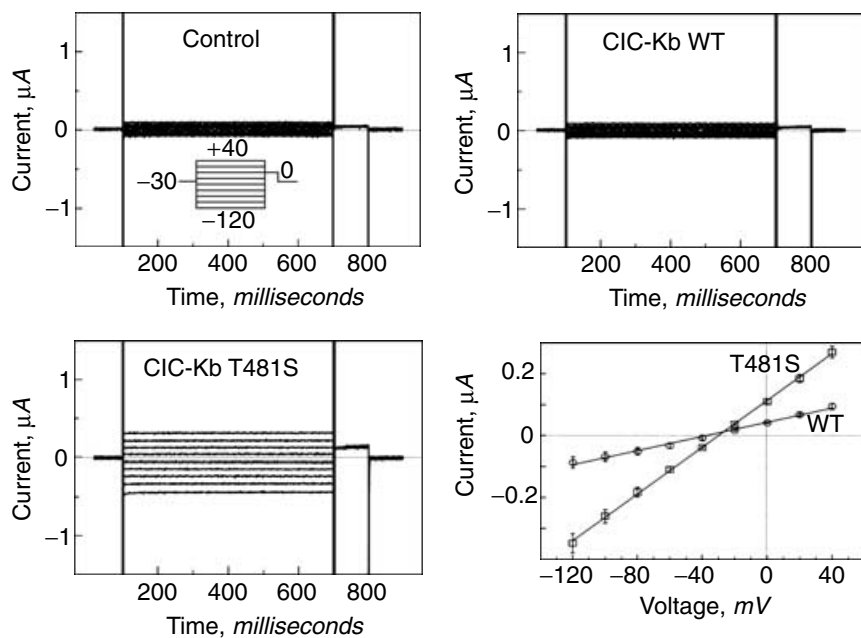
Fig. 1. Currents induced by ClC-Kb wild-type (WT) and ClC-Kb T481S in absence of barttin. A noninjected oocyte served as control. As indicated in the upper left panel, ion currents were activated by rectangular voltage steps from −120 mV to +40 mV starting from a holding voltage of −30 mV. A statistical analysis of the current/voltage (I/V) relationship derived from more than seven oocytes injected with 10 ng ClC-Kb wildtype- or ClC-Kb T481S cRNA is shown in the lower right panel.

in relative light units. Noninjected oocytes and oocytes expressing ClC-Kb with an intracellular, amino-terminal HA-epitope (ClC-KbHA$_i$) served as control. The experiments were repeated in two different batches of oocytes derived from different frogs. For each data point, approximately 20 oocytes derived from the same preparation were individually analyzed, and the relative light units are given as mean ± SEM. To exclude saturation of the chemiluminescence reaction, we used as a positive control ClC-5 with an extracellular HA epitope, which gave rise to much higher luminescence signals (data not shown).

RESULTS

Six ClC-Kb allelic variants, whose frequency among apparently healthy subjects was found to exceed 5% [10], were chosen for electrophysiologic evaluation after expression in *Xenopus* oocytes: ClC-Kb L27R (leucine at amino acid position 27 exchanged for arginine), -G214A, -I419V, -T481S, -T562M, and -E578K. With the exception of the glutamate to lysine exchange at position 578 (E578K), similar amino acid variations either were present in the ClC-Kb orthologs of other species (L27R, G214A, T481S, T562M), or the exchange did not influence charge or polarity of the respective amino acid (I419V). As expected, similar to ClC-Kb wild-type protein, which previously was shown to be nonfunctional after heterologous expression in *Xenopus* oocytes [13], ClC-KbL27R, -G214A, -I419V, -T562M, and -E578K did not induce currents different from noninjected oocytes when analyzed by the two-electrode voltage clamp technique. However, small but significant currents with a linear voltage dependence and a reversal potential close to

the chloride equilibrium potential were observed after expression of ClC-KbT481S (Fig. 1).

Sufficient insertion of ClC-K chloride channels into the plasma membrane is dependent on the presence of an auxiliary protein, the ClC-K beta subunit barttin. As reported previously, coexpression of wild-type ClC-Kb and barttin resulted in small linear chloride currents without notable voltage-dependence of gating [4, 5]. Linear currents with comparable reversal potentials and current amplitudes were recorded after expression of ClC-KbL27R, -G214A, -I419V, -T562M, and -E578K, together with barttin. However, simultaneous expression of barttin and ClC-KbT481S resulted in huge currents exceeding the wild-type currents by a factor of about 20 (Fig. 2). Similar to the chloride currents mediated by wild-type ClC-Kb and barttin, the ClC-KbT481S currents displayed no rectification behavior and reversed at −30 mV, the equilibrium potential of chloride, thus suggesting that the T481S amino acid exchange, apart from the current amplitude, did not interfere with the chloride permeation properties of ClC-Kb. To investigate this point in more detail, we determined the anion selectivity profile of ClC-Kb wild-type and −T481S chloride channels in presence of barttin. Consistent with previous findings [4, 5], anion substitution experiments revealed chloride>bromide>iodide conductance- and permeability sequences for ClC-Kb wild-type channels. As determined by measurements of current amplitudes at +40 mV and of reversal potentials after substitution of 80 mmol/L chloride with bromide or iodide, congruent conductance and permeability sequences, respectively, were observed for ClC-KbT481S (Fig. 3). Given the structural similarity between threonine and serine, both nucleophilic amino acids, which only differ by an additional methyl group in the side chain of
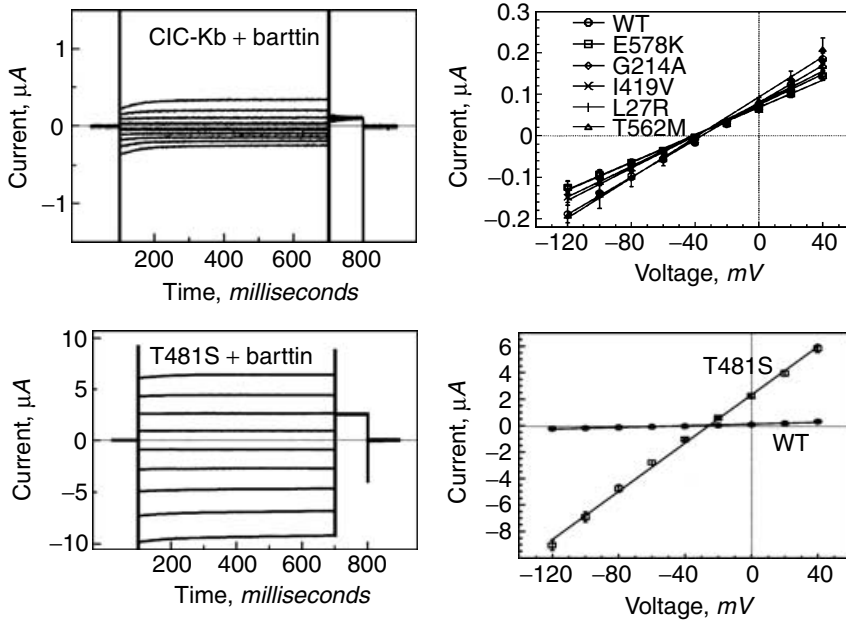
Fig. 2. Currents induced by ClC-Kb wild-type and different ClC-Kb single amino acid variants in presence of barttin. Linear chloride currents without voltage dependent gating in the physiological voltage range between −80 and −20 mV were induced by all ClC-K variants investigated. Please note the different scaling between the upper and lower panels. The I/V relationships in the upper and lower right panels were derived from $N \geq 7$ oocytes.
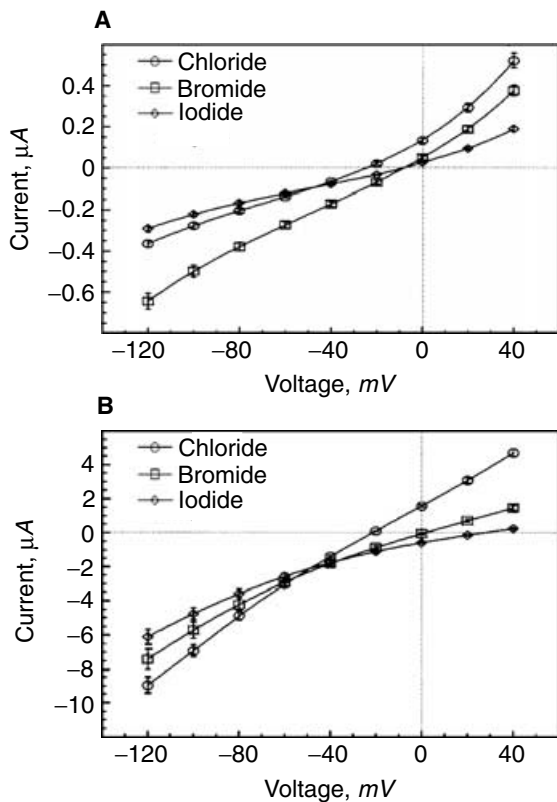
Fig. 3. Anion selectivity of ClC-Kb wild-type and ClC-Kb T481S channels. I/V relations were determined after replacement of 80 mmol/L chloride by bromide or iodide. Conductance sequences were derived from current levels at +40 mV, permeability sequences from the shift of current reversal potentials. The smaller shift of the reversal potential in presence of iodide observed in ClC-Kb wild-type expressing oocytes may be due to the low contribution of ClC-Kb wild-type–induced chloride conductance to the overall conductance of the oocyte membrane. Reduced inward current levels at −120 mV are explained by an inhibition of ClC-Kb wild-type and ClC-Kb T481S by extracellular iodide.

threonine, it is amazing to observe such a pronounced effect of the T481S amino acid exchange on chloride current amplitude. Interestingly, this threonine is conserved at homologous positions at the beginning of the P-helix among all members of the human CLC family [11]. Comparison of ClC-K sequences across different species, however, revealed an analogous substitution by serine in ClC-K genes cloned from rabbit [14] and *Xenopus* laevis [15]. This raised the question if chloride channel activation by the switch from threonine to serine in the N-terminal part of the P-helix is limited to ClC-Kb or is a more general phenomenon also affecting other members of the CLC family. To clarify this point, we introduced homologous mutations into ClC-Ka (ClC-Ka T481S), its rat ortholog ClC-K1 (ClC-K1 T481S), ClC-2 (ClC-2 T520S), and ClC-5 (ClC-5 T518S). As expected, mutation of threonine to serine at amino acid position 481 in the closely ClC-Kb-related ClC-Ka and ClC-K1 chloride channels caused a dramatic activation of current amplitude again without notable effects on basic biophysical properties of the chloride currents. In case of ClC-K1, which is exceptional within the ClC-K channel group as it induces chloride currents even in absence of the beta subunit barttin [13, 16], the T481S-induced amplification of current amplitude in presence of barttin overstrained the capacity of our measuring system. However, in absence of barttin we observed an increase of current amplitude from 183 ± 21 nA to 8223 ± 593 nA at +40 mV for ClC-K1 wild-type and –T481S, respectively ($N = 7$). By contrast, reduced amplitudes were measured for the inwardly rectifying chloride currents induced after expression of ClC-2 and the outwardly rectifying chloride currents induced by ClC-5, when the corresponding threonine (at amino acid positions 520 and 518
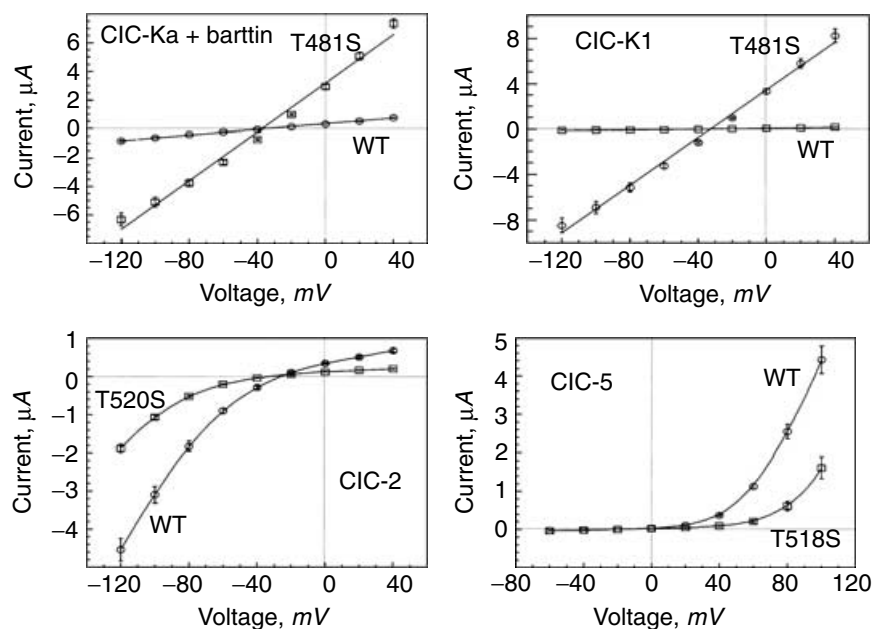
Fig. 4. **Effect of threonine to serine mutation at corresponding amino acid positions of human ClC-Ka, rat ClC-K1, rat ClC-2, and human ClC-5.** ClC-Ka currents (upper left panel) were measured in presence of barttin, whereas ClC-K1 currents (upper right panel) were recorded after isolated expression of ClC-K1. Similar to the ClC-K constructs, currents for ClC-2 (lower left panel) and ClC-5 (lower right panel) were determined after expression of 10 ng cRNA for each construct.

for ClC-2 and ClC-5, respectively) was mutated to serine (Fig. 4).

We have previously shown that ClC-Kb–mediated chloride currents feature a pronounced sensitivity toward changes of extracelluar pH [5]. Extracellular alkalinization to pH 8.0 strongly activates chloride current amplitude, whereas a nearly complete channel inhibition is observed after extracellular acidification to pH 7.0. A shift in the pH sensitivity, resulting in channel activation at the perfusion solution pH of 7.4, might thus underlie the amplification of ClC-Kb current amplitude after introducing the T481S mutation. To clarify this point, we determined the pH sensitivity of ClC-KbT481S in comparison with ClC-Kb wild-type currents. As shown in Figure 5, a steep pH dependence with a Hill coefficient approximating two was observed for both ClC-Kb- and ClC-KbT481S-induced currents. A slightly reduced maximal inhibition by extracellular acidification indeed was calculated for ClC-KbT481S (84% vs. 93% for ClC-Kb wild-type). However, different from ClC-Kb, which showed half maximal activity at pH 7.6, half maximal activation of ClC-KbT481S currents required a pH of 7.9, thus ruling out a shift of the pH optimum to more neutral values as mechanism for T481S-dependent channel activation.

Current activation could also result from increased trafficking to the plasma membrane of the T481S mutant as compared to ClC-Kb wild-type channels. Consistent with our previous findings [5], immunofluorescence staining of epitope-tagged ClC-Kb- and barttin constructs expressed in *Xenopus* oocytes revealed pronounced intracellular staining of ClC-Kb, with only weak signals in the plasma membrane. An identical distribution pattern was observed for ClC-KbT481S (Fig. 6). Quantification of ClC-Kb wild-type and T481S surface expression by

a luminometric surface ELISA revealed faint signals in absence of barttin, which increased by a factor of five in presence of barttin. In both cases, however, surface expression was not significantly different between ClC-Kb wild-type and ClC-Kb T481S. A mere trafficking effect, therefore, is unlikely to explain the channel activation by the T481S variation.

DISCUSSION

In this report we describe an unexpectedly pronounced effect of a relatively common genetic polymorphism on the activity of the ClC-Kb chloride channel. We showed that in contrast to the more distantly related CLC channel family members ClC-2 and ClC-5, a comparable degree of current activation occurred for the highly ClC-Kb–related chloride channels ClC-Ka and ClC-K1 after replacement of the corresponding threonine by serine. In search of the mechanism for current activation, a shift of the ClC-Kb pH optimum to the physiologic extracellular pH of 7.4 was excluded. Moreover, two independent experimental approaches failed to prove increased membrane insertion of ClC-Kb T481S as compared to the wild-type protein. A mere increase of ClC-Kb T481S membrane abundance thus is unlikely to explain all of the effect on channel activity. Because macroscopic current amplitude results from the number of ion channel proteins within the plasma membrane and the open probability and conductance of each ion channel, the latter finding suggested that the T481S-dependent current amplification, rather, is caused by an increase in ClC-Kb open probability or single-channel conductance. Due to rather low functional expression in heterologous expression systems, even in presence of the beta subunit barttin,
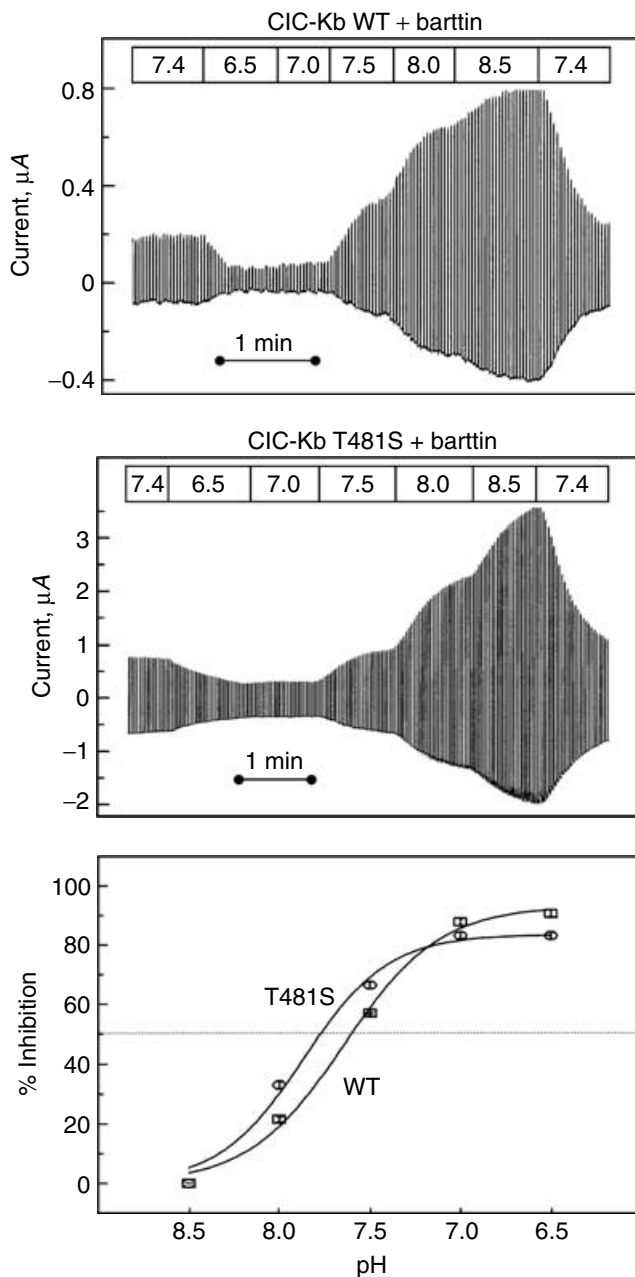
Fig. 5. pH dependence of ClC-Kb wild-type and ClC-Kb T481S-induced currents after coexpression with barttin. Currents were induced by repetitive voltage steps from −50 to 0 mV. Relative inhibition of current levels was calculated in relation to currents at pH 8.5, and the data were fitted with a modified Hill equation (see **Methods** section). Half maximal inhibition is indicated by the dotted line.

single-channel characteristics of ClC-Kb chloride channels unfortunately are still unascertained. However, with regard to the sequence similarity to ClC-1 and ClC-2, which along with the ClC-K channels form a separate branch of the CLC chloride channel family, it seems reasonable to speculate about analogous concepts of single-channel activity also applying to ClC-K channels. Extensive biophysical studies mainly on the skeletal mus-

cle chloride channel ClC-1 or ClC-0, its ortholog from the electric organ of the marine electric ray *Torpedo marmorata*, have proposed that CLC channels form homodimers with each subunit containing its own pore [1, 17]. The activity of both pores is regulated by two different gating processes, a fast gating process in which they open and close independently of one another ("fast gate"), and a slower gating process that controls both pores simultaneously ("slow gate" or "common gate"). In view of the recently enlightened crystal structure of a bacterial ClC channel [11], it was suggested that the helices at the central dimer interface participate in the common gating process [17, 18]. Importantly, the T481S amino acid exchange investigated in this study affects the N-terminal portion of the P-helix, which, together with the H-helix, forms the very core of the channel dimer interface. The T481S mutation, hence, could interfere with the common gating process and by this change current amplitude. This notion indeed is supported by the finding that an exchange of the corresponding threonine to alanine in ClC-1 (T539A) shifts the channel open probability due to alterations in the common gating process [18]. If applied to ClC-K channels, this suggests a mechanism for the T481S mutation by which current amplitude is altered by affecting the channel open probability. As to demand for such a mechanism, differences in intrinsic pore properties, such as the relative permeability for different anions, were not observed between ClC-Kb wild-type and –T481S chloride channels.

Numerous naturally occurring mutations associated with different monogenic diseases have been identified in several members of the CLC chloride channel family, which all have in common that they cause a more or less complete loss of ion channel function [1, 19]. The T481S mutation described here is the first natural CLC channel mutation leading to a pronounced gain of channel function. Unlike the rare ClC-Kb loss of function mutations, which show a clear association with autosomal-recessive Bartter syndrome, the T481S gain of function mutation with a prevalence of about 20% in our healthy control population apparently is not associated with a defined monogenic disease. As an important component of the renal tubular chloride reabsorbing machinery, however, increased ClC-Kb channel function might predispose to alterations in salt handling along the nephron. In combination with other genetic or environmental factors, this might contribute to salt-sensitive increases in extracellular fluid volume and, by this, affect blood pressure. Consistent with this hypothesis, an increased ClC-K2 channel activity by overexpression of ClC-K2 (the rat ortholog of ClC-Kb) in the inner medulla was suggested to contribute to elevated blood pressure in Dahl rats, an animal model for salt-sensitive hypertension [20]. However, the basolateral chloride conductance is unlikely to be rate limiting in the process of tubular transepithelial chloride
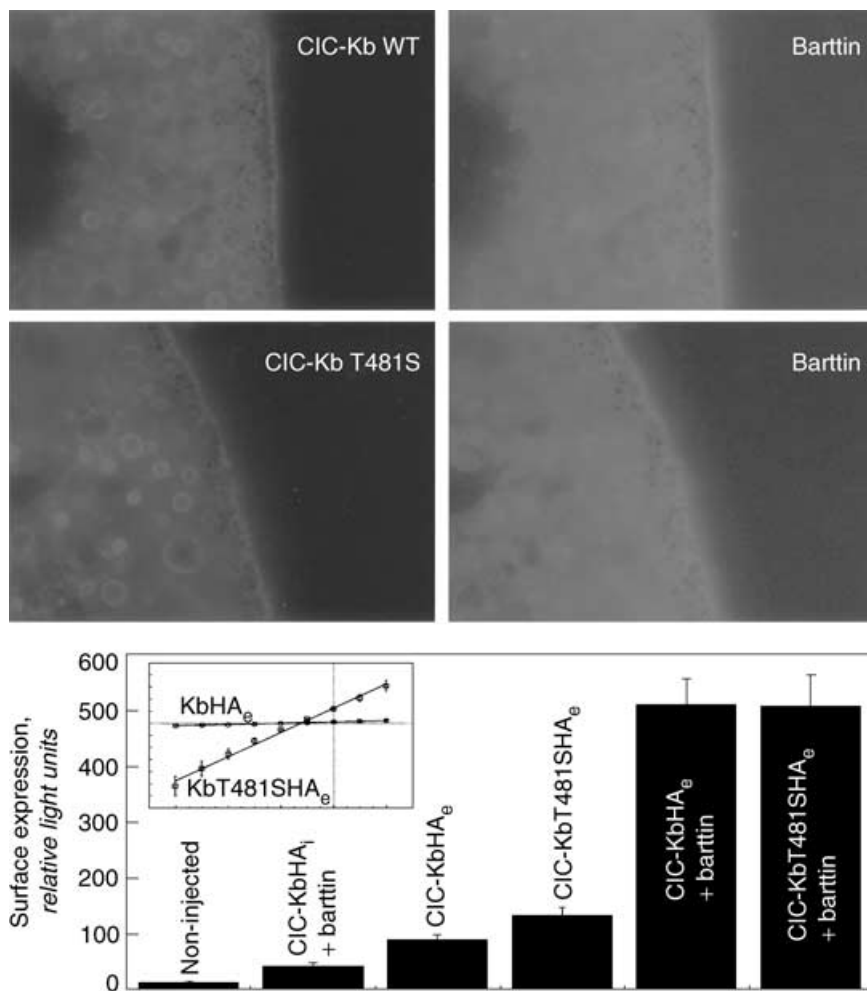
Fig. 6. Immunolocalization and surface expression of ClC-Kb wild-type and ClC-Kb T481S coexpressed with barttin in *Xenopus* oocytes. The N-terminal HA epitope and the C-terminal V5 epitope used for immunodetection of ClC-Kb and barttin, respectively, did not interfere with ion channel function. A costaining of ClC-Kb and barttin on the same oocyte slices is shown in the left and right panels, respectively. ClC-Kb constructs with an extracellular HA epitope were used for luminometric surface quantification of ClC-Kb (ClC-KbHA$_e$) and ClC-Kb T481S (ClC-KbT481SHA$_e$) in absence and presence of the ClC-K beta subunit barttin. Noninjected oocytes and oocytes expressing an intracellularly tagged ClC-Kb construct (ClC-KbHA$_i$) together with barttin served as negative control. Corresponding I/V curves for ClC-KbHAe (KbHAe) and ClC-KbT481SHA$_e$ (KbT481SHAe) in presence of barttin are shown in the inset.

reabsorption. Additional factors thus might be required for the ClC-Kb T481S variation to be effective on the overall chloride-reabsorbing capacity.

CONCLUSION

We showed that genetic heterogeneity of ClC-Kb correlates with functional heterogeneity. This allocates ClC-Kb to a set of genes potentially relevant for salt-sensitivity of blood pressure regulation.

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