Original Paper

A Newly Cloned CIC-3 Isoform, CIC-3d, as well as CIC-3a Mediates Cd^{2+} -Sensitive Outwardly Rectifying Anion Currents

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Key Words

CIC-3d • CIC-3a • Anion current • Cadmium

Abstract

Background: CIC-3, a member of the CIC family, is predicted to have six isoforms, CIC-3a to -3f, with distinct N- and C-terminal amino acid sequences. There have been conflicting reports on the properties of CIC-3a (also known as the N-terminal short form of CIC-3) and CIC-3b (the N-terminal long form of CIC-3) as plasmalemmal Cl^- channels. Meanwhile, little is known about other isoforms. The amino acid sequence of CIC-3d (a C-terminal variant of the short form) listed in the NCBI database was derived from the genomic sequence, but there has been no experimental evidence for the mRNA. **Methods:** PCR-cloning was made to obtain the full coding region of CIC-3d from mouse liver. Its molecular expression on the plasma membrane was microscopically examined in HEK293T cells transfected with GFP-tagged CIC-3d. Its functional plasmalemmal expression and the properties of currents were studied by whole-cell recordings in the cells transfected with CIC-3d. **Results:** The cloned CIC-3d was found to be the only isoform which has an N-terminal amino acid sequence identical to CIC-3a. When introduced into HEK293T cells, a minor fraction of exogenous CIC-3d proteins was detected at the plasma membrane, and activation of anion currents was observed at neutral pH under normotonic conditions. The properties of CIC-3d currents were found to be shared by CIC-3a-mediated currents. Also, both CIC-3d and -3a currents were found to be sensitive to Cd^{2+} . CIC-3d overexpression never affected the endogenous activity of acid- or swelling-activated anion channels. **Conclusion:** We thus conclude that plasmalemmal CIC-3d, like CIC-3a, mediates Cd^{2+} -sensitive outwardly rectifying anion currents and that CIC-3d is distinct from the molecular entities of acid- and volume-sensitive anion channels.

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Introduction

Members of the ClC family are expressed in a wide range of organisms. Mammals express nine members of the ClC family. Among them, ClC-1, -2, -Ka and -Kb are expressed at the plasma membrane and function as Cl⁻ channels, whereas others (ClC-3 to -7) localize mainly to the intracellular endosomal-lysosomal system and may function predominantly as Cl⁻/H⁺ antiporters [1].

ClC-3 is a ubiquitously expressed ClC member. In mice, four variants of the ClC-3 mRNA (*Clcn3*), which encode four different proteins possessing distinct N- and C-terminal amino acid sequences, have been reported: *Clcn3a*, *b*, *c* and *e* (GenBank Accession # NM_007711.3, _173873.1, _173876.3 and _173874.1). ClC-3a corresponds to the N-terminal short form of ClC-3 and consists of 760 amino acids. ClC-3b (also called the N-terminal long form or ClC-3A) and ClC-3c have an additional 58 and 31 amino acids, respectively, in their N-termini. ClC-3e, originally called ClC-3B [2], has the same N-terminus as ClC-3b and a distinct C-terminus from ClC-3a, -3b and -3c. Considering that this C-terminal variant of ClC-3b exists, C-terminal variants of ClC-3a and -3c possessing the e-type of C-terminus may be predicted to exist, and may be called ClC-3d and -3f, respectively. However, mRNAs for ClC-3d and -3f have not yet been reported. The first purpose of the present study was to clone the ClC-3d mRNA from mouse liver.

Intracellular ClC-3 may be a Cl⁻/H⁺ antiporter that serves as an anionic shunt for the establishment of a proton gradient generated by vacuolar ATPase activity [3]. However, the biological function of plasmalemmal ClC-3, if any, has been a matter of debate for many years. ClC-3a was originally reported to be PKC- and Ca²⁺-sensitive anion channel with moderate outward rectification when transfected into *Xenopus* oocytes and CHO cells [4, 5]. Subsequently, ClC-3a was reported to exhibit activity of a swelling-activated anion channel with PKC sensitivity and mild outward rectification when expressed in NIH/3T3 cells [6]. In contrast, more recently, ClC-3a was reported to exhibit activity of a PKC-insensitive anion channel with strong outward rectification when overexpressed in other mammalian cell lines [7-9]. Also, ClC-3a was reported to behave as an acid-activated anion channel [10]. On the other hand, ClC-3b was reported to function as a Ca²⁺/calmodulin-dependent protein kinase II α (Ca/CaMKII α)-activated Cl⁻ channel that exhibits moderate outward rectification when stably transfected in tsA cells [11]. Furthermore, the C-terminal variant of ClC-3b, ClC-3e (ClC-3B), was reported to function as a CFTR-regulated outwardly rectifying Cl⁻ channel when co-transfected with EBP50 in C127 cells [2] and to interact with PDZ-containing proteins at the C-terminal PDZ-binding domain [2, 12]. However, nothing has been reported about the electrophysiological characteristics of other isoforms of ClC-3. The second purpose of the present study was to examine the properties of anion currents mediated by ClC-3d expressed at the plasma membrane, comparing them with those of ClC-3a.

The volume-sensitive outwardly rectifying (VSOR) anion channel, the large-conductance maxi-anion channel, and the acid-sensitive outwardly rectifying (ASOR) anion channel are known to be widely expressed in mammalian cells, but their molecular entities have not so far been identified [13, 14]. The third purpose of this study was to compare the ClC-3d current with the currents of these anion channels and to test a possible involvement of ClC-3d in these anion channels.

Materials and Methods

Cell culture

Human embryonic kidney HEK293T cells and mouse mammary C127 cells were grown in DMEM (Nissui, Tokyo, Japan) supplemented with 100 U/ml penicillin *plus* 100 μ g/ml streptomycin as well as with 10% fetal calf serum (FCS) and fetal bovine serum (FBS), respectively, at 37°C in a humidity-controlled incubator with 5% CO₂ (MCO-96; Sanyo, Tokyo, Japan).

Cloning and vector construction

To clone an uncharacterized variant of the ClC-3 gene (*Clcn3*), we used the following PCR primer set with the sequences 5'-tcctgaaaggccggttagca-3' (forward) and 5'-accaggtacagctaacagactc-3' (reverse). The forward primer was designed to target a sequence in exon 1, which is common among *Clcn3a*, *b* and *e*, and the reverse primer to target a sequence in the last exon (exon 15), which is common to all variants. cDNAs were prepared from the mouse liver. PCR products were cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA) and sequenced. We then identified the cDNA of *Clcn3d*. We also cloned *Clcn3a* using the same primer set.

To generate expression vectors for electrophysiological studies in HEK293T cells, 5'UTR sequence of cDNA was replaced by Kozak translation initiation sequence by PCR using the primer sets of 5'-acaaatggaggcagcatt-3' (forward) and 5'-catggtgtgatcactagt-3' (reverse) for *Clcn3d*, and 5'-acaaatggaggcagcatt-3' (forward) and 5'-catggtgtgatcgaattcc-3' (reverse) for *Clcn3a*. Then the cDNAs of *Clcn3d* and *Clcn3a* were subcloned into the Sac1-Sac2 and Sac2 sites, respectively, of CMV-pIRES2-EGFP vector (Clontech, Mountain View, CA, USA). The *Clcn3d/a* mutant with a point mutation (E224A) in the pore-forming region was generated by PCR-mediated mutagenesis in CMV-pIRES2-EGFP vectors as follows: 5'-gcaggtcccctggtacatgttgc-3' (forward) and 5'-ttttcctaaactcaaactgatgc-3' (reverse).

To observe subcellular distribution of ClC-3d or ClC-3a protein, we generated two types of tag-fused constructs, GFP tag at the C terminus and HA tag at the N terminus. These GFP-tagged constructs (EGFP in the C-terminus) were generated by PCR-mediated mutagenesis in CMV-pIRES2-EGFP vector (forward: 5'-atggtgagcaaggcgaggag-3', reverse: 5'-aagaattgtgctattcaacaacg-3' for *Clcn3d*; forward: 5'-atggtgagcaaggcgaggag-3', reverse: 5'-gttgaacattattgaagc-3' for *Clcn3a*). GFP-tagged E224A mutants of ClC-3a/d were also generated in the same manner as their wild types. To generate N-terminal HA-tagged *Clcn3d*, a cassette including HA epitope sequence with Kozak sequence (forward strand: ctagcacaacatgtaccatagcagctgccagactacgtgagct, reverse strand: cagcgtagtctgggacgtcgatgggtacatggtgtg) was inserted into the Nhe1-Sac1 site of CMV-pIRES2-EGFP vector. The Sac1 site was added to 5' end of coding sequence of *Clcn3d* by PCR in pGEM®-T Easy vector. Then, it was subcloned into the Sac1-Sac2 site of CMV-pIRES2-EGFP vector in frame with the HA epitope sequence. N-terminal HA-tagged ClC-3a was generated by PCR-mediated mutagenesis using the primer set of 5'-gtcccagactacgtacaaatggaggcagcatt-3' (forward) and 5'-gtcgtatgggtacatggtgtgatcgaattcc-3' (reverse) in pGEM®-T Easy vector and then subcloned into the Sac2 site of CMV-pIRES2-EGFP vector.

RT-PCR

To examine expression of ClC-3d mRNA in mouse brain, heart and liver, semi-quantitative RT-PCR was performed. Each tissue was obtained from euthanized mouse and grinded in the mortar. Total RNA was isolated using Sepasol RNA I reagent (Nacalai Tesque, Kyoto, Japan). Contamination of genomic DNA in total RNA was removed by DNase1 treatment (RT-grade: Nippon Gene, Tokyo, Japan). The cDNA was synthesized using oligo-dT primer (Invitrogen/Life Technologies, Carlsbad, CA, US) and SuperScript III First-Strand Synthesis System (Invitrogen/Life Technologies). PCR was carried out using KOD+ DNA polymerase (TOYOBO, Tokyo, Japan) with a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems/Life Technologies, Foster City, CA, USA). To amplify *Clcn3d*, a primer set was designed to the exon 1/4 junction (forward: 5'-tgagctgcaaggaaactc-3') and exon 14 (reverse: 5'-gctttagtgtcgtgagatgc-3'). A primer set for all variants of *Clcn3* (forward: 5'-gcagccattactgctgtga-3', reverse: 5'-actgcagccataagaggaac-3') was used to estimate the total expression level of *Clcn3*. GAPDH (forward: 5'-atggtctacatgttccagt-3', reverse: 5'-ccttcacaatgccaaag-3', product size 392 bp; or forward: 5'-gcacagtcaaggccgagaat-3', reverse: 5'-ttcaccacctggagaaggc-3', product size 151 bp) was also amplified as an internal control. It must be pointed out that precise assessment of the expression level is difficult, because of the length of the PCR-amplified fragment (2276 bp).

Overexpression of *Clcn3* in HEK293T

Clcn3a/d-expression vectors (0.8 µg/ml) were transfected into HEK293T cells using Lipofectamine 2000 (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Transfection reagent was removed 3 h after transfection, and transfected cells were cultured for 2 days at 37°C before use. CMV-pIRES2-EGFP vector alone was used for the mock-transfected control.

Observations of ClC-3 localization

Subcellular localization of GFP-tagged ClC-3d or ClC-3a proteins was observed in living cells using an epifluorescence microscope (IX70: Olympus, Tokyo, Japan) and a confocal laser microscope (A1R: Nikon, Tokyo, Japan). Cells were counterstained with Cell Mask™ Orange Plasma Membrane Stain (1:1000 in culture medium; Molecular Probes/Life Technologies, Eugene, OR, USA) for 15 min and washed twice with culture medium. Subcellular localization of HA-tagged ClC-3 proteins was detected by immunocytochemistry under a confocal laser microscope (A1Rsi: Nikon). Cells were fixed with 4% paraformaldehyde and then incubated with 1 µg/ml anti-HA antibody (H3663: Sigma-Aldrich, St. Louis, MO, USA). Alexa 594 conjugated secondary antibody (goat anti-mouse antibody A11020: Molecular Probes/Life Technologies) was used at 1:800 concentration.

siRNA transfection

Murine C127 cells sparsely seeded on 12-well plate were transfected with siRNA against mouse *Clcn3* (siRNA ID: MSS203003: Invitrogen/Life Technologies) by incubating with 100 µl of siRNA-containing medium which was prepared by mixing 95 µl of serum-free OPTI-MEM (GIBCO, Grand Island, NY, USA), 4 µl of transfection reagent (HiPerFect: Qiagen) and 1 µl of distilled water containing 20 µM siRNA. One day after transfection, the cells were washed with culture medium and grown in the same medium for 2 days at 37°C before use for RNA isolation or patch-clamp experiments. Stealth RNAi™ siRNA Negative Control (Invitrogen/Life Technologies) was also transfected into C127 cells as a control.

Validation of knockdown efficiency

The effect of siRNA-mediated knockdown on *Clcn3* expression was assessed by RT-PCR. Total RNA was isolated from control or gene-specific siRNA-transfected cells using Sepasol RNA I reagent (Nacalai Tesque). Contamination of genomic DNA in total RNA was removed by DNase1 treatment (RT-grade). The cDNA was synthesized using oligo-dT primer (Invitrogen/Life Technologies) and GoScript™ Reverse Transcription System (Promega). Semi-quantitative RT-PCR was carried out using KOD⁺ DNA polymerase (TOYOBO) with a Gene Amp PCR System 9700 thermal cycler. Quantitative RT-PCR was also carried out using Power SYBR® Green PCR Master Mix (Applied Biosystems/Life Technologies) with 7300 Real Time PCR system (Applied Biosystems/Life Technologies). Primer sets for *Clcn3* and GAPDH described in *RT-PCR* were used in these experiments.

Electrophysiology

Whole-cell or inside-out patch voltage-clamp was performed with an EPC10 amplifier controlled via PATCHMASTER software (HEKA Elektronik, Lambrecht/Pfalz, Germany) or with an Axopatch 200A or 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA) controlled via pClamp 9.2 software (Molecular Devices). For patch-clamping in cells transfected with plasmid DNA or expression vector, cells exhibiting GFP fluorescence were selected under a fluorescence microscope. Patch pipettes were made from borosilicate glass capillaries, and the pipette resistance before patch formation was 2–3 MΩ. The current signals were filtered at 1 kHz and sampled at 5 kHz. Liquid junction potentials were calculated with JPCalc in pCLAMP10 software (Molecular Devices) and corrected on-line. The series resistance during the recording was kept at <10 MΩ and compensated for by 75–80%. The input resistance was maintained at ~5 GΩ and >2 GΩ during recordings of ClC-3 currents and other currents, respectively. When extracellular Cl⁻ was replaced with I⁻ or Br⁻, a 3 M KCl-2% agar bridge was used as the reference electrode. All the current recordings were performed at room temperature (22–26°C).

For recording of whole-cell currents through ClC-3d, -3a and their mutants, the following isotonic solutions were used. The extracellular bath solution contained (in mM): 135 *N*-methyl-*D*-glucamine (NMDG)-Cl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 40 mannitol (pH 7.4, 310 mosmol/kg-H₂O). For halide selectivity measurements, 135 mM Cl⁻ in the bath solution was replaced with equimolar I⁻ or Br⁻. The permeability ratio for halide anions was estimated using the Goldman-Hodgkin-Katz equation:

$$P_{\text{X}}/P_{\text{Cl}} = \{ [\text{Cl}^-]_o^0 \exp(-F\Delta E_{\text{rev}} / RT) - [\text{Cl}^-]_o \} / [\text{X}^-]_o$$

where ΔE_{rev} is the shift in the reversal potential caused by the replacement of Cl⁻ with the test anion X⁻. $[\text{Cl}^-]_o^0$ is the initial standard concentration of extracellular Cl⁻ (141 mM), and $[\text{Cl}^-]_o$ is the remaining extracellular Cl⁻ after the replacement (6 mM). F, R, and T have their usual thermodynamic meanings. When Ca²⁺ was removed from the bath solution, equimolar Mg²⁺ and 0.1 mM ethylene glycol tetraacetic

acid (EGTA) were added to the solution. When 3 mM CdCl₂ was added to the bath solution, the NMDG-Cl concentration was reduced to maintain the osmolality identical. The patch pipette solution contained (in mM): 143 NMDG-Cl, 5 HEPES, 3 Na₂ATP, 1 MgCl₂ and 1 EGTA with 0.2 CaCl₂ (estimated free [Ca²⁺] = 15 nM; pH 7.4, 270 mosmol/kg-H₂O). In some experiments, *O,O'*-bis(2-aminophenyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid (BAPTA) was added to the pipette solution in place of EGTA and CaCl₂. The osmolality of solutions was measured using an OM802 freezing-point depression osmometer (Vogel, Giessen, Germany).

Recording of whole-cell currents of the VSOR anion channel was performed under hypotonic stimulation, as previously described [15], using the following isotonic and hypotonic solutions. The bathing solution contained (in mM): 110 CsCl, 5 MgSO₄, 10 HEPES, and 100 mannitol (pH 7.5 adjusted with CsOH, 330 mosmol/kg-H₂O). To make the hypotonic bathing solution, the concentration of mannitol was reduced to 45 mM (pH 7.5, 275 mosmol/kg-H₂O). The pipette (intracellular) solution contained (in mM): 110 CsCl, 2 MgSO₄, 2 Na₂ATP, 0.3 Na₃-GTP, 10 HEPES, 1 EGTA, and 50 mannitol (pH 7.3 adjusted with CsOH, 300 mosmol/kg-H₂O).

Recording of whole-cell currents of the ASOR anion channel was carried out when exposing cells to acidic bathing solution, as previously reported [16], with the following isotonic solutions. The bathing solution contained (in mM): 110 CsCl, 5 MgSO₄, 12 HEPES, 7 Tris, and 90 mannitol (pH 7.5 adjusted with CsOH, 330 mosmol/kg-H₂O). To make acidic bathing solution, HEPES was replaced with MES (pH 4.5, 330 mosmol/kg-H₂O). The pipette (intracellular) solution contained (in mM): 110 CsCl, 1 MgSO₄, 1 Na₂-ATP, 15 Na-HEPES, 10 HEPES, 1 EGTA, and 50 mannitol (pH 7.4 adjusted with CsOH, 300 mosmol/kg-H₂O).

Recording of inside-out single-channel currents of the maxi-anion channel that is characterized by a large single-channel conductance of ≥300 pS [17] was done upon patch excision, as previously described [18], using the following isotonic Ringer solution in the pipette and bath solutions (in mM): 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5 Na-HEPES, 6 HEPES and 5 glucose (pH 7.4, 290 mosmol/kg-H₂O).

Drugs

5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), phloretin, phorbol 12-myristate 13-acetate (PMA), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), BAPTA and the active form of CaMKIIα were purchased from Sigma-Aldrich. 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid (DCPIB) was from Tocris Bioscience (Bristol, UK). Thapsigargin was from Wako Chemicals (Osaka, Japan). EGTA was from Dojindo (Kumamoto, Japan).

Results

Cloning of *Clcn3d*

Clcn3a consists of 12 exons (exon 1, 4-13, and 15), whereas *Clcn3b* and *Clcn3e* contain one (exon 2) and two (exon 2 and 14) additional exons, respectively. To clone the full coding region of novel variants of *Clcn3* with parts of the 5' and 3' UTRs, we used a PCR primer set containing a forward primer for exon 1 and a reverse primer for exon 15. Of several cDNA clones from mouse liver, we found one clone possessing exon 14 (Fig. 1A). This is in contrast to *Clcn3a*, which does not have exon 14. However, no other differences were found between the isolated clone and *Clcn3a*. For example, the 5' end of the isolated clone consisted of exon 1 and exon 4, just like in *Clcn3a* (Fig. 1B). The isolated clone is distinct from *Clcn3e*, which is also known to possess exon 14, because it lacks exon 2 (Fig. 1B). These sequence features reveal that it represents a 3' end variant of *Clcn3a*, *Clcn3d*, and encodes the predicted isoform d of the ClC-3 protein, ClC-3d. ClC-3d was found to be the only isoform which has an *N*-terminal amino acid sequence identical to ClC-3a. As is the case for ClC-3e, insertion of exon 14 not only adds a 76 bp sequence but also generates a frameshift in exon 15, resulting in a different amino acid sequence of the *C*-terminal ends of ClC-3d and ClC-3a, as schematically depicted in Figure 1C.

PCR using a primer set specific to *Clcn3d* revealed that the expression level of *Clcn3d* is relatively high in the liver and low but present in the brain, whereas it was not detected in the heart (Fig. 2). Based on normalization against the amount of GAPDH, *Clcn3d* is more than 13 times more abundant in the liver compared to the brain, and is expressed poorly in the heart.

Fig. 1. Cloning of CIC-3d. Upper-case and lower-case letters designate the coding and UTR sequences, respectively. (A) Sequence of isolated *Cln3d*. Exon 14 is indicated by an underline. (B) Nucleotide sequences of the 5' end of *Cln3a*, *Cln3b/e*, and the isolated clone. The isolated clone possesses an exon junction identical to that of *Cln3a*. Exon 1, exon 2 and exon 4 are designated in grey non-italic, black non-italic and black italic, respectively. The start codon (ATG) is indicated by underlines. (C) Schematics of CIC-3a and -3d proteins. CIC-3a and CIC-3d contain 13 and 61 amino residues, respectively, with no similarities in the C-terminus beyond CBS2. The amino acid sequence that is part of the CBS2 domain is indicated by underlines.

Fig. 2. RT-PCR for expression of mRNAs of CIC-3d and CIC-3 including all variants in mouse heart, brain and liver. Data represent three similar experiments.

Subcellular localization of exogenous CIC-3d protein
In HEK293T cells transfected with C-terminal GFP-tagged CIC-3d (CIC-3d-GFP), GFP signal was predominantly observed within the cells (Fig. 3A: green). Some of the CIC-3d-GFP

Fig. 3. Subcellular distribution of ClC-3-GFP fusion proteins transfected into HEK293T cells. (A) Epifluorescence microscopic observation of wild-type ClC-3d-GFP-transfected cells. A fraction of the ClC-3d-GFP fusion protein is located peripherally in association with the plasma membrane. (B and C) Confocal laser microscopic observations of cells transfected with wild-type ClC-3d-GFP and ClC-3a-GFP, respectively. The plasma membrane was stained with Cell Mask™ Orange (in red). Note that the ClC-3d signal is located not only in the intracellular granular component but also at the plasma membrane (B), whereas the ClC-3a signal is mostly located in the intracellular large vesicles (C). (D) Epifluorescence microscopic observations of ClC-3d E224A-GFP-transfected cells. (E and F) Confocal laser microscopic observations of cells transfected with ClC-3d E224A-GFP and ClC-3a E224A-GFP, respectively. *Arrows* indicate GFP signal looked to be located at the plasma membrane, and *arrowheads* indicate GFP signal looked to be located on the membrane of intracellular vesicles. Scale, 10 μ m.

resided in the granular component. The remaining ClC-3d was located in the cell periphery close to the plasma membrane. To obtain precise localization, we visualized the plasma membrane by confocal laser microscopy using the Cell Mask™ Orange Plasma Membrane Stain (Fig. 3B: red). The optical sections suggested that a minor fraction of ClC-3d was actually located at the plasma membrane (at *arrows* in Fig. 3B). On the other hand, overexpression of ClC-3a-GFP induced the formation of large intracellular vesicles, possibly lysosomes, and most of the ClC-3a protein accumulated in these vesicular membranes (at *arrowheads* in Fig. 3C), consistent with a previous report [8]. Similar intracellular localization of ClC-3d and -3a was also observed in HEK293T cells overexpressing with N-terminal HA-tagged-ClC-3d

and -3a (data not shown), suggesting that intracellular distribution patterns of ClC-3d and -3a proteins are not influenced by the position of tags. The E224A mutants of these proteins showed similar localization patterns to the wild-type ClC-3d (Fig. 3D - F). A minor fraction of ClC-3a/d E224A looked to be also located on the plasma membrane (at *arrows* in Fig. 3E and F). As reported in a previous paper [8], overexpression of the E224A mutant of ClC-3a did not induce lysosome-like vesicle formation (Fig. 3F).

Steep outward rectification of ClC-3d and ClC-3a currents

Next we tried to record the current through ClC-3d proteins expressed at the plasma membrane of ClC-3d-overexpressing HEK293T cells. We used NMDG-Cl-based bath and pipette solutions for this purpose (see Materials and Methods for details). Our whole-cell patch voltage-clamp recording revealed steep outwardly rectifying currents in ClC-3d-overexpressing cells (Fig. 4). The current responses to step pulses exhibited little time-dependent activation and inactivation (Fig. 4Ab). The current density at +100 mV reached 15–20 pA/pF (Fig. 4Ba: *open squares*), but the current decreased greatly at lower voltage levels, and significant currents were seen only in the voltage range of >+40 mV, compared to the currents generated in mock-transfected cells (Fig. 4Aa and 4Ba: *open circles*). In order to record this current consistently, it is important to check the high input resistance (~5 GΩ) of the cell in the voltage range of <+40 mV and also to keep a low series resistance (<10 MΩ) during the recording. The process of relaxation of current activation just after the beginning of test voltage pulses of >+40 mV was not seen, nor were there tail currents after the end of the pulses within the limit of time resolution of our recording (~1 ms), implying that activation and deactivation of the channel activity of ClC-3d are almost instantaneous; there were only residual uncompensated capacitive surges. For comparison, we also tried to examine the current through ClC-3a, the prototypical short form of ClC-3, and we found virtually the same current characteristics as those of ClC-3d, except for the much smaller average current amplitude in ClC-3a-overexpressing cells (Fig. 4Ad and 4Bb: *open squares*) presumably due to more limited expression at the plasma membrane (see above). These characteristics of the ClC-3d and -3a currents are consistent with those of the ClC-3a current reported by Li et al. [7, 8].

A glutamate residue on the extracellular side of the Cl⁻-conducting pore of ClC channels is known to be important for fast gating, anion selectivity, voltage dependence, and extracellular Cl⁻ and pH dependence of ClC channels [19]. Neutralization of this 'gating glutamate' with alanine in ClC-3, -4 and -5 has been reported to change drastically the current rectification [8, 20]. Indeed, in our experiments with the same E224A point mutation in ClC-3d and -3a, there was loss of the steep outward rectification and gain of an inwardly rectifying current component (Fig. 4Ac, 4Ae, 4Ba and 4Bb: *filled diamonds*), reproducing previous reports on ClC-3a [8, 9]. This further confirms that the steep outwardly rectifying currents in the wild-type ClC-3d- and -3a-overexpressing cells are indeed generated by the overexpressed proteins in the plasma membrane.

Cl⁻ > I⁻ selectivity of ClC-3d and ClC-3a currents

We next examined the halide selectivity of ClC-3d, ClC-3a and their E224A mutants. In both ClC-3d- and ClC-3a-overexpressing cells, the steep outwardly rectifying current induced by a ramp voltage pulse from -100 mV to +100 mV (Fig. 5A: *pale grey trace*) was reduced by half in the voltage range >+40 mV when most of the extracellular Cl⁻ was replaced with I⁻ (Fig. 5A: *black trace*, and Fig. 5B: *left grey* and *white columns*; $P < 0.01$ by paired t test). When the extracellular I⁻ was next replaced with Br⁻, the current amplitude was restored to nearly the same level as in the initial Cl⁻-rich condition (Fig. 5A: *dark grey*). When the current density at +100 mV was compared, the current in the presence of Br⁻ was statistically not different from that in the presence of Cl⁻ in ClC-3d-overexpressing cells (Fig. 5B: *right grey column*), while it was larger by 10% in ClC-3a-overexpressing cells (Fig. 5B: *right white column*; $P < 0.01$ by paired t test). Although the permeability ratios of I⁻ and Br⁻ with respect to Cl⁻ could not be determined from the reversal potentials because significant currents were seen only

Fig. 4. Voltage dependence of the whole-cell currents through ClC-3d, ClC-3a and their mutant channels. (A) Representative current traces obtained from HEK293T cells overexpressing GFP only (a; *Mock*), ClC-3d (b), the E224A mutant of ClC-3d (c), ClC-3a (d) and the E224A mutant of ClC-3a (e). The currents were elicited by applying test voltage pulses (0.5 s duration) ranging from -100 mV to +100 mV in 20 mV increments with fixed pre-pulses (0.1 s duration) to -100 mV and post-pulses (0.1 s duration) to -80 mV. The holding potential was 0 mV. (B) Current-voltage (*I-V*) relationships for the whole-cell currents elicited by the test pulses.

Ba shows the relationships obtained from mock-transfected cells (*open circles*; $n = 7$), ClC-3d-overexpressing cells (*open squares*; $n = 8$) and ClC-3d E224A mutant-overexpressing cells (*filled diamonds*; $n = 9$). Bb shows those from ClC-3a-overexpressing cells (*open squares*; $n = 8$) and ClC-3a E224A mutant-overexpressing cells (*filled diamonds*; $n = 8$). Note that the scale of the y-axis in Bb is different from that in Ba. The current amplitude was measured 20 ms after the beginning of the test pulse. Each symbol represents the mean $\pm$ SEM of the current amplitude (given as current density, pA/pF). Asterisks in Ba indicate a significant difference in the amplitude of the currents elicited by the same voltage pulse in ClC-3d-overexpressing cells and in mock-transfected cells ($P < 0.05$ by unpaired *t* test).

at $>+40$ mV, the result indicates the conductance selectivity sequence estimated from the slope conductance for ClC-3d and ClC-3a at large positive potentials should be $\text{Br}^- \geq \text{Cl}^- > \text{I}^-$.

When the E224A mutation was introduced into ClC-3d, the same ramp voltage pulse in the normal Cl^- -rich condition elicited a current with a greatly reduced, nearly linear outward component and an additional inwardly rectifying component (Fig. 5C: *pale grey*). Replacement of Cl^- with I^- caused the reappearance of a large outwardly rectifying component and reduction of the inward component (Fig. 5C: *black*), indicating that the inward flux of extracellular anions into the cells was enhanced over the whole voltage range. Subsequent replacement with Br^- reduced the outward component and increased the inward component (Fig. 5C: *dark grey*), but compared to the current in the initial Cl^- -rich condition, larger outward and smaller inward components were still apparent. The conductance selectivity sequence estimated from the slope conductance for the E224A mutant of ClC-3d at large positive potentials is $\text{I}^- > \text{Br}^- > \text{Cl}^-$. In the mutant-expressing cells, shifts in the reversal potential could be evaluated because the increase in the inward component varied with the extracellular anion used for replacement, and small leftward shifts in the reversal potential were reproducibly observed upon replacement of Cl^- with I^- and Br^- (Fig. 5C: *right panel*). In the ClC-3d mutant-expressing cells, the reversal potential of the current in the initial Cl^- -rich condition was 0.55 ± 0.46 mV ($n = 8$), consistent with the Nernst potential for Cl^- in this experimental condition (0.8 mV), and the shifts from the initial reversal potential caused by

Fig. 5. Halide selectivity of ClC-3d, ClC-3a and their mutant channels. (A) Current traces obtained from a ClC-3d-overexpressing cell with a ramp voltage pulse from -100 mV to +100 mV (2 s duration) in the presence of Cl⁻-rich extracellular solution before replacement of Cl⁻ (pale grey), after replacement of the extracellular Cl⁻ with I⁻ (black) and after replacement with Br⁻ (dark grey). The traces were obtained from the same cell. (B) Comparison of the current amplitude at +100 mV in the presence of extracellular I⁻ vs. that in the presence of Cl⁻, and in the presence of Br⁻ vs. in the presence of Cl⁻. Normalized current densities, compared to those before replacement of Cl⁻, are indicated in the bar graph. Dark columns give the means ($\pm$ SEM) for data obtained from ClC-3d-overexpressing cells and white columns give them for ClC-3a-overexpressing cells. The numbers in parentheses indicate the numbers of cells examined. Asterisks indicate significant changes in current density after anion replacement ($P < 0.05$ by paired t test). (C) Current traces obtained with the same ramp pulse protocol, from a cell overexpressing the E224A mutant of ClC-3d. The right panel shows an expansion of the traces around their x-axis intersections, from the section of the left panel indicated within the dotted square. The dotted lines over the current traces in the right panel indicate the results of linear fitting. The leftward shifts of the reversal potential after replacement of extracellular Cl⁻ with I⁻ and Br⁻ were consistently observed in all cases.

replacement with I⁻ and Br⁻ were -4.19 ± 2.48 mV and -5.23 ± 0.84 mV, respectively (both were significant at $P < 0.05$ by one-way repeated measure ANOVA, $n = 8$), giving permeability ratios for I⁻ and Br⁻ relative to Cl⁻ ($P_{\text{I}}/P_{\text{Cl}}$ and $P_{\text{Br}}/P_{\text{Cl}}$) of 1.23 ± 0.13 and 1.24 ± 0.04 . Similar shifts in the reversal potential were also seen in the ClC-3a mutant-expressing cells (data not shown, $n = 8$). Thus, the E224A mutation exhibits the permeability sequence for ClC-3d and ClC-3a to $\text{I}^- \approx \text{Br}^- > \text{Cl}^-$.

Weak effects of anion channel blockers on ClC-3d and ClC-3a currents

We also examined the effects of anion channel blockers on ClC-3d and ClC-3a currents. A general anion channel blocker, the carboxylate analog NPPB, was found to cause weak, but significant, suppression (20%) of ClC-3d currents when used at 300 μM (Fig. 6A: right panel and Fig. 6B: leftmost grey column; $P < 0.01$ by paired t test). However, the same concentration of NPPB had no significant effect on ClC-3a currents (Fig. 6B: leftmost white column), as observed in a previous report [7]. It is possible that the effect of NPPB on ClC-3d currents was more noticeable because the cell surface expression level of the channel was higher in ClC-3d-overexpressing cells than in ClC-3a-overexpressing cells (Figs. 3 and 4B). Nevertheless, another classic global anion channel blocker, the stilbene-derivative DIDS, had no significant effect on ClC-3d currents at 300 μM ($96.6 \pm 3.3\%$ of the initial amplitude before DIDS, $n = 8$).

It should be noted that DCPIB, a relatively specific blocker of the VSOR anion channel [21], had no significant effects on either ClC-3d or -3a currents at 10 μM (Fig. 6B: 2nd group of columns from left). Moreover, another VSOR channel blocker, phloretin [22], also had no effects on ClC-3d or -3a currents when used at 30 μM (Fig. 6B: 3rd group of columns from left).

Fig. 6. Effects of anion channel blockers, activation of Ca^{2+} -dependent kinases and Ca^{2+} depletion on ClC-3d and -3a currents. (A) An example of the effect of 300 μM NPPB on the ClC-3d current. The effect was observed 5 min after bath application of NPPB. (B) Summary of the effects of 300 μM NPPB, 10 μM DCPIB, 30 μM phloretin and 1 μM PMA on ClC-3d and -3a currents. These drugs were applied by bath perfusion, and their effects were assessed after 5 min. Peak current densities at +100 mV (in %), normalized to the current density before drug application, are given in the bar graph. Dark columns give the means ($\pm$ SEM) for data obtained from ClC-3d-overexpressing cells, and white columns give them for ClC-3a-overexpressing cells. The numbers in parentheses indicate the numbers of cells examined. Asterisk indicates significant suppression of the current ($P < 0.05$ by paired t test). (Ca) Comparison of the I - V relationships for ClC-3d currents in the presence (open diamonds; $n = 7$) and absence (open squares; $n = 8$) of the active form of

CaMKII α (0.2 $\mu\text{g}/\text{ml}$) in the patch pipette solution. There were no significant differences in the current amplitude (by unpaired t test) over the whole range of membrane voltages examined. (Cb) Comparison of the I - V relationship in the Ca^{2+} depleted condition (open diamonds; $n = 8$) to that in the normal Ca^{2+} condition (open squares; $n = 9$). Ca^{2+} was depleted both intracellularly and extracellularly by 30 min pretreatment of the ClC-3d-overexpressing cells with 1 μM thapsigargin, use of a perfusion solution from which Ca^{2+} was removed (see Materials and Methods) and inclusion of 10 mM BAPTA in the pipette solution. Each data point represents the mean $\pm$ SEM (vertical bar). There were no significant differences between the I - V relationships (by unpaired t test).

Lack of modulation of ClC-3d and ClC-3a currents by Ca^{2+} -dependent kinases

ClC-3 was originally reported as a channel greatly inhibited by activation of PKC [5], and the phosphorylation site of ClC-3 by PKC was subsequently identified [23]. However, our results demonstrate that acute application of 1 μM PMA to activate PKC had no effect on either ClC-3d or ClC-3a currents (Fig. 6B: rightmost group of columns). Moreover, it was reported that ClC-3 activity requires the phosphorylation of ClC-3 by CaMKII α instead [11] and that this mechanism underlies the Ca^{2+} -dependent modulation of excitatory postsynaptic potentials [24] and long-term potentiation [25] in hippocampal neurons. Our results, however, indicate that ClC-3d currents could be clearly observed without intentionally activating Ca^{2+} -dependent kinases and that the inclusion of the active form of CaMKII α in the pipette solution did not enhance the current amplitude further (Fig. 6Ca). In addition, even when Ca^{2+} in both the extracellular and intracellular environments was eliminated by pretreating the cells for 30 min with an ER Ca^{2+} pump inhibitor, thapsigargin (1 μM), removing it from the bath solution (and adding 0.1 mM EGTA) and including 10 mM BAPTA in the pipette solution, the amplitude of the ClC-3d current was unchanged (Fig. 6Cb). Thus,

Fig. 7. Effects of extracellular Cd^{2+} on ClC-3d and -3a currents. (A) Sample traces of ClC-3d currents before and 2 min after application of 1 mM Cd^{2+} , and 2 min after washout of Cd^{2+} . (B) I - V relationships for ClC-3d (a; $n = 7$) and ClC-3a (b; $n = 7$) currents before (open squares) and 2 min after application of 1 mM Cd^{2+} (filled squares). Each data point represents the mean $\pm$ SEM (vertical bar). (C) Mean peak current densities (with SEM bars) at +100 mV in the presence and after washout of Cd^{2+} (in %), normalized to the current density before Cd^{2+} application, are indicated as in Figure 6B. Asterisks indicate significant suppression of the currents during Cd^{2+} application ($P < 0.01$ by paired t test). (D) Concentration dependence of the Cd^{2+} -induced suppression of ClC-3d currents. Normalized mean current densities at +100 mV at plateau levels are plotted against the extracellular Cd^{2+} concentrations. The numbers in parentheses indicate the numbers of cells examined. Asterisks indicate significant suppression of the currents ($P < 0.01$ by paired t test). The solid curve represents a fit with the Hill equation for the % of current observed at +100 mV in the presence of Cd^{2+} compared to that before Cd^{2+} application, indicating the half-maximal suppression at 1.8 μM and the Hill coefficient of 1.1.

our results confirm that the activity of plasmalemmal ClC-3 does not require activation of Ca^{2+} -dependent processes in the cells.

Extracellular Cd^{2+} suppresses ClC-3d and ClC-3a currents

Group 12 (IIB) metal ions, like Zn^{2+} and Cd^{2+} , are known to block ClC-1 and ClC-2 [26, 27], but whether these may also block other ClC members is currently unknown. We examined the effects of extracellular Cd^{2+} on ClC-3d and -3a currents and found that bath application of 1 mM Cd^{2+} quickly suppressed both ClC-3d and -3a currents by approximately 40% within 1–2 min of its application (Fig. 7A: middle panel; Fig. 7Ba, 7Bb and 7C: left group of columns; $P < 0.01$ by paired t test). The Cd^{2+} -induced partial suppression could be reversed when washout was quickly made (Fig. 7A: rightmost panel, and Fig. 7C: right group of columns). The suppression was not enhanced further even when Cd^{2+} was applied at 3 mM (Fig. 7D: rightmost point). Dose dependence of the Cd^{2+} -induced suppression of ClC-3d currents indicates that the degree of suppression was unchanged in the range of 0.1 to 3 mM and the half-maximal suppression was attained at 1.8 μM (Fig. 7D). Thus, ClC-3d has a fairly high sensitivity to Cd^{2+} , but the Cd^{2+} effect is inherently partial.

Independence of ClC-3d from ASOR, VSOR and maxi-anion channel proteins

The molecular correlates of the acid-activated ASOR anion channel and two types of volume-regulated anion channel (VRAC) [13], the VSOR and maxi-anion channels, have not

Fig. 8. Lack of effects of CIC-3d overexpression on ASOR and VSOR anion channel currents. (A) Whole-cell currents of ASOR anion channels activated by extracellular acidification ($pH\ 4.5$) in mock-transfected and CIC-3d-transfected HEK293T cells. (B) Whole-cell currents of VSOR anion channels activated in control and CIC-3d-transfected cells by replacement of the isotonic bath solution (Iso) with a hypotonic (Hypo) one. Left and middle panels: Representative records of anionic currents before ($pH\ 7.5$ or Iso) and during exposure to acidic or hypotonic solution. The currents were elicited by application of alternating pulses from 0 to ± 40 mV or of step pulses from -100 to $+100$ mV in 20-mV increments. Expanded traces of current responses to step pulses under acidic or hypotonic stimulation are also presented (bottom panels). Right panels: Comparison of I - V relationships for basal currents (filled symbols), ASOR currents (open symbols in top panel) and VSOR currents (open symbols in bottom panel) between the control cells (squares) and the CIC-3d-transfected cells (circles). Each data point represents the mean $\pm$ SEM (vertical bars). The data for ASOR or VSOR currents recorded in the control and CIC-3d-transfected cells are not significantly different from each other at every voltage, whereas basal currents recorded in CIC-3d-transfected cells were significantly larger than those in the control cells at positive potentials over $+20$ or $+40$ mV, as indicated by asterisks ($P < 0.05$ by unpaired t test).

yet been identified. Since CIC-3a was previously suggested to correspond to the molecular entity of the ASOR anion channel [10] or VSOR anion channel [6, 23], we examined the possibility that CIC-3d is responsible for the endogenous ASOR or VSOR current in HEK293T cells. As shown in Figure 8A, reduction of extracellular pH from 7.5 to 4.5 induced activation of anionic currents in mock-transfected control HEK293T cells (left panel). The acid-sensitive currents were characterized by time-dependent activation in response to step pulse application (Fig. 8A: left panel) and a steep outward rectification (Fig. 8A: left and right panels), in accord with previous findings on ASOR anion channel currents [16]. ASOR channel currents that were essentially the same were observed in CIC-3d-transfected cells (Fig. 8A: middle and right panels). As shown in Figure 8B, application of hypotonic solution activated anionic currents of a VSOR-type channel that were characterized by inactivation kinetics and

Fig. 9. Lack of effects of siRNA-mediated knockdown of CIC-3 on maxi-anion channel currents in C127 cells. (A and B) Representative time course of maxi-anion channel activation in the inside-out patch excised from a control C127 cell transfected with non-targeting (negative control) siRNA (A) and a C127 cell transfected with CIC-3 gene-specific siRNA (B) at 0 to ± 25 mV. Arrow indicates the time at which the patch was excised. Right panels represent voltage- and time-dependent inactivation of the steady-state current recorded at *asterisks* in the left panels in response to voltage pulses (500 ms) from 0 to ± 50 mV in 10-mV increments. (C) Comparison of the mean patch currents recorded at +25 mV between C127 cells transfected with non-targeting siRNA (control) and those with CIC-3 gene-specific siRNA. Each column represents the mean $\pm$ SEM (vertical bar). (D) Expression of CIC-3 mRNA in C127 cells transfected with non-targeting (control) siRNA or CIC-3 gene-specific siRNA. GAPDH was used as an internal control. M: Marker. Data represent duplicate experiments.

moderate outward rectification [28, 29], in both control and CIC-3d-transfected HEK293T cells. The amplitude and other properties of the ASOR and VSOR channel currents recorded in CIC-3d-transfected cells were indistinguishable from what was observed in control cells. In addition, 1 mM Cd^{2+} , which could partially suppress CIC-3a and CIC-3d currents (Fig. 7), was found to fail to inhibit both the ASOR and VSOR currents in HEK293T cells. At +100 mV, the ASOR currents were 116.1 ± 17.8 and 109.1 ± 17.1 pA/pF ($n = 7$; $P > 0.5$), and the VSOR currents were 126.3 ± 38.1 and 161.0 ± 48.3 pA/pF ($n = 7$; $P > 0.5$) in the absence and presence of 1 mM Cd^{2+} , respectively. Thus, it is obvious that the CIC-3d protein is a molecular entity independent of the ASOR and VSOR anion channels.

We next examined whether CIC-3d overexpression introduces functional expression of the maxi-anion channel into HEK293T cells, which do not have any endogenous maxi-anion channel activity. Excision-induced activation of large-conductance single-channel events was never observed in either control HEK293T cells or CIC-3d-transfected cells (data not shown, $n = 10$). We then examined whether any variant of CIC-3 is related to the molecular entity of the swelling- or excision-activated large-conductance maxi-anion channel endogenously expressed in mouse mammary C127 cells [30, 31]. As shown in Fig. 9 (A, B), currents were similarly activated after membrane patches were excised from control C127 cells transfected with non-targeting siRNA (A: *left panel*) and from C127 cells transfected with siRNA targeting all variants of CIC-3 (B: *left panel*). The currents of inside-out patches from both cells consisted of multiple large-conductance unitary steps (with the single-channel

conductance of ~ 400 pS) and displayed voltage- and time-dependent inactivation at both positive and negative potentials over ± 10 mV (Fig. 9A and 9B: *right panels*). The properties of these single-channel currents were identical to those of maxi-anion channels observed in the patches excised from non-transfected C127 cells [30, 31]. The mean peak patch currents recorded at +25 mV were indistinguishable between control and ClC-3 siRNA-transfected cells (Fig. 9C), although expression of ClC-3 mRNA was markedly reduced (by $85.3 \pm 7.8\%$, $n=4$) by transfection with ClC-3 siRNA (Fig. 9D). Knockdown efficacy of $>80\%$ reduction was confirmed by quantitative RT-PCR (data not shown). Taken together, it appears that any variants including ClC-3d are independent of the maxi-anion channel protein.

Discussion

Anion channels play essential roles in diverse cellular processes such as excitation, contraction, salt and water transport, volume regulation, pH regulation, proliferation, migration, apoptosis, necrosis, and ischemic injury [32]. Of the various anion channel types, the molecular entities corresponding to the following three types of broadly expressed anion channel have not been identified [13]: the swelling-activated VSOR anion channel, the acid-activated ASOR anion channel and the swelling- or excision-activated large-conductance maxi-anion channel. Since ClC-3 is ubiquitously expressed [5, 33], it would be reasonable to assume that some isoforms of ClC-3 might be related to these unidentified anion channels. In fact, Hume and coworkers [6, 23, 34] proposed that guinea pig and human short-form ClC-3 (corresponding to ClC-3a) is the long-sought VSOR anion channel. However, a large number of contradictory or inconsistent data have been reported, as summarized previously [13]. Most critically, patch-clamp experiments performed using *Clcn3*^{-/-} mice demonstrated that VSOR-type swelling-activated anionic currents were unaffected in acinar cells [35, 36] and cardiomyocytes [37, 38]. On the other hand, Matsuda, Lamb and coworkers reported that human short-form ClC-3 (corresponding to ClC-3a) is responsible for the native ASOR-type acid-activated anion channel currents [10]. At variance with this hypothesis were results from our recent studies [14, 39] that showed that ASOR anion channel currents were not affected by either siRNA-mediated knockdown of ClC-3 or overexpression of ClC-3a in human epithelial cells. However, there remains a possibility that other isoforms of ClC-3, especially some uncloned one, might correspond to the ASOR, VSOR and/or maxi-anion channel molecules. Thus, the present study was started to clone the ClC-3 isoform d, the full coding region of which was not yet identified, and then a possibility of its involvement in these anion channels was tested after characterizing the current mediated by this isoform.

Overexpression of ClC-3d cloned from mouse liver (Fig. 1) in HEK293T cells, however, provoked a different type of anion channel current characterized by almost instantaneous activation and deactivation kinetics (Fig. 4A), steep outward rectification which was abolished by the E224A mutation (Fig. 4B), and an anion selectivity sequence of $\text{Cl}^- > \text{I}^-$ which became $\text{I}^- > \text{Cl}^-$ in the E224A mutant (Fig. 5). It was found that these ClC-3d current characteristics are shared by ClC-3a currents (Figs. 4 and 5). Furthermore, ClC-3d currents were not sensitive to a stilbene-derivative anion channel blocker (DIDS) to which ASOR, VSOR and maxi-anion channel currents are, more or less, sensitive [16, 28, 30]. Finally, in the present study, both ClC-3a- and ClC-3d-induced currents were consistently found to show weak but significant inhibition by Cd^{2+} (Fig. 7), whereas Cd^{2+} failed to suppress ASOR currents and VSOR currents in HEK293T cells, as previously shown for VSOR currents in T_{84} cells [40]. Thus, the current characteristics of ClC-3d and -3a proteins we observed are clearly different from those of ASOR, VSOR and maxi-anion channels. Indeed, overexpression of ClC-3d failed to affect the ASOR and VSOR anion currents (Fig. 8). Also, ClC-3d overexpression never introduced functional expression of maxi-anion channels into HEK293T cells, which lack endogenous activity of this channel. Moreover, siRNA-mediated down-regulation of *Clcn3* failed to suppress the endogenous activity of the maxi-anion channel in mouse mammary C127 cells (Fig. 9). Taken together, these results lead us to conclude that ClC-3d is unrelated to the molecular entities corresponding to the ASOR, VSOR and maxi-anion channels.

The characteristics of ClC-3d and -3a currents revealed in the present study are consistent with those observed on the ClC-3 current in some reports [7, 8], but in contrast to those in the original report [5] and in some other reports [6, 9, 11]. However, the characteristics revealed in our study are also quite similar to those of the currents mediated by ClC-4 and -5 [20], which belong to the same branch of the ClC family as ClC-3. Anionic currents induced by overexpression of ClC-3a or ClC-3d were found to be independent of PKC activation (Fig. 6B). These observations are consistent with the results obtained for ClC-3a by Weinman and coworkers [7] but not with those obtained by Kawasaki et al. [5]. In the present study, ClC-3d currents were found to be independent of CaMKII α activation and insensitive to Ca²⁺ depletion (Fig. 6C). These results are in contrast to the result obtained for the long form of ClC-3 (ClC-3b) by Huang et al. [11], even though a putative CaMKII α phosphorylation site (S109) in the *N*-terminal region of ClC-3b is conserved in ClC-3d (as S51). Consistent with our conclusion that neither ClC-3a nor ClC-3d is related to the VSOR anion channel (see above), the anionic currents induced by overexpression of ClC-3a or ClC-3d were insensitive to two blockers of the VSOR anion channel that are of different type, DCPIB and phloretin (Fig. 6B). The phloretin insensitivity is in contrast to the result in a previous report for ClC-3a by Matsuda et al. [9]. ClC-3a-induced currents were insensitive to a broad-spectrum anion channel blocker, NPPB (Fig. 6B), as reported previously [7]. ClC-3d-induced currents, however, exhibited weak sensitivity to a high concentration (300 μ M) of NPPB (Fig. 6B). It is possible that this minor difference in NPPB sensitivity between ClC-3a and ClC-3d is due to the different amino acid sequences in their *C*-terminal regions (Fig. 1C). The Cd²⁺ sensitivity of ClC-3 (Fig. 7) is a new finding not reported previously. Cd²⁺ is known to interact with cysteine and histidine residues in ClC-1 [26] and cysteine-256 in ClC-2 [27], causing reduction of these Cl⁻ channel currents. Thus, it is possible that Cd²⁺ binds to similar residues in ClC-3, although cysteine-256 in ClC-2 is not conserved in ClC-3. Also, overall similarity between ClC-3a and ClC-3d currents may suggest that the distal *C*-tail sequence, which is only the portion different between molecular structures of ClC-3a and ClC-3d, does not have functional relevance to the channel activity. From these results, we conclude that ClC-3d, just like ClC-3a, functions as a Cd²⁺-sensitive outwardly rectifying anion channel in a manner independent of intracellular Ca²⁺-dependent processes.

Physiological/pathophysiological roles of the ClC-3d anion channel remain to be identified in future studies. Since *Clcn3d* was found to be highly expressed in the liver (Fig. 2), it is possible that ClC-3d is important in metabolic activity. In this regard, it is noteworthy that ClC-3 null mice exhibit a phenotype with metabolic abnormalities including poor growth and reduced body fat [36, 41]. *Clcn3d* was also detected in the brain in mice, though at a lower level than in the liver (Fig. 2). In regard to neuronal activity, Nelson and coworkers [24, 25] recently found that there was loss of Cl⁻-dependent changes in synaptic strength and excessive induction of long-term potentiation in the hippocampus in ClC-3 null mice. Further investigation is needed to clarify the extent to which loss of the ClC-3d anion channel is responsible for these metabolic and neuronal abnormalities in ClC-3 null mice, in which all isoforms of ClC-3 are knocked out.

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Conflict of Interest

None declared.

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