Identification of an acid-activated Cl\(^-\) channel from human skeletal muscles

MASANOBU KAWASAKI, TOSHIKO FUKUMA, KAZUSHI YAMAUCHI, HISATO SAKAMOTO, FUMIAKI MARUMO, AND SEI SASAKI

Second Department of Internal Medicine, Tokyo Medical and Dental University, Tokyo 113–8519, Japan

Kawasaki, Masanobu, Toshiko Fukuma, Kazushi Yamuchi, Hisato Sakamoto, Fumiaki Marumo, and Sei Sasaki. Identification of an acid-activated Cl\(^-\) channel from human skeletal muscles. Am. J. Physiol. 277 (Cell Physiol. 46): C948–C954, 1999.—CIC-4 gene was isolated as a putative Cl\(^-\) channel. Due to a lack of functional expression of CIC-4, its physiological role remains unknown. We isolated a human CIC-4 clone (hCIC-4sk) from human skeletal muscles and stably transfected it to Chinese hamster ovary cells. Whole cell patch-clamp studies showed that the hCIC-4sk channel was activated by external acidic pH and inhibited by DIDS. It passed a strong outward Cl\(^-\) current with a permeability sequence of I\(^-\) > Cl\(^-\) > F\(^-\). The hCIC-4sk has consensus sites for phosphorylation by protein kinase A (PKA); however, stimulation of PKA had no effect on the currents. hCIC-4sk mRNA was expressed in excitable tissues, such as heart, brain, and skeletal muscle. These functional characteristics of hCIC-4sk provide a clue to its physiological role in excitable cells.

human CIC-4sk; CICN4; outwardly rectifying chloride channel; acidification

OUTWARDLY RECTIFYING Cl\(^-\) CHANNELS (ORCC) PLAY ROLES IN CELL VOLUME REGULATION (8, 27, 40), DRIVING THE FLUID SWELLING (9). ClC-3 is thought to be important in cell disease (24, 31). ClC-5 may be involved in vacuolar Cl\(^-\) transport, contributing to their acidification in renal tubules. In contrast, two laboratories had already isolated the CIC-4 DNA clone; however, both failed to express it functionally (16, 39). It remains unknown whether the CIC-4 channel is indeed an ORCC as CIC-3 and -5.

In the present study, we isolated CIC-4 (hCIC-4sk) cDNA clone from human skeletal muscle and established stably transfected mammalian cell lines [Chinese hamster ovary (CHO) cells] expressing hCIC-4sk channels. We characterized the expressed hCIC-4sk channel using a patch-clamp technique. We observed outward Cl\(^-\) currents that were activated by extracellular strong acidification.

MATERIALS AND METHODS

Isolation of human CIC-4 cDNA clones and DNA sequencing. We performed a PCR cloning strategy using HIT-T15, a Syrian hamster pancreatic β-cell, as a source of mRNA. One microgram of HIP-T15 cell total RNA was reverse transcribed at 42°C for 60 min and then heated at 94°C for 5 min (Avian myeloblastosis virus RNA-dependent DNA polymerase; Boehringer Mannheim). The synthesized cDNA was used for subsequent PCR in the following profile: 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, 35 cycles. PCR primers used were as follows: sense strand CCGATCCGGNATHCCNGARHTNAARAC and antisense strand CCGAATTCTGNA-CNARNNGNCYCTTYT (where N = A/C/G/T; H = A/C/T; R = A/G; and Y = C/T). The PCR product of expected size (318 bp) was cut with EcoRI and BamHI, ligated into EcoRI and BamHI cut pSPORT1 (BRL), and then sequenced. The sequence revealed the existence of a new CIC channel; however, it was highly homologous to human retina CIC-4 (gene symbol: CICN4; 90.1%). Northern blot analysis using this PCR clone (HIT38) as a probe revealed abundant expression in rat brain and rat skeletal muscle. Therefore, a human skeletal muscle library in λgt11 was screened for the isolation of a full clone. The library was screened under high stringency (6× sodium chloride-sodium phosphate-EDTA, 5× Denhardt’s solution, 1% SDS, 100 µg/ml salmon sperm DNA, and 50% formamide at 42°C) using an HIT 38 insert labeled with [α-32P]dCTP (3,000 Ci/mmol; Amersham). Two clones with inserts of 3.5 and 4.5 kb were subcloned into pUC 118, designated as hCIC-4sk. Nested deletion clones were prepared using the Erase-A-Base system (Promega) and were sequenced using T7 DNA polymerase by the chain termination method or the dideoxy chain termination method using fluorescence-labeled primers on an automated sequencer. Antisense strand was sequenced using synthetic primers. hCIC-4sk-expressing cell line The Sal I and blunt-cut fragment of CIC-4sk was ligated into a mammalian expression vector, pMAM2-BSD. CHO-K1 cells (obtained from Japanese Collection of Research Bioresources) were grown in Ham’s F-12 Nutrient Mix (GIBCO) supplemented with 10% FBS at 37°C in 5% CO\(_2\). Conventional intranuclear microinjection of the expression plasmid vector was carried out using an Eppendorf transjector 5246 and micromanipulator 5171, attached to a Zeiss inverted phase-contrast microscope (30). Cells containing stably integrated copies of transfected recombinant plasmid were selected by adding blastidin S (BSD) to the growth media at a concentration of 10 µg/ml. After selection for 2 mo, BSD-resistant cell clones were isolated and transferred to separate culture dishes for expansion and analysis. Stable expression of the transfected plasmid was confirmed by Northern blot analysis. Total RNA was isolated...
from ~10^7 cells of each BSD-resistant clone treated with or without 2 µM dexamethasone for 24 h (22). Twenty micrograms of each sample of total RNA were resolved in a formaldehyde-0.7% agarose gel and were blotted onto a nylon membrane. The hybridization was performed overnight with 10^6 counts·min^-1 of ClCN4 cDNA probe labeled with [α-^32^P]dCTP (Amersham) by random priming (Promega). A human multiple tissue Northern blot with 2 µg of poly(A)^+ RNA/tissue (Clontech) was also hybridized under the same condition. Hybridization was visualized by autoradiography.

The RT-PCR reaction was performed to distinguish hClC-4 from other Cl^- channels. Total RNAs were isolated from each cell of the CIC-3-expressing cell (C21), hClC-4sk-expressing cell (C53), and CIC-5-expressing cell (J27). Total RNA (1 µg) was reversetranscribed at 50°C for 30 min and then heated at 94°C for 5 min (Avian myeloblastosis virus RNA-dependent DNA polymerase; Boehringer Mannheim). The synthesized cDNA was used for subsequent PCR in the following profile: 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, 40 cycles. Specific sense and antisense primers for hClC-4sk transcript were designed as follows: sense, 5'-ATGTTGATGAGCCGAGCCTGTCGATGAGCC-3'; antisense, 5'-AGAAGTTGCCCCGCAAACCTGCATTGCTGATGAC-3'. The expected size of the PCR product is 316 bp for the hClC-4sk transcript. PCR products were analyzed on a 1.2% agarose gel. To identify the products as a partial fragment of the hClC-4sk, they were subcloned into pCR 2.1 (Invitrogen) and sequenced using the dideoxy chain termination method using fluorescence-labeled primers on an automated sequencer (ABI 377).

Electrophysiological characterization. Conventional patch-clamp techniques were used to record whole cell currents from CIC-4- or CIC-4 E224A-transfected CHO cells. A mutation of E224A in CIC-4sk was made using a PCR mutation strategy (Stratagene). All transfected cells for expression studies were cultured in a dexamethasone-free medium. The CIC-4sk-transfected cells treated with dexamethasone produced huge currents and a burst in many occasions. To obtain stably whole cell currents in broad voltage ranges and for a long time period, we had to lower the expression level of hClC-4sk. Currents were recorded at room temperature (20–24°C) with an EPC-7 patch-clamp amplifier (List-Electronic; West Germany), and the data were stored on a DAT recorder (DAT-200; Sony, Tokyo, Japan). Records were sampled at 2,000 points/s and were analyzed on a Compaq ProLinea 4/50 computer using Axon version 6.0 software. The obtained data were transferred to a Macintosh 550c computer and were analyzed using Excel 2.2 software. In the whole cell configuration, the pipette solution contained (in mM): 130 potassium glutonate, 20 KCl, 5 PIPES, 1 EGTA, and 100 µg/ml nystatin (stock solution 25 mg/ml in DMSO; see Ref. 23), pH 7.2. The bath solution contained (in mM) 130 tetraethy lammonium chloride, 1 MgCl2, 10 PIPES, 1 EGTA, and 0.51 CaCl2 (pH 7.20, 6.0, and 4.5). In the study of anion selectivity, the relative anion permeabilities were determined on the basis of the relative current after the replacement of Cl^- with other anions using an SF-77B Perfusion Fast-Step (Warner Instrument). The bath solution was 120 mM NaF for F^-, 120 mM NaCl for Cl^- (pH 4.5). The toxicity of the standard pipette and bathing medium was measured by freezing-point depression and was adjusted to 280 mosmol with maltose. All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted. Data were filtered at 1 kHz and were sampled at 2 kHz.

RESULTS

Isolation of hClC-4sk. Figure 1 shows the primary structure of hClC-4sk deduced from the nucleotide sequence of the cDNA isolated from the human skeletal muscle cDNA library. The hClC-4sk Cl^- channel is composed of 760 amino acid residues. The nucleotide sequence of hClC-4sk had seven nucleotides that were different from that of the ClCN4 isolated from a retina cDNA library (39); as a result, it possessed four different amino acids (residues R178A, Y498I, Y499I, and N659K, see Fig. 1). The overall amino acid identities to the ClCN3 and ClCN5 were high (78% amino acid sequence identity to ClCN3; 78% to ClCN5). Two potential N-glycosylation sites are found at residues 119 and 421. There are four consensus sequences for phosphorylation sites for protein kinase C at positions Thr43, Thr39, Thr362, and Ser462 and two potential phosphorylation sites for protein kinase A at positions Thr362 and Thr363 (21). Full-length hClC-4sk cDNA probe hybridized with several bands, mainly at 4.4 and 7.5 kb, under a high stringent condition in human tissues (Fig. 2A). The expression was observed to be highest in the skeletal muscle. Moderate expression was also observed in the liver, lung, pancreas, and testis at much lower levels. These results were consistent with those of van Slengenhorst et al. (39).

Functional expression of hClC-4sk in CHO cells. CHO-K1 were stably transfected with the coding sequence of the cDNA of hClC-4sk using an expression vector pMAM2-BS2. After the selection with BSD for 2 mo, 85 clonal cell lines derived from the transfected cells were examined for hClC-4sk mRNA expression, and two clonal cell lines (clone 11 and clone 53) were selected by Northern blot analysis (Fig. 2B). We did RT-PCR to reveal a level of gene expression of hClC-4sk from CIC-4-transfected cells in the absence of dexamethasone (Fig. 2C). As negative controls, we used CIC-3- and CIC-5-transfected cells to show the primer set specific for hClC-4sk. To identify the products as a partial...
fragment of the hClC-4sk, they were subcloned and sequenced (4/4).

To examine the expression of hClC-4sk channels on the cell surface, we assayed the transfected cells by the patch-clamp technique in the whole cell configuration. In a physiological condition (pH 7.2, 280 mosmol), CIC-4sk-transfected cells showed a very small current (165 ± 33.8 pA; mean ± SE at +100 mV membrane potential, n = 10). Nontransfected (134 ± 61 pA, n = 9) and vector-alone-transfected (167 ± 53 pA, n = 6) CHO-K1 cells produced a similar level of current amplitude (Fig. 3). In the previous reports of other laboratories, CICN4 channels were not functionally expressed in Xenopus oocytes and mammalian cells (16, 39). The present result at pH 7.2 was consistent with those previous reports.

Recently, an immunohistological study demonstrated that the CIC-5 channel protein was colocalized with H+-ATPase at intracellular vesicles in renal proximal tubule (15). Their localization suggested that the CIC-5 channel probably plays a role in vesicular acidification. Intravascular environment is maintained at pH of 4.5–5.0 in mammalian cells (28). It would be possible that CIC-3, CIC-4, and CIC-5 are regulated by extracellular pH (pHo). We examined the effect of acidification on the hClC-4sk-expressing cells. When pH of the bath solution was changed from pH 7.2 to 4.5, an outwardly rectifying current appeared in hClC-4sk-transfected cells (1,383 ± 187 pA; n = 10/10), whereas no significant current appeared in nontransfected (134 ± 34 pA; n = 7) and vector-alone-transfected (215 ± 108 pA, n = 6) cells (Fig. 3). The acidic pH-induced currents were activated instantaneously by depolarizing voltage with further slow activation. Stepping back to the holding potential caused deactivation instantaneously. The predicted reversal potential of the current activated by acidic solution was near the equilibrium potential for Cl−, suggesting that the current was carried mainly by Cl−. Steady-state current-voltage curves of activated channel currents revealed a strong outward rectification (Fig. 3, bottom right) that is stronger than that of CIC-3 and -5 in CHO cells (19, 30). When hClC-4sk-expressing cells were exposed to pH 6.0, hClC-4sk channels were activated by this pHo in some cells (n = 5/9), and current amplitudes were about one-half that of pHo 4.5 (682 ± 176 pA; Fig. 3). In the remaining inactivated cells (4/9), reducing bath pH further to <5.0, the channels were activated (n = 4/4). When increasing the pHo value above 6.5, the CIC-4 was always inactive.

Relative anion selectivity to Cl−, I−, and F− was compared (Fig. 4). In the whole cell currents, solutions

---

Fig. 2. A: Northern blot analysis of hClC-4sk expression in different human tissues. Poly(A)+ RNA (~2 µg/lane) from various human tissues were loaded in each lane and subsequently hybridized with the full-length hClC-4sk cDNA probe. Markers of transcript size (in kb) are indicated. B: stable expression of hClC-4sk. Northern blot analysis of transfected cell lines for expression of hClC-4sk (top). Every lane on an agarose gel contained 10 µg of total RNA from each transfected cell line. -, Pretreatment with 2 mM dexamethasone (Dex) for 1 day; +, without pretreatment. Lane 1, clone 11 Dex (−); lane 2, clone 53 Dex (−); lane 3, clone 11 Dex (+); lane 4, clone 53 Dex (+). Total RNAs transferred to the nylon membrane were stained by ethidium bromide and visualized by ultraviolet light (bottom). C: expression of hClC-4sk without dexamethasone detected by RT-PCR. hClC-4sk PCR products could be obtained from the hClC-4-transfected cells without dexamethasone. RT-PCR for hClC-4sk at 40 cycles revealed undetectable level of gene expression from CIC-3- and CIC-5-transfected cells.
containing iodides gave current amplitudes $1.42 \pm 0.36$ (at 100 mV; $n = 4$) times larger than those with Cl$^-$. The current reversed at more negative potentials when Cl$^-$ was replaced by equimolar I$^-$. F$^-$ was less permeable than Cl$^- (0.26 \pm 0.05)$. Replacement of extracellular Cl$^-$ with F$^-$ shifted the reversal potential to more positive values. The anion selectivity sequence of hClC-4sk currents was I$^- >$ Cl$^- >$ F$^- (7)$. The anion permeability sequence of the hClC-4sk was identical to that of the rat CIC-3 and rat CIC-5 (18, 25, 30). Extracellular 1 mM DIDS, an inhibitor of Cl$^-$ channels, decreased the conductance by $77.5 \pm 4.6\% (n = 3)$. Thus the CIC-4 channel is a DIDS-sensitive ORCC.

Figure 5 showed representative sequential traces of hClC-4sk current regulated by pH. In nontransfected cells, when pH of the bathing solution was changed from pH 7.2 to 4.5 using the triple-barreled method, endogenous Cl$^-$ currents evoked by pulse stimuli remained very small. In contrast, on exposure to the bath solution of pH 4.5, an outwardly rectifying Cl$^-$.current appeared from the hClC-4sk-expressing cell within a few minutes [mean time to half-maximal activation = 79.2 $\pm$ 6.8 (SD) s, $n = 6$; Fig. 5B, top]. Treatment of pH 8.0 bath solution sharply decreased the currents (Fig. 5B, middle), and this inhibitory effect quickly disappeared after changing the bath solution from pH 8.0 to 4.5 (mean time to half-maximal activation = 11.6 $\pm$ 5.8 s, $n = 6$), indicating that the underlying mechanisms for the initial and second activation by acid pH may be different. Next, we tested the effects of activators of protein kinase A on a low pH-induced Cl$^-$.current. 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate (200 µM) with 1 mM IBMX and 10 µM forskolin at pH 4.5 had no significant effect on the current (Fig. 5B, bottom), suggesting that the pH-sensitive hClC-4sk channel was not regulated by protein kinaseA. To confirm that the Cl$^-$ currents observed in hClC-4sk-transfected cells indeed passed through the hClC-4sk channels, we introduced a point muta-
Fig. 5. Effect of pH on the hClC-4sk channel currents. A: nontransfected; B: CIC-4. Representative sequential traces of whole cell currents from hClC-4sk-transfected cells sequentially exposed to pH 7.2, 4.5, and 8.0. Labeled arrows indicate changes of bath solution pH. "Forskolin" indicates the application of forskolin mixture. Holding potential was clamped at ±50 mV.

Fig. 6. Mutant expression of hClC-4sk E224A. A: representative trace of the hClC-4sk E224A-transfected CHO cells on exposure to the pH 7.2 bath solution. Holding potential was changed from −100 to +100 mV stepped by +20 mV. B: current-voltage relationships of hClC-4sk E224A channel. Whole cell currents measured between −100 and +100 mV. Ensemble averages of hClC-4sk channel currents were constructed for each of the data points plotted (n = 3).
tion, E224A, to hClC-4sk cDNA and characterized the functional properties of hClC-4sk E224A. The E224A mutant-transfected cells generated inwardly instead of outwardly rectifying Cl\(^-\) currents, even at pH\(_o\) 7.2 (n = 3, Fig. 6).

**DISCUSSION**

The ClCN4 gene was isolated from the Xp22.3 region using a positional cloning strategy (39). A deletion of the Xp22.3 region caused a psychomotor delay and mental retardation in the patient (3). The ClCN4 gene possibly contributes to the pathogenesis of these neurological disorders. In the present study, human CIC-4 cDNA was isolated from a human skeletal muscle cDNA library by a sequence homology-based strategy. We successfully obtained stable functional expression in CHO cells.

The present nucleotide sequence of hClC-4sk showed seven nucleotides in the ORF-coding sequence different from that of ClCN4. These nucleotide mutations changed four amino acids of the ClCN4 amino acid sequence. However, the amino acids at these four positions of hClC-4sk were conserved in those of rat and mouse ClC-4 and human genomic DNA. The human retina cDNA alone is different. Probably, its nucleotide sequence differences are caused by sequencing or cloning errors.

In this study, the hClC-4sk channel was activated by extracellular acidic pH. On exposure to acidic bath solutions, hClC-4sk generates a very strong outwardly rectifying Cl\(^-\) current (Fig. 3). Mock-transfected CHO cells did not produce Cl\(^-\) currents in the acidic bath solutions. The E224A mutation altered its channel rectification from strongly outward to inward (Fig. 6). These data proved that hClC-4sk actually generated Cl\(^-\) currents. The properties of the ClC-4 whole cell currents, namely strong outward rectification, DIDS sensitivity, anion conductivity, and insensitivity to protein kinase A, were consistent with the previous reports of ClC-3 and ClC-5, except for activation by acidification (9, 19, 20, 30). Thus the CIC-4 channel is an actual member of the ORCC subfamily.

If we assume that CIC-4 is a vacuolar Cl\(^-\) channel, the expected orientation of the hClC-4sk channel within a vacuolar membrane would make it better suited for Cl\(^-\) efflux rather than Cl\(^-\) influx for vacuole acidification. Thus it is necessary for the CIC-4 to pass through influx of Cl\(^-\). However, the hClC-4sk inward current was inactive in the present study. Native vesicular Cl\(^-\) conductance was regulated by protein kinase A (2, 26). hClC-4sk was not regulated by protein kinase A (Fig. 5) like other members of the ORCC subfamily (ClC-3 and ClC-5; see Refs. 20 and 30). It would be possible that a regulatory factor(s) is lacking to obtain inward currents and protein kinase A activation properties in CHO cells. With regulator(s), the hClC-4sk channel may play a role of vacuolar acidification in excitable cells.

pH\(_o\) can modulate the channel activity of some Cl\(^-\) channels. Acidic pH\(_o\) diminished time-dependent inactivation of CIC-1 inward currents; thus, the steady-state component was enhanced (29). Hyperpolarization-activated Cl-2 currents were stimulated by acidic pH\(_o\) (32, 34). Reducing pH\(_o\) from 7.3 to 6.5 decreased inwardly rectifying Cl\(^-\) currents from the epithelial cells of the choroid plexus (18). These reports showed that pH\(_o\) modulated channel activity. During the revision of this draft, an expression study of ClC-4 was published by Friedrich et al. (12). Interestingly, they showed that the ClC-4 channel was inhibited by external low pH and has an anion permeability sequence of Cl\(^-\) > I\(^-\). In contrast, the hClC-4sk channel was activated by external low pH in an all-or-none fashion. It has an anion conductivity sequence of I\(^-\) > Cl\(^-\). The reason for the functional discrepancies between their results and ours is not clear. The expression system (Xenopus oocyte vs. CHO-K1 cells) and experimental condition (perforated vs. cell-attached whole-cell current) are different. It is possible that the CIC-4 channel is modulated by endogenous intracellular factors.

The time course of the initial activation of hClC-4sk by acidic pH was always longer than that of the second activation. When extracellular acidic solution was first exposed to the hClC-4sk-expressing cells, they always generated Cl\(^-\) currents after a few minutes. Alkaline pH\(_o\) of 8.0 quickly shut down the outwardly rectifying Cl\(^-\) current, which had already been activated by acidic pH\(_o\) (Fig. 5). Blockade of hClC-4sk by an increase in pH\(_o\) was again quickly recovered by a low pH\(_o\). The slow initial activation and the fast pH regulation afterward are possibly caused by different regulatory mechanisms. The pH gradient along the exocytotic pathway may be important for the sorting proteins and vesicle trafficking (6, 25). We speculate that the CIC-4 channels might localize in organelles at neutral pH. Decreasing pH\(_o\) may activate the exocytotic pathway, allowing the sorting of vesicles containing the CIC-4 channels into the plasma membrane. A number of the hClC-4sk channels increased, and it produced Cl\(^-\) currents. Once hClC-4sk channels were put on the plasma membrane, they should be quickly regulated by pH\(_o\), suggesting that the CIC-4 channel protein may be directly modified by extracellular acidification through possible protonation of an external amino acid residue.

We thank K. Hayashi for help with cloning of hClC-4sk. This work was supported by grants-in-aid from the Ministry of Education, Science, and Culture, Japan.

Address for reprint requests and other correspondence: M. Kawasaki, Second Dept. of Internal Medicine, Tokyo Medical and Dental University, 1–5–45 Yushima, Bunkyo-ku, Tokyo 113–8519, Japan.

Received 4 January 1999; accepted in final form 17 August 1999.

**REFERENCES**


