A Novel Human Cl− Channel Family Related to Drosophila flightless Locus*

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Makoto Suzuki‡ and Atsuko Mizuno
From the Department of Pharmacology, Jichi Medical School 3311-1, Yakushiji, Minamikawachi, Tochigi 329-0498, Japan

Large conductance chloride (maxi-Cl−) currents have been recorded in some cells, but there is still little information on the molecular nature of the channel underlying this conductance. We report here that tweety, a gene located in Drosophila flightless, has a structure similar to those of known channels and that human homologues of tweety (hTTYH3) are novel maxi-Cl− channels. hTTYH3 mRNA was found to be distributed in excitable tissues. The whole cell current of hTTYH3 was large enough to be discriminated from the control but emerged only after treatment with ionomycin. Analysis of pore mutants suggested that positively charged amino acids contributed to anion selectivity. Like a maxi-Cl− channel in situ, the hTTYH3 single channel showed 26-picosiemen linear current voltage, complex kinetics, and pH, ionic, and cell volume regulation. Cl− channels play roles in the stabilization of membrane potential, transport, and cell volume regulation. Cl− channels are observed in many tissues and are thus variably regulated by voltage, calcium, pH, or cell volume. There are three types of Cl− channels based on their conductance: small (<10 pS), medium (10–100 pS), and large (>100 pS, maxi-Cl−) conductance channels. Small and medium conductance channels are found ubiquitously, even in oocytes, whereas large conductance channels are rarely found. To our knowledge, less than 12 cells possessing maxi-Cl− channels have been found over the past 40 years. A maxi-Cl− channel in muscle or in other tissues is activated directly by cytosolic Ca2+ (1, 2), whereas activity of the channel in other tissues is independent of Ca2+ (3, 4). GTP-binding protein (5), cell volume (3), and protein kinases (6) have been reported to modify the function of a maxi-Cl− channel. However, because of their rarity, the physiological roles of maxi-Cl− channels remain obscure.

The molecular structures of Cl− channels are diverse, with different transmembrane segments (TMSs) such as cystic fibrosis transmembrane regulator (7) and CIC (8) with 10 or 12 TMSs. Aquaporin-6 encodes a unique acid-dependent Cl− channel with 6 TMSs (9). The CICA family, with 5 TMSs, encodes medium conductance Ca2+-activated Cl− (CaC) channels (10, 11). Thus, it is possible that there exists a Cl− channel with more variable TMSs.

On the other hand, cDNA encoding ion channels is generally expressed in ovary cells, such as Chinese hamster ovary (CHO) and Xenopus oocytes. They possess Cl− channels endogenously. CaC currents are frequently observed as endogenous currents of these cells. The CaC current is driven by small or medium conductance Cl− channels activated by a high concentration of intracellular Ca2+, is outwardly rectifying, and is inhibited by 4,4′-disothiocyanato-stilbene-2,2′-disulfonic acid (DIDS) and niflumate (3). Human embryonal kidney (HEK) cells have also been used for the expression of cDNA encoding CaC (10), but they possess endogenous maxi-Cl− channels regardless of the concentration of Ca2+ (12).

To try to find a novel ion channel, we used the BLAST program to search for proteins that have four or more TMSs as predicted by hydrophobicity and a gene related to behavior abnormality. By using a cluster of leucine residues as a transmembrane probe, we found a human gene (httyh3) that has homology to Drosophila tweety (dtyth1) located in the flightless locus (13), which is related to behavior abnormality. flightless is a mutant of Drosophila melanogaster. The flightless gene is molecularly characterized by four transcription units within it, which are named tweety (twe), fli, dodo (dod), and penguin (pen). These genes are required for normal flight. We therefore investigated the localization and expressed currents of hTTYH3 in the present study.

MATERIALS AND METHODS

Isolation and Detection of hTTYH3—Human TTYH3 cDNA (AL350584) was purchased from Incyte Genomics. Mutants were made by using PCR (QuickChange, Stratagene). Northern blot analysis was performed with a membrane (human multiple tissues Northern blot, Clontech, Tokyo, Japan) according to the manufacturer’s protocol using a PstI fragment as a probe. Mouse RNA was isolated by using the guanidine thiocyanate method with organic extraction (Trizol, Invitrogen). A mouse TTYH3 (mTTYH3) fragment was cloned by PCR followed by guest on November 4, 2016 http://www.jbc.org/ Downloaded from
by reverse transcription (RT) using a primer set (5'-AAAGCTGTTGCGGCTACCAAA-3' and 5'-CTATGCTGCAAGGACATGCAGAG-3') that detects an 84-amino acid fragment from S positions 310–394. RT-PCR detections of human, hamster (Cricetulus griseus), and mouse TTYH3 (hmcTTYH3) exons in the cell lines and tissues were performed by conventional methods. Human endothelial cells (number 375) and smooth muscle cells (number 716) were purchased from Applied Cell Biology Research Institute. Neuronal cells (PC12 and N2A) were gifts from K. Shimazaki (Department of Neurophysiology, Jichi Medical School, Japan).

The mTTYH3 protein was detected in mouse tissues. Specific antibodies were raised against the C-terminal region of MRKLYLATSQ, which was common in human, hamster, and mouse clones. An antigen (1:100) was used to conjugate with keyhole limpet hemocyanin (KLH), and the antigen was intramuscularly injected into New Zealand White rabbits, and this was followed by biweekly booster injections of the same dose of the antigen in Freund’s incomplete adjuvant. Serum in which the titer was over 10,000 times higher than that of the control was obtained 8 weeks later.

The immunized rabbit serum was passed through a column (Amersham Biosciences) and affinity-purified by a kit (Pierce) according to the manufacturer’s protocol. Extracts of subconfluent HEK293 or CHO cells (in 60-mm dishes) with or without transfection of hTTYH3 cDNA were lysed by gentle sonication in 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20 (pH 8.0), and protease inhibitors. Digestion was performed with the proteins at a concentration of 20 mg/ml and incubated with N-glycanase (0.5 units/20 μl) for 18 h at 37°C. Western blot analysis with a blocking test by an excess of antigen (1 μg/ml) was performed to evaluate specificity. Immune complexes were detected by horseradish peroxidase-labeled anti-rabbit IgG by using an ECL detection system (PerkinElmer Life Sciences). Histologic staining was performed by conventional methods and detected by fluorescent isothiocyanate (FITC)-labeled anti-rabbit IgG (Dako, Kyoto, Japan). The tissues were viewed through a microscope (BX-5, Olympus, Tokyo, Japan), and photographs were stored in a computer.

**Expression of cDNA**—A plasmid expressing green fluorescence protein (pEGFP-N1, Clontech, Tokyo, Japan) or red fluorescence protein (pDsRed1-N1, Clontech) was used as a marker for transfection. 0.5 μg of pEGFP-N1 and 1 μg of hTTYH3 or mTTYH3 cDNA or its mutants were transfected into mammalian cells grown on coverslips using (pDsRed1-N1, Clontech) was used as a marker for transfection. 0.5 μg of pDsRed1-N1 and 1 μg of hTTYH3 or mTTYH3 cDNA or its mutants were transfected into mammalian cells grown on coverslips using FuGENE6™ (Roche Applied Science). Growth and transfection were performed in 10% fetal calf serum-containing media. The cells were used without any further treatment for patch clamp samples. The experiments were performed at 45–72 h after the transfection.

**Electrolytes**—Patch clamp recordings were carried out according to conventional methods (14). The experiments were performed at room temperature (20–25°C). Microelectrodes were made from borosilicate capillary tubes (Drummond Scientific, Broomall, PA) that were coated by beeswax before fire-polishing. They had a resistance of 10 megohms. Series resistance in a whole cell configuration was around 300 megohms. The GFP-positive cells were visualized by fluorescence measurement (CAM 2000 System, Jaco, Tokyo, Japan) with an emission of 490 nm. Currents were recorded with an EPC-9 patch clamp amplifier (HEKA, Pfalz, West Germany). Applied voltage and sampling (10 kHz) were controlled by a computer system (HEKA, Pfalz, West German). Samples were filtered through 1 kHz, and analysis was performed using the software Patch Analyst Pro. The bath solution contained 140 mM NaCl (or TEA-Cl or sodium gluconate), 1.0 mM MgCl₂, and 3 mM HEPES (pH 7.4). The whole cell patch pipette contained a filtered solution of 140 mM CsCl, 1.0 mM MgCl₂, and 3 mM HEPES (pH 7.2) with or without 0.5 mM CaCl₂. To calculate the ratio of X⁻ to Cl⁻ permeability in a whole cell configuration, the current-clamp mode was used to measure reversal potential. Then the reversal potential was compensated by liquid junction potential, and the ratio was calculated by using an equation for single channel conductance analysis, the pipette solution was changed to 140 mM NaX, 1.0 mM MgCl₂, and 3 mM HEPES (pH 7.4). Single channel recording was performed in inside-out patches, and then reversal potential was calculated to obtain the selective permeability. To obtain the desired Ca²⁺ concentration, Ca/EGTA concentration in the solution for the cytoplasmic side was adjusted with appropriate amounts of KOH, MgCl₂, and HEPES, keeping a constant ionic strength (Σi) of 15. A hypotonic solution was made by addition of distilled water to the bath solution. Osmolality was measured using an osmometer (One-Ten Osmometer, Fiske, MA).

The data were analyzed using one-way analysis of variance, and the significance was calculated using Bonferroni’s analysis. p ≤ 0.05 was considered statistically significant.

**RESULTS**

**Isolation and Characterization of hTTYH3 Protein**—The sequence of the purchased cDNA (AL530584) had significant homology to ttyh1 and ttyh2 (NM209659 and NM032646) and was named ttyh3. The gene ttyh3 is located in 7p22.3. Other family members of ttyh3 were found by using the BLAST program; one was found in Drosophila (CG3638) and two were found in Homo sapiens (ttyh1 and ttyh2). hTTYH3 mRNA encodes 480 nucleotides and 523 amino acids. hTTYH3 is thought to have 5 TMSs (16), whereas hTTYH3 is thought to possess 6 TMSs. Both were predicted by the Susui program (GenomNet, Kyoto University) (Fig. 1A). The third TMS (TMS3) was predicted in hTTYH3 but not in hTTYH1. The cluster of leucine used for the search was found in TMS4. To determine the membrane topology, a marker (His6) was inserted between TMS3 and TMS4 (amino acids 199–209) and between TMS5 and TMS6, which were expressed in mammalian cells and then detected with an FITC-labeled antibody against His6 (data not shown). The mutants containing the marker in the given positions (amino acids 202, 205, 208, and 257, Fig. 1A, green triangles) were positive, and the other mutants (red triangles) were negative for binding when the antibody was incubated outside the cells. However, addition of His6 may change the transmembrane topology.

Detection of an ttyh3 product and glycosylation scanning were performed with an antibody raised against C-terminal peptides. The amino acid sequence of the C-terminal region used for the antibody is a common sequence among hamsters, mice, and humans. Western blot analysis showed positive signal in expressed CHO cells (Fig. 1B), whereas immunoreactivity was not detected in control CHO cells. On the other hand, immunoreactivity was faintly detected by Western blotting and clearly detected by immunohistochemistry in HEK cells (Fig. 1C), suggesting that hTTYH3 is an endogenous Cl⁻ channel. Incubation of the same membrane with an excess of antigen (1 μg/ml) abolished the signals, indicating that the antibody is specific for hTTYH3 detection. Possible glycosylation sites (Asn-Xaa-Thr/Ser, red circle) were found at the positions 128, 144, and 353. Glycosylation scanning was performed by using N-glycanase digestion. The protein of hTTYH3 expressed in CHO was incubated with N-glycanase, resulting in digestion of the upper band, but the sequence-predicted 58-kDa band remained. Thus, the upper band in CHO was a glycosylated form of hTTYH3. When threonine was substituted by alanine at the three sites, Thr at position 353 was the only site glycosylated. Thr at 353 was therefore located in an extracellular domain. Therefore, the structure of hTTYH3 is like that illustrated in Fig. 1A (lower panel), regardless of whether TMS3 (white column) penetrates the membrane or not.

The putative structure of hTTYH3 may be comparable with that of the Ca²⁺-activated large conductance potassium channel (17, 18) (maxi-K⁺, Fig. 1A, lower). The pore region in maxi-K⁺ determines potassium ion selectivity located between TMS5 and TMS6. The position of the pore region in hTTYH3 is predicted to be similar to that of maxi-K⁺, where positively charged amino acids, arginine and histidine, are commonly conserved in the TTYH family as a glutamate- or Asp-rich domain. Knockout of the maxi-K⁺ channel gene resulted in a "flightless" in Drosophila; both genes are related to behavior abnormality.
Localization of hTTYH and mTTYH3—hTTYH3 and mTTYH3 are distributed mainly in excitable tissues. hTTYH3 mRNA of 4.8 kb was found by Northern blotting in the brain, heart, skeletal muscle, colon, spleen, kidney, and peripheral blood leukocytes (Fig. 2A). Possible transmembrane segments (TMS1–6, boxes), marker for membrane topology (triangle), pore region (P) with negatively charged amino acids (blue circle), possible glycosylation sites (red circles), and Glu/Asp-rich C-terminal region (yellow) are illustrated. The primers for RT-PCR are indicated by arrows. The lower schema shows the proposed hTTYH3 structure in Drosophila Flightless encoding a large conductance channel (maxi-Cl) in comparison to the structure of a large conductance Ca2+-activated K+ channel (maxi-K) found in Drosophila slowpoke. B, expression of exogenous hTTYH3 protein by Western blot. Upper panel, the protein (20 μg) in each lane was separated by conventional SDS-PAGE. The immune complexes with the antibody (1:1000) were detected by horseradish peroxidase-labeled anti-rabbit IgG (1:10,000) by using an ECL detection system. The upper arrow indicates the glycosylation form of hTTYH3, and the lower arrow indicates the predicted 58 kDa of hTTYH3. Left panel, lane CHO, control CHO; hTTY3, hTTYH3-transfected CHO; + glycanase, digestion with N-glycanase of hTTYH3-transfected CHO; number-T/A, mutant possessing Ala substituted with Thr at the position of the number was transfected into CHO. Right panel, lane HEK, control HEK; hTTY3, hTTYH3-transfected HEK. Lower panel, the panel on the left shows the same membrane as above. The membrane was washed in SDS containing boiled DW and re-probed with the same antibody with an excess amount of antigen. C, histologic staining was performed and detected by FITC-labeled anti-rabbit IgG. The image of the cells was viewed with fluorescence microscopy (left panels) or with Nomarski optics (right panels). CHO cells were transfected by hTTYH3 (upper panels). Endogenous hTTYH3 was detected in HEK cells (middle panels). The immunoreactivity remarkably decreased in HEK cells incubated with an excess amount of antigen (Ag) (lower panels).

Fig. 1. Amino acid alignment of the hTTYH family. A, amino acid alignment of the human TTYH family (hTTYH1–3, hTTYH1, NM020659; hTTYH2, NM020646; hTTYH3, AL530584) is shown. Possible transmembrane segments (TMS1–6, boxes), marker for membrane topology (triangle), pore region (P) with negatively charged amino acids (blue circle), possible glycosylation sites (red circles), and Glu/Asp-rich C-terminal region (yellow) are illustrated. The primers for RT-PCR are indicated by arrows. The lower schema shows the proposed hTTYH3 structure in Drosophila Flightless encoding a large conductance channel (maxi-Cl) in comparison to the structure of a large conductance Ca2+-activated K+ channel (maxi-K) found in Drosophila slowpoke. B, expression of exogenous hTTYH3 protein by Western blot. Upper panel, the protein (20 μg) in each lane was separated by conventional SDS-PAGE. The immune complexes with the antibody (1:1000) were detected by horseradish peroxidase-labeled anti-rabbit IgG (1:10,000) by using an ECL detection system. The upper arrow indicates the glycosylation form of hTTYH3, and the lower arrow indicates the predicted 58 kDa of hTTYH3. Left panel, lane CHO, control CHO; hTTY3, hTTYH3-transfected CHO; + glycanase, digestion with N-glycanase of hTTYH3-transfected CHO; number-T/A, mutant possessing Ala substituted with Thr at the position of the number was transfected into CHO. Right panel, lane HEK, control HEK; hTTY3, hTTYH3-transfected HEK. Lower panel, the panel on the left shows the same membrane as above. The membrane was washed in SDS containing boiled DW and re-probed with the same antibody with an excess amount of antigen. C, histologic staining was performed and detected by FITC-labeled anti-rabbit IgG. The image of the cells was viewed with fluorescence microscopy (left panels) or with Nomarski optics (right panels). CHO cells were transfected by hTTYH3 (upper panels). Endogenous hTTYH3 was detected in HEK cells (middle panels). The immunoreactivity remarkably decreased in HEK cells incubated with an excess amount of antigen (Ag) (lower panels).
brane was incubated with a 32P-labeled hTTYH3 fragment. The blot analysis of hTTYH3 in human tissues are shown. The membrane is composed of tissue cDNA in the upper panel, and β-actin cDNA was used for the control amplification of tissue cDNA in the lower panel. RT-PCR analysis of denoted antigen (Ag) was performed by conventional method. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for the control amplification of tissue cDNA. Positive signals are indicated by triangles.

Whole Cell Recordings of hTTYH3-transfected CHO Cells—We first selected HEK293 cells for the expression of hTTYH3, because HEK293 cells were used for discovery of the ClCA family (10). When transiently expressed in HEK293 cells, hTTYH3 was associated with a prominent Cl− current that was activated by ionomycin (10−6 M). The current was much less marked in GFP vector (mock)-transfected cells. However, a similar large conductance Cl− single channel (12) was detected in the following investigation. Therefore, we used CHO cells.

The basal current in hTTYH3-transfected CHO cells was different from that in untransfected control cells. Although ionomycin induced an outward-rectified current in mock-transfected cells, it induced an inward rectified current in hTTYH3-transfected cells (Fig. 4A). The current was not altered by substitution of sodium with TEA but was altered by substitution of Cl− with gluconate in the bath solution, suggesting that the current is a current driven by an anion. The permeability ratio of Cs+/Cl− was nominally zero and that of gluconate/Cl− was 0.33 (15).

To investigate the ion selectivity, we used whole cell patches with a pipette solution containing 0.5 mM CaCl2, because this large amount of Ca2+ was required to stabilize the current-voltage relation. The ionic selectivity was altered in hTTYH3 pore mutants by exchanges of charged amino acids (Fig. 4B). Mutant H370D showed a different selectivity, in which gluconate/Cl− decreased from 0.3 to 0.12 and Cs+/Cl− increased to 0.1. Mutant R366Q was more permeable to cations. The reversal potential with isosmotic NaCl/CsCl shifted to 12.5 mV (mean of eight), suggesting significant permeability to Na+. Assuming that TEA and gluconate are impermeable ions, the Na+/Cs+/Cl− permeability ratio was calculated to be 0.7:0.25:1. Thus, positively charged amino acids at positions 366 and 370 composed the pore of hTTYH3, playing a role in anion selectivity.

Fig. 2. Localization of hTTYH3 and mTTYH3. A, results of Northern blot analysis of hTTYH3 in human tissues are shown. The membrane was incubated with a 32P-labeled hTTYH3 fragment. The arrow indicates hTTYH mRNA of 4.8 kb. B, RT-PCR analysis of denoted mouse tissues and cell line. The primer sets were designed commonly to mouse tissues and cell line. The primer sets were designed commonly to

Fig. 3. Immunohistochemical staining of representative tissues. Immunoreactivity was detected in tissues. The primary C-terminal antibody was diluted 1:100 and detected with FITC-labeled anti-rabbit antibody (1:1000). Thalamic portions of the brain, heart, renal cortex, and skeletal muscle are shown. Negative controls with an excess of antigen (Ag) are shown on the right. They were viewed through a microscope (×200), and the photos were converted into white and black images (Photoshop version 7, Macintosh). Positive signals are indicated by triangles.
Nif, niflumic acid; Cont, control hTTYH3-transfected cells; blockers were added to the cytosolic side. Recorded in an inside-out configuration. Single channel traces at **, p < 0.01. Right, effects of blocking reagents on the hTTYH3 current are shown as the magnitude of the current at +100 mV (n = 8). ***, p < 0.001. A, hTTYH3 in CHO cells and effects of blocking reagents. The open probability was plotted against blocking reagents. A, left, effects of blocking reagents on the hTTYH3 single channel. Single channel traces at −30 mV were recorded in an inside-out configuration. Blockers were added to the cytosolic side. Cont., control hTTYH3-transfected cells; Nif, niflumic acid; Zn, ZnCl₂; solid and dashed lines, opened and closed states, respectively.

FIG. 4. Whole cellular current of hTTYH3 in CHO cells and effects of blocking reagents. A, currents were recorded from −100 to +90 mV at steps of 10 mV in control CHO cells (left) and cells treated with ionomycin (center). The hTTYH3-transfected cells were treated with ionomycin (right). B, the current-voltage relation was obtained by ramp pulses from −120 to 120 mV. Left, representative current-voltage relation of control cells and wild hTTYH3-transfected cells treated with ionomycin (NaCl). Then the bath solution was changed to TEACl (TEA) or sodium gluconate (Gluconate). The current-voltage relation was obtained from H370D (center) or from R366Q (right) mutant-transfected cells. C, left, effects of blocking reagents on the hTTYH3 current were shown at −90 mV and at −10 mV in control CHO cells (TEA or Gluconate). The current-voltage relation was obtained from H370D (center) or from R366Q (right) mutant-transfected cells. The current-voltage relation was obtained by ramp pulses from −120 to 120 mV. Right, effects of blocking reagents on the hTTYH3 current were shown at −90 mV and at −10 mV in control CHO cells (TEA or Gluconate). The current-voltage relation was obtained from H370D (center) or from R366Q (right) mutant-transfected cells. The current-voltage relation was obtained by ramp pulses from −120 to 120 mV. Left, representative current-voltage relation of control cells and wild hTTYH3-transfected cells treated with ionomycin (NaCl). Then the bath solution was changed to TEACl (TEA) or sodium gluconate (Gluconate). The current-voltage relation was obtained from H370D (center) or from R366Q (right) mutant-transfected cells. C, left, effects of blocking reagents on the hTTYH3 current were shown at −90 mV and at −10 mV in control CHO cells (TEA or Gluconate). The current-voltage relation was obtained from H370D (center) or from R366Q (right) mutant-transfected cells. The current-voltage relation was obtained by ramp pulses from −120 to 120 mV. Right, effects of blocking reagents on the hTTYH3 current were shown at −90 mV and at −10 mV in control CHO cells (TEA or Gluconate). The current-voltage relation was obtained from H370D (center) or from R366Q (right) mutant-transfected cells. The current-voltage relation was obtained by ramp pulses from −120 to 120 mV.

Single Channel Analysis of hTTYH3—hTTYH3 was characterized by single channel analysis. Maxi-Cl⁻ channel activity was observed within 30 s after detachment of the membrane when bath solution contained 0.1 mM of Ca²⁺ (Fig. 5A). Such a large conductance was observed in hTTYH3-transfected cells but not in non-transfected cells. The current-voltage relation was linear in symmetrical Cl⁻ solution (n = 12) with slope conductance of 260 pS (Fig. 5B). Open probability was not dependent on voltage of less than 0 mV but decreased at voltage over 60 mV (Fig. 5C). The distribution of current amplitudes fitted well to Gaussian’s analysis, indicating a subconductance of 50 pS (Fig. 5D). The channel had a long open lifetime as was observed in the trace at +40 mV, whereas it also had a fluctuating rapid opening as was observed in the trace at −60 mV. Long opening and rapid (but transient) fluctuations were observed in the same patch membrane. The hTTYH3 channel therefore involved a multiopeing mechanism. Open-closed lifetimes fit better to two kinetic states (τo1 = 52, τo2 = 510 ms, τc1 = 60 and τc2 = 250 ms, mean of 12) rather than 1.

As in the case of a maxi-K⁺ channel (17) and CIC channel (10), a high concentration of cytosolic Ca²⁺ was essential for hTTYH3 activation. The open probability was plotted against Ca²⁺ concentration, resulting in an ED₅₀ of about 2 μM (Fig. 5E). The range of Ca²⁺ concentrations is similar to that for maxi-K⁺ activation (18). The value 2 μM is within the physiologic range of Ca²⁺ in excitable cells in which hTTYH3 is detected. Thus, like the maxi-K⁺ channel, hTTYH3 may play a role in Ca²⁺ signal transduction.

However, it took around 10 s for activation when the cytosolic side of the isolated membrane was exposed to high Ca²⁺ solution. Thus, protein phosphorylation or another complex mechanism might be involved in the activation. To search for activators, activity of hTTYH3 was observed in a cell-attached configuration with stably transformed CHO cells, and the presence of hTTYH3 channels in the attached membrane was determined later in an inside-out configuration in high Ca²⁺ solution. Although protein kinase sites on the amino acid sequences were predicted, direct opening of the hTTYH3 channel by protein kinase A (dibutyryl cAMP, 10⁻⁵ M) or kinase C (phorbol ester, 10⁻⁸ M) was not observed. Addition of staurosporine (10⁻⁶ M), GTPγS (10⁻⁵ M), or okadaic acid (10⁻⁵ M) to the bath solution did not activate the hTTYH3 channel (data not shown). Therefore, the physiological role of hTTYH3 remains obscure and awaits in vivo study.

Expression and Activation of hTTYH1 and hTTYH2 Channels—To elucidate the physiological role of the TTYH family, we examined the functional expression of other members, hTTYH1 and hTTYH2. Based on the above results, we tested whether hTTYH1 and hTTYH2 were also expressed as evoked
channels (Fig. 6). Activity of the hTTYH1 single channel was sometimes observed in cell-attached patches, showing slope conductance of 280 pS. However, the hTTYH2-induced channel appeared only after treatment with ionomycin. hTTYH2 was expressed as a Ca$^{2+}$-dependent inward-rectified Cl$^{-}$ channel of 120 pS in an inward direction and of 45 pS in an outward direction. In a cell-attached configuration, reversal potential of hTTYH1 and hTTYH2 was around −30 mV, which is similar to the Cl$^{-}$ equilibrium potential. By substitution of NaCl with TEA-Cl in a pipette solution, the permeability ratio, $P_{Na}/P_{Cl}$, was calculated to be less than 0.1 for hTTYH1 and 0.1 for hTTYH2.

We next tested the influence of a hypotonic solution on hTTYH1 and hTTYH3 channels. In a whole cell configuration with CsCl in the pipette solution without Ca$^{2+}$ ($pCa = 7.2$), the current by hTTYH1 was usually silent but remarkably activated when the bath osmolarity was changed to a hypotonic solution of 250 mOsm (Fig. 7A). The current was further activated in a hypotonic solution of 220 mOsm. A whole cellular current-voltage relation of hTTYH1 in a hypotonic solution was not obtained because the magnitude of the current was unsteadily high. Because the current at 0 mV was stable even in a hypotonic solution, this prominent current was not due to leakage but due to fluctuated opening and closing of the large channel. The current magnitude at 100 mV was measured ($n = 6$, Fig. 7B). The evoked current at 220 mOsm was diminished by 50 μM GdCl$_3$ and abolished by 10 μM DIDS. On the other hand, the current of hTTYH3-expressed cells was significantly activated only when exposed to a solution of 220 mOsm. The evoked current was blocked by 50 μM GdCl$_3$. This magnitude of activation in a hypotonic solution was, however, observed in GFP-transfected control CHO cells (19).Thus, we conclude that hTTYH1 is involved in a swelling-activated Cl$^{-}$ channel, whereas hTTYH3 may not be involved. Thus, the common feature of these three channels is large conductance.

**DISCUSSION**

In this study, we showed that the hTTYH family possesses 5 or 6 transmembrane segments encoding a large conductance Cl$^{-}$ channel. The structure of hTTYH3 was estimated by computer-based hydrophobic analysis, addition of tag alignment, and glycosylation scanning. The results of a mutation study suggested that the structure of this novel maxi-Cl$^{-}$ channel is similar to the structure of known cation channels, in which a selective pore is located between TMS5 and TMS6 and a regulatory domain is located in the C terminus. An ion channel with the pore domain between TMS5 and TMS6 is a prototype structure of cation-permeable channels, such as the K$^{+}$ channel encoded by the Kv family and the Ca$^{2+}$ channel encoded by the Trp family (20). These molecules have been discovered from abnormal behavior of the fruit fly, *Drosophila*. The hTTYH family may also become a prototype of the Cl$^{-}$ channel, because hTTYH1–3 showed comparable structure and function.

Discrimination between endogenous and exogenous CaC currents was usually difficult. Fortunately, hTTYH3 encoded a characteristic large conductance channel, which was not observed in CHO cells. The magnitude of the endogenous current evoked by ionomycin does not exceed 1 nA at 100 mV in CHO cells (21, 22), compared with that shown around 4 nA in hTTYS-transfected CHO cells. hTTYH1 and hTTYH2 induced...
currents that were different in conductance and in dependence of Ca\(^{2+}\). hTTYH1 encoded a Ca\(^{2+}\)-independent large current around 5 nA that was induced by a hypotonic solution. This swelling-activated current was neither observed endogenously (19) nor in hTTYH3-transfected CHO cells. Furthermore, the results of the study using mutants strongly suggested that the large conductance was induced by exogenous, not endogenous, hTTYH3 cDNA because exogenous mutants altered the whole cell current. Therefore, hTTYH-induced currents observed in this study were endowed exogenously by cDNA.

The channel encoded by hTTYH3 showed characteristics similar to those of maxi-Cl\(^-\) channels in situ. First, most Cl\(^-\) channels are blocked by niflumic acid at a concentration of 300 \(\mu\)g/ml (3). Activity of maxi-Cl\(^-\) is, however, blocked by DIDS or SITS (4, 5, 23). Second, voltage-dependent suppression at high depolarization is observed in hTTYH3 and in some CaC channels (1, 24, 25). However, the voltage-dependent suppression of the maxi-Cl\(^-\) channel is characterized as a “bell-shaped” relation; suppression to the opening is observed in highly depolarized and in deep repolarized potentials (26). The current-voltage relation of the hTTYH3 channel did not show bell-shaped suppression because suppression at deep negative potential was not observed. Furthermore, it is still not clear why open probability of the channel was decreased despite the fact that the whole cellular current was not decreased at a highly depolarized potential. The depolarization might increase the number of open channels but decrease the probability of opening. A subunit might be needed to solve the discrepancy in voltage dependence. Third, maxi-Cl\(^-\) channels have complex mechanisms of conductance (1, 27) and lifetime kinetics (25, 28). Fourth, the hTTYH3 channel displayed a permeability sequence of I\(^-\) > Br\(^-\) > Cl\(^-\), which was observed in skeletal muscle (29) and corresponds to Eisenman’s sequence. The ionic environment within the channel is, however, influenced by the cationic environment, which alters the selective order of Cl\(^-\) channels (26), suggesting that this order of permeability may not be specific for the hTTYH3 structure.

hTTYH3 may encode a Ca\(^{2+}\)-activated maxi-Cl\(^-\) channel in the excitable membrane. A maxi-Cl\(^-\) channel in worm, amphibian, or rat lactotrophs was found to be activated directly by cytosolic Ca\(^{2+}\) (2, 25, 28). However, there is argument against a direct ligand-type Ca\(^{2+}\) dependence in the activation of maxi-Cl\(^-\) channels. Some maxi-Cl\(^-\) channels are activated by polymyxin B, an inhibitor of protein kinase C (6), G-protein (24), or okadaic acid (30). Although we used various reagents to investigate the regulation, none of them activated the hTTYH3 channel. Further studies are required to clarify the regulation.

hTTYH3 cDNA as well as protein was found in HEK293 cells. However, Ca\(^{2+}\)-dependent maxi-Cl\(^-\) channels in HEK cells were not discovered previously. The Ca\(^{2+}\)-independent maxi-Cl\(^-\) channel of HEK cells (12) exhibits an outward-rectified property. It is similar in conductance but not similar in characteristics to the hTTYH3 channel. Results of Western blot analysis suggested that the amount of endogenous hTTYH3 protein was small, and this might have been the reason why an
hTTYH3-like channel was not discovered in previous electrophysiologic studies. An hTTYH3-like channel may not be expressed in the plasma membrane of HEK cells in situ.

On the other hand, the hTTYH1-like Ca\(^{2+}\)-independent large conductance Cl\(^-\)/HCO\(_3\)-sensitive maxi-Cl\(^-\) channel has been frequently observed in mammalian cells such as HEK293 cells (12), smooth muscle (23, 31), T-lymphocytes (32), endothelial cells (3), and cardiac myocytes (27). The maxi-Cl\(^-\) channels in the latter two are activated in hypotonic media. The physiological role of a swelling-activated anion channel has been described in detail (8). A hypothesis solution induces gain of cell volume, which is restored by K\(^+\) and Cl\(^-\) exit from the cell interior in concert. Volume-sensitive (swelling-activated) Cl\(^-\) channels play a cardinal role in this phenomenon. A direct contribution of volume-sensitive Cl\(^-\) channels to the physiological situation has also been described. Recently, Bell et al. (33) suggested that a volume-sensitive maxi-Cl\(^-\) channel in macula densa cells plays an important role in tubulo-glomerular feedback. This finding provides a new insight into the physiological role of the volume-sensitive maxi-Cl\(^-\) channel as a regulator of body fluid homeostasis.

The C-terminal region of hTTYH1 is shorter than those of the other two. Based on these comparisons, hTTYH3 is structurally and functionally more similar to hTTYH2 than to hTTYH1. Thus, more members similar to hTTYH1 may be discovered in the future. Investigation of this family will reveal the molecular mechanism and the physiologic role of the large conductance Cl\(^-\) channel family irrespective of calcium dependence.

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Note Added in Proof—Sequences of isolated clones used in the expression study have been deposited in GenBank\textcopyright (hTTYH3, AB162931; hTTYH2, BC005168; hTTYH1s, AB162929; and mTTY1s, AB162930). The hTTYH1s, encoding the volume-sensitive Cl\(^-\) channel, is a C-terminal spliced variant of ttyh1 (NM020659). Exon 11 is skipped in hTTYH1s. Expression of hTTYH1 did not show the volume-sensitive Cl\(^-\) current.

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Makoto Suzuki and Atsuko Mizuno

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