Molecular identification of HSPA8 as an accessory protein of a hyperpolarization-activated chloride channel from rat pulmonary vein cardiomyocytes

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**Running title:** HSPA8 is an accessory protein of CLCN2 in rat heart

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**Abstract**

Pulmonary veins (PVs) are the major origin of atrial fibrillation. Recently, we recorded hyperpolarization-activated Cl\(^-\) current (\(I_{\text{Cl,h}}\)) in rat PV cardiomyocytes. Unlike the well-known chloride channel protein 2 (CLCN2) current, the activation curve of \(I_{\text{Cl,h}}\) was hyperpolarized as the Cl\(^-\) ion concentration ([Cl\(^-\)]) increased. This current could account for spontaneous activity in PV cardiomyocytes linked to atrial fibrillation. In the current study, we aimed to identify the channel underlying \(I_{\text{Cl,h}}\). Using RT-PCR amplification specific for Clcn2 or its homologues, a chloride channel was cloned from rat PV and detected in rat PV cardiomyocytes using immunocytochemistry. The gene sequence and electrophysiological functions of the protein were identical to those previously reported for Clcn2, with protein activity observed as a hyperpolarization-activated current by the patch-clamp method. However, the [Cl\(^-\)] dependency of activation was entirely different from the observed \(I_{\text{Cl,h}}\) of PV cardiomyocytes: the activation curve of the Clcn2-transfected cells shifted toward positive potential with increased [Cl\(^-\)], whereas \(I_{\text{Cl,h}}\) of PV and left ventricular cardiomyocytes showed a leftward shift. Therefore, we used mass
spectrometry to explore the possibility of additional proteins interacting with CLCN2 and identified an individual 71 kDa protein, HSPA8, which was strongly expressed in rat PV cardiomyocytes. With co-expression of HSPA8 in HEK293 and PC12 cells, the CLCN2 current showed voltage-dependent activation and shifted to negative potential with increasing [Cl\textsuperscript{−}]. Molecular docking simulations further support an interaction between CLCN2 and HSPA8. These findings suggest that CLCN2 in rat heart contains HSPA8 as a unique accessory protein.

Ectopic excitability originating in pulmonary veins (PVs) causes symptomatic atrial fibrillation (AF) (1, 2). A number of researchers have recorded spontaneous excitability in isolated PV tissues. Several potential causes of spontaneous activity have been reported in these cases, including independent pace-making activity in muscular tunics of guinea-pig PVs (3), α- and β-adrenergic stimulation by noradrenaline (3–5), Ca\textsuperscript{2+} overload in intracellular storage sites induced by digitalis (6), and intracellular Ca\textsuperscript{2+} leakage (7). The progression of atrial fibrosis is also considered to be an essential factor in structural remodeling in AF (8–12). Genome-wide analysis indicated that chromosomal loci associated with Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels and hyperpolarization-activated chloride channel protein 2 (CLCN2) channel (18), have been identified in mammalian cardiomyocytes. CLCN2 is activated by both hyperpolarization and hypoosmotic stress. Interestingly, Duan et al. detected a minor splice variant of CLCN2 that is unique to the rat heart (19). However, like the full-length protein, this variant was activated by both hyperpolarization and hypoosmolality. In contrast, \textit{I\textsubscript{Cl,h}} was accepted as a novel type of hyperpolarization-activated Cl\textsuperscript{−} current, because its response to intracellular Cl\textsuperscript{−} and extracellular osmolarity differed from those of CLCN2 (15).

In the current study, we attempted to identify the Cl\textsuperscript{−} channel responsible for \textit{I\textsubscript{Cl,h}} using a molecular cloning and proteomic analysis-based approach. Our results revealed that a member of the heat shock protein (HSP) 70 superfamily, HSPA8, acts as an auxiliary protein of the CLCN2 Cl\textsuperscript{−} channel. Thus, we propose that CLCN2, in association with HSPA8, is a leading candidate for the molecular architecture underlying \textit{I\textsubscript{Cl,h}}. 
Results

Functional cloning of full-length Clcn2 from rat PV cardiomyocytes

To identify a Cl− channel that could be attributed to the previously reported $I_{\text{Cl,h}}$ in rat PV cardiomyocytes (15), we attempted to isolate complementary DNA (cDNA) corresponding to the Cl− channel by RT-PCR. Total RNA extracted from rat PV cardiomyocytes was reverse-transcribed and amplified using a set of specific primers (Full-rCLCN2-F/Full-rCLCN2-R, Table 1). Based on the hypothesis that the Cl− channel-encoding gene of interest should be homologous to Clcn2, the primers were designed to span the region encompassing the start and end codons of Clcn2 (i.e. the complete coding sequence). As shown in Figure 1A, the reverse-transcript from the small intestine indicated that the intrinsic full-length Clcn2 gene was 2811 bp in length. Transcripts from PV cardiomyocytes were apparently identical in size to this Clcn2 sequence. Gel bands corresponding to the Clcn2 cDNA amplicons transcribed from PVs were digested, purified, and inserted into the pcDNA3.1 expression vector using appropriate restriction enzymes. Sanger sequencing confirmed that the inserted cDNA sequences were identical to the published Clcn2 sequence (Figure S1). The smaller transcripts below the Clcn2 bands (Figure 1A) are products of non-specific amplification.

The electrophysiological properties of the cloned Clcn2 gene were analyzed in HEK293 cells using the patch-clamp method. Without CLCN2 transfection, time-dependent current was negligible (Figure S2A). To evaluate the effects of different Cl− ion concentrations ([Cl−]) on the voltage-gating, steady-state activation under various [Cl−] (40, 100, or 150 mM) was analyzed by measuring the amplitude of the tail current at 40 mV. The relationship between the test potentials and the relative amplitude of the tail current was fitted with the Boltzmann equation and plotted (Figure 1C). As [Cl−] increased, the steady-state activation curves shifted toward positive potential and maintained similar slopes. The $V_{1/2}$ and the slope factors were $-72.3 \pm 6.6$ mV and $-29.6 \pm 3.3$, respectively, at a [Cl−] of 40 mM (n = 5), $-58.3 \pm 15.4$ mV and $-30.6 \pm 9.5$, respectively, at a [Cl−] of 100 mM (n = 7), and $-52.1 \pm 10.4$ mV and $-28.3 \pm 7.2$, respectively, at a [Cl−] of 150 mM (n = 7) (Figure 1D, E). There was statistical difference in $V_{1/2}$ (Kruskal-Wallis test, $p = 0.0025$), but no difference in slope factors. These results are consistent with previous reports (20). The time-course of activation is summarized in Figure 1F with fast and slow time constants ($\tau_{\text{fast}}$ and $\tau_{\text{slow}}$). In some cases, the time constants had to be interfered by the apparent inactivation, most likely as a result of Cl− depletion.

Cell localization of CLCN2 in rat PV and LV cardiomyocytes

CLCN2 is reported to be expressed in the rat left ventricle (LV) (21). We confirmed the cellular localization of CLCN2 in LV and PV cardiomyocytes using immunocytochemistry. Figure 2 shows representative confocal images of cardiomyocytes isolated from these tissues and labeled with a set of
CLCN2 (green) and Na-K pump (red), or RyR (green) and CLCN2 (red) antibodies. Immunostaining of Na-K pump indicates a plasma membrane pattern while RyR represents typical cell structure of cardiomyocyte. Overlay image of CLCN2 with Na/K pump reveals that the Cl− channel expresses on the cell surface in addition to intracellular spaces. CLCN2 also expressed in the periphery of LV myocyte.

Hyperpolarization-activated Cl− current in rat LV cardiomyocytes

Next, we examined whether the Cl− current in LV myocytes also exhibited I_{Cl,h}-like properties. The effects of different [Cl−]i (40, 100, or 150 mM; n = 8, 7, 6 for each, respectively) on voltage-gating was evaluated using the patch-clamp method. As [Cl−]i increased, the steady-state activation curves shifted toward negative potential with more gradual slopes. The V_{1/2} and the slope factors were −75.0 ± 7.4 mV and −15.7 ± 5.5, respectively, at a [Cl−]i of 40 mM, −89.4 ± 10.2 mV and −22.2 ± 5.5, respectively, at a [Cl−]i of 100 mM, and −104.3 ± 10.8 mV and −26.2 ± 7.1, respectively, at a [Cl−]i of 150 mM (Figure 3B–D). The values of both V_{1/2} and slope factor were decreased as [Cl−]i increased. The time-course of activation at −120 mV was analyzed by fitting the data to a first order biexponential function. Both τ_{slow} and τ_{fast} were plotted (Figure 3E). τ_{slow} values were 1346.6 ± 1150.0 ms, 915.4 ± 869.5 ms, and 636.5 ± 489.8 ms at [Cl−]i of 40 mM, 100 mM, and 150 mM, respectively (Figure 3E, filled circles), and significantly decreased by increasing [Cl−]i (p = 0.004).

Molecular identification of HSPA8 as an accessory protein of CLCN2

The electrophysiological function of endogenous CLCN2 in rat LV and PV cardiomyocytes was observed as I_{Cl,h}. The observed [Cl−]i-dependent gating of I_{Cl,h} was largely different from the properties of the well-known CLCN2 current (ClC-2). When Clcn2 is introduced alone in HEK293 cells, the steady-state activation curve of ClC-2 shifts toward negative potential with increasing [Cl−]i, (22, 23). We hypothesized that CLCN2 is regulated by an unidentified molecule, which would modulate the [Cl−]i-dependency of the channel. We therefore explored CLCN2-binding proteins by immunoprecipitation. Solubilized membrane proteins from rat PV and LV cardiomyocytes were precipitated with an anti-CLCN2 antibody and separated by electrophoresis. Precipitants were detected at ~90, ~70, ~50, and ~25 kDa on Coomassie-stained gels (Figure 4A). CLCN2 was identified as the band running at ~90 kDa. A band of weak intensity at ~180 kDa was presumed to be residual CLCN2 dimer. Bands at ~50 and ~25 kDa are consistent with the heavy and light chains of immunoglobulin used in the immunoprecipitation. However, the residual ~70 kDa bands (marked with arrows in Figure 4A) are unknown proteins, indicating an accessory protein of CLCN2. These bands were excised, digested with trypsin, and eluted with 0.5% trifluoroacetic acid. The eluted peptides
were then analyzed using mass spectrometry. Of the eluted peptides, 62 were identified as being of rat origin (Figure S3). Only eight of the peptides had been recognized as a part of functional protein and all corresponded to constitutive heat shock protein HSPA8 (71 kDa) of rats (Figure 4B and Figure S3). Immunoblotting of the precipitate indicated that HSPA8 corresponded to the CLCN2-interacting protein (Figure 4C, left), and the interaction between HSPA8 and CLCN2 was confirmed by reverse co-immunoprecipitation using an anti-HSPA8 antibody (Figure 4C, right). The molecular interaction was confirmed by immunoblotting more than three times in both PV and LV samples. Figure 4C is the representative examples. Hspa8 was abundantly expressed in rat PV cardiomyocytes (Figure 5A), and was successfully subcloned from rat PV cardiomyocytes (Figure S3) using specific primers (Table 1). Immunocytochemistry discovered that HSPA8 was present on the cell surface of PV and LV cardiomyocytes (Figure 5B, C), implying co-localization with CLCN2.

Functional characterization of HSPA8 by patch-clamping in HEK293 and PC12 cells

Cloned Hspa8 was co-transfected into HEK293 cells with Clcn2, and the CIC-2 current was recorded using the conventional whole-cell patch-clamp method (Figure 6). At a [Cl\(^-\)] of 40 mM, the \(V_{1/2}\) was \(-71 \pm 20.0\) mV and the slope factor was \(-23.9 \pm 3.1\) (n = 5). The \(V_{1/2}\) was not statistically changed (p = 0.38) while the slope factor was affected by transfection of cloned Hspa8 (p = 0.03). On the other hand, with a [Cl\(^-\)] of 150 mM, the voltage-dependency of the CIC-2 current produced by co-transfected Hspa8 and Clcn2 appeared not to fit a single Boltzmann function, but was satisfactorily approximated by the sum of the two components. The \(V_{1/2}\) and slope factor values for one component were \(-120.4 \pm 12.3\) mV and \(-12.9 \pm 6.2\), respectively. They were significantly affected by co-transfection of HSPA8 (p = 0.004 for \(V_{1/2}\) and p = 0.009 for slope factor with Mann-Whitney test). The \(V_{1/2}\) and slope factor values for the other component were \(-25.1 \pm 25.4\) mV and \(21.2 \pm 8.6\), respectively, without statistical difference from those values in the absence of Hspa8 (p = 0.126 and 0.247 for \(V_{1/2}\) and slope factor). The relative magnitude of the two components are 0.38 ± 0.14 for the former (low-voltage) and 0.62 ± 0.14 for the latter (high-voltage) component, respectively (n = 5, Figure 6A – C). Reportedly, rat CLCN2 binds to another heat shock protein, human HSP90, in HEK293 cells (24). To diminish the effect of human HSP90 on voltage-dependent gating, we carried out an identical patch-clamp protocol using the rat-derived cell line PC12. The cells are known to possess voltage-gated K\(^+\) currents (25) while Cl\(^-\) current was undetectable as indicated in Figure S2B. When Clcn2 was transfected alone into PC12 cells, the \(V_{1/2}\) and slope factor values of the CIC-2 current were \(-55.1 \pm 9.4\) mV and \(-19.5 \pm 1.8\), respectively, at a [Cl\(^-\)] of 40 mM, and \(-56.3 \pm 24.7\) mV and \(-22.7 \pm 5.9\), respectively, at a [Cl\(^-\)] of 150 mM (n = 4 for each, Figure 7A – C). Statistical difference between [Cl\(^-\)] of 40 and 150 mM in both \(V_{1/2}\) and slope factor were unobserved. By contrast,
when Hspa8 was overexpressed with Clcn2 in PC12 cells, the activation curves deviated from a single Boltzmann function, and were fitted to the sum of two Boltzmann components, i.e. low and high voltage-activated components (Figure 7D–F). At a [Cl\(^{-}\)] of 40 mM (n = 6), the \(V_{1/2}\) and slope factor values of the low voltage-activated component were −110.9 ± 12.7 mV and −14.9 ± 7.9, respectively. The values for the high voltage-activated component were −40.3 ± 23.5 mV and −14.8 ± 3.0, respectively. When [Cl\(^{-}\)] was increased to 150 mM (n = 5), the activation of the low voltage-activated component shifted toward negative potential (p = 0.002). The \(V_{1/2}\) and slope factor values of the low voltage-activated component at a [Cl\(^{-}\)] of 150 mM were −182.0 ± 13.6 mV and −22.9 ± 6.1, respectively, while those of the high voltage-activated component were −17.1 ± 10.2 mV and −12.5 ± 4.5, respectively. Statistical analysis revealed that the \(V_{1/2}\) of low-voltage component after the co-transfection was substantially shifted toward negative potential by increasing [Cl\(^{-}\)] (p = 0.002) while the other component was unaffected (p = 0.09). The relative magnitudes of the low voltage-activated component are overviewed in Figure 7G. To evaluate the effect of HSPA8 on time-dependent kinetics, we measured the time constant of the activated currents, and tail currents subsequent to the activation by hyperpolarization to -160 mV. The influence of the presence or absence of HSPA8 were compared in both condition of [Cl\(^{-}\)] = 40 and 150 mM. As a result, there are no significant impact of HSPA8 on the time constants (Figure 7H, I). Electrophysiological properties other than the voltage-dependencies and time constants were also evaluated (Figure 8). Consistent effects of Hspa8 expression were not uncoverd in those parameters.

**Homology modeling of protein structures and protein-protein docking simulations**

To compare the protein-protein docking simulation between CLCN2 and either HSP90 or HSPA8, protein structures of rat CLCN2, rat HSPA8 and human HSP90 were homologically simulated using the homology-modeling server SWISS-MODEL. The Cl\(^{-}\) channel structure from human CLC-1 (26) was used as a template for the model of rat CLCN2. The QMEAN score and sequence identity of the model were −1.43 and 53.71%, respectively. QMEAN is a type of Z-score: values around zero indicate good agreement between model and experimental structures of similar size, and scores of −4.0 or less indicate models of low quality (27). A sequence identity of greater than 40% is preferable when building an appropriate model. The X-ray crystallography structure of bovine HSPA8 was used as the a template for rat HSPA8 (28). The QMEAN score and sequence identity were −0.70 and 99.64%, respectively. Human HSP90 was modeled based on data from cryo-EM (29). The QMEAN score and sequence identity of the model were −1.74 and 86.58%, respectively. Protein-protein docking between each subunit of the homodimeric CLCN2 (green and cyan) and its interaction proteins (wheat and olive) were built in 1:1 stoichiometry using ClusPro 2.0. The weighted energy of the top-ranked cluster was −1690.2 C for the CLCN2/HSP90
interaction, and \(-1497.8\) C for the CLCN2/HSPA8 interaction, indicating enough energies to form protein-protein interactions. The highest priority models for CLCN2/HSPA8 and CLCN2/HSP90 are represented in Figures 9 and 10, respectively. The anion selectivity filter is indicated with red color in Figure 9A and 10A, the estimated molecular surfaces of both models are overviewed in Figure 9B and 10B, and the electrostatically tight interaction between CLCN2 and HSP90 or HSPA8 is shown in a blue-red color gradient in Figure 9C and 10C. According to the simulation model, HSP90 (Figure 9A-C) and HSPA8 (Figure 10A-C) bind to CLCN2 in different ways. HSP90 surrounds CLCN2 and binds to the lateral side of each CLCN2 subunit in 1:1 stoichiometry. The dimer interface remains unaffected (Figure 9C). In contrast, HSPA8 protrudes straight from the boundary part between the two CLCN2 subunits, and tightly binds to the interface of the homodimer, i.e. one HSPA8 binds to both CLCN2 subunits (Figure 10C).
Discussion

Major findings

This study demonstrates that CLCN2 is functionally expressed in cardiomyocytes and interacts with HSPA8 at the cell membrane. Because other possible proteins that attributes to the hyperpolarization-activated Cl− current is undetectable, the CLCN2-HSPA8 complex is a leading candidate for the molecular architecture of the previously reported $I_{\text{Cl,h}}$. The CIC-2 current induced by the co-transfection of Clcn2 and Hspa8 into HEK293 and PC12 cells showed unique [Cl−]-dependent properties. Voltage-dependent activation revealed two components with Boltzmann fitting, i.e. low and high voltage-activated component. Statistical analyses revealed that essential impact of Hspa8 is appear to be the low voltage-activated component, which shifts to hyperpolarized potentials by increasing [Cl−]. $V_{1/2}$ of the high voltage-activated component and other physiological parameters in the presence of Hspa8 are hardly different from those in the absence of it.

It is known that as [Cl−] increases, the steady-state activation curve of CLCN2 shifts toward positive potential (Figure 1C–E) (20), and that such [Cl−] dependency is caused by the association of HSP90 with CLCN2. Hinzpeter et al. reported that human HSP90 binds to rat CLCN2 in HEK293 cells (24), and that pharmacological inhibition of HSP90 reduced CIC-2 currents and impaired the [Cl−]-dependent rightward shift of the fractional conductance. Previously, we recorded a CIC-2-like current, $I_{\text{Cl,h}}$, and in the current study, we confirmed the expression of CLCN2 in the cells (Figures 1–5). Interestingly, the activation curve of $I_{\text{Cl,h}}$ shifted toward negative potential with increasing [Cl−] in PV cardiomyocytes. Here, another CLCN2 interacting partner, HSPA8, was identified (Figure 4). Homology modeling of the protein structure and protein-protein docking simulations demonstrated that both heat shock proteins, human HSP90 and rat HSPA8, interact with the same channel-pore subunit, CLCN2, but in different ways (Figures 9 and 10). Each HSP90 binds to one of the dimeric subunits of CLCN2, without affecting the interface of the homodimer (Figure 9), whereas HSPA8 binds to both CLCN2s at the boundary between the homodimer subunits. These structural differences may underlie the different [Cl−]-dependencies, i.e. the CIC-2 current produced by CLCN2/HSP8 shows a [Cl−]-dependent leftward shift in the activation curve, as was observed for $I_{\text{Cl,h}}$ in LV and PV cardiomyocytes (Figure 3) (15). However, in the present study, we could not perfectly reproduce the [Cl−]-dependent leftward shift of the activation curve when Hspa8 and Clcn2 were co-transfected into HEK293 or PC12 cells (Figure 6, 7). This may indicate that HSPA8 and endogenous CLCN2-interacting proteins, such as HSP90, hinder each other when associating with CLCN2, thereby counteracting the [Cl−]-dependent shift of the activation curve. Theoretically, two Boltzmann components suggest that HSPA8 and the other CLCN2-interacting molecules produce two CIC-2 currents with different kinetic properties, one of which is activated at negative potential while the other is activated at more positive
potential under high [Cl\(^-\)]\(_i\) conditions (Figure 7D – G). In this respect, the present study suggests that HEK293 cells use HSP90 as a CLCN2 binding partner, while PC12 cells also contains a subunit that is functionally distinct from HSPA8.

CLCN2 belongs to the CLC Cl\(^-\) channel/transporter family, which includes homodimeric channels with two independent Cl\(^-\) permeation pathways. The negatively-charged carboxyl side chain of a glutamate residue located within the permeation pathway forms a protopore (30). The CLC family possesses two different ionic gates: a fast-acting protopore gate and slow-acting common gate. The fast gate controls each protopore, and a common gate simultaneously controls both protopores. Detailed mechanisms of these gates are still being debated. It was recently suggested that the voltage-dependent activation of the protopore gates is not governed by the movement of voltage-sensitive domains (31); instead, the hyperpolarization pushes the intracellular Cl\(^-\) ions into binding sites within the pore, resulting in the opening of the protopore gate. This hypothesis favors the [Cl\(^-\)]\(_i\)-dependent rightward shift of the activation curve of the CIC-2 current observed in HEK293 cells. Indeed, the CLCN2/HSP90 association (observed in Figure 9) hardly obstructs the nature of the protopores. At the same time, it has been reported that neutralization of E217, the gating glutamate of guinea-pig CLCN2, results in CIC-2 channels without a protopore gate, and that the activation curve of these channels shows a rightward shift with decreasing [Cl\(^-\)]. (32). These findings indicate that [Cl\(^-\)]\(_i\) affects the protopore gate and the common gate in opposite ways, and that the overall [Cl\(^-\)]\(_i\)-dependency usually masks the effect on the protopore gate in the case of CIC-2 current. Accordingly, we speculate that HSPA8 affects the [Cl\(^-\)]\(_i\)-dependency by enhancing the [Cl\(^-\)]\(_i\)-dependent property of the common gate. Structural analysis (Figure 10) indicates that the CLCN2/HSPA8 complex interacts with the dimeric interface across both individual subunits. It is likely that the common gate is stabilized by the CLCN2/HSPA8 interaction, and its characteristics may be unmasked. This finding is consistent with our speculation, and we may safely conclude that HSPA8 is an auxiliary subunit that modulates the [Cl\(^-\)]\(_i\)-dependency of CLCN2.

To the best of our knowledge, this is the first report to identify HSPA8 as an accessory subunit of an ion channel. HSPA8 is a clathrin-uncoating ATPase (33) and is classified as a member the HSP70 family (34). Most HSPs are stress-inducible, whereas HSAP8 constitutively localizes to the cytoplasm and lysosome. As a chaperone protein, HSPA8 is substantially recruited in the cell-cycle G1/S transition (35, 36), and positively regulates chaperone-mediated autophagy (37–39). With these cellular functions, HSPA8 is thought to play a crucial role in cell differentiation and embryonic development (40–42). Additionally, the ability of HSPA8 to interact with ion channels on the cell membrane has been reported in the process of ubiquitin-mediated
proteasomal degradation. For example, HSPA8 cooperates with co-chaperons, such as carboxyl-terminus heat shock cognate 70-interacting protein and Bag1, in the ubiquitination of CFTR (43), Kv1.5 (44), and the human ether a go-go-related gene K+ channel (44). In the current study, expression of HSPA8 was nearly restricted to the cell surface, and a corresponding co-chaperone was not observed in the Coomassie-stained SDS-PAGE gels (Figure 4A). Thereby, HSPA8 is supposed to bind directly to CLCN2 on the membrane, and regulate channel properties rather than engage the maturation of the channel. Hinzpeter et al. (24) reported that the association of HSP90 with ClC-2 results in higher channel activity as a result of increased cell-surface channel expression, facilitation of channel opening, and enhanced channel sensitivity to intracellular [Cl−]. In the present study, Clcn2 was transiently transfected with or without co-transfection of Hspa8, and the level of gene expression was not stably controlled. The density of ClC-2 current in HEK293 and PC12 cells varied from cell to cell (Figure 8); thus, we could not determine if HSPA8 aids in the expression of CLCN2 channels. It should be noted that HEK293 and PC12 cells possess CLCN2-interacting proteins, and therefore, the positive effect of HSPA8 on channel expression may have been masked by these endogenous molecules.

Accessory proteins for CLCN2 appear to differ by cell type. In glial cells, CLCN2 interacts with GlialCAM (45), which was originally identified as a major partner of MLC1, the protein responsible for autosomal-recessive inheritance disease megalencephalic leukoencephalopathy with subcortical cysts (MLC) (46). GlialCAM directs the CLCN2 channel to the cell-cell junctions, enhances the current amplitude (47), and changes the activation properties by affecting the common gate (48). We propose that CLCN2 requires accessory proteins for proper expression and function at cell membranes, and that HSPA8 fills this role in the heart.

Limitations

It was considerably challenging to reproduce the same time-dependent kinetics in ClC-2 current in different biological materials, such as Xenopus oocytes, cultured cells and isolated cardiomyocytes. Reportedly, time constant in activation currents of ClC-2 is < 200 ms for HEK293 (49), ~2000 ms for cardiomyocytes (18, 19), and >> 5000 ms for Xenpus oocytes (50). In other words, the activation kinetics prolongs as cell-size increased. One of the reasons is consider to be the amount of cytoskeleton inside cells. Ahmed N, et al. have reported that disruptions of the cytoskeletons dramatically enhanced the activation process of the current (50). Further efforts will be required to determine molecular reasons for cell-type dependent differences in the time-dependency of the current.

Conclusions

This study identified an accessory protein of the ClC-2 channel in rat cardiomyocytes. This regulatory protein is likely to be responsible for the molecular
characteristics of \( I_{\text{Clh}} \) in voltage-dependent gating. The results may contribute to the development of drugs that pharmacologically suppress the hyperpolarization-activated current that precedes diastolic depolarization in arrhythmia.

**Materials and methods**

*Ethical Approval*

The protocols used in this study were approved by the Animal Ethics Committees of the Yamagata University Faculty of Medicine, Japan, and the Akita University School of Medicine, Japan. All experiments were performed at Yamagata University or Akita University under personal and project licenses following appropriate institutional reviewing.

*Preparation of rat hearts*

Male Wistar rats (8–14 weeks) were purchased from Charles River Laboratories, or CLEA Japan, Inc. To isolate cardiomyocytes from PV and LV, rats were anesthetized by intraperitoneal injection of mixed anesthesia (0.15 mg/kg of medetomidine, 2.0 mg/kg of midazolam, and 2.5 mg/kg of butorphanol). After checking suppression of the nociceptive reflex, the chest cavity was opened under artificial respiration and the aorta was cannulated *in situ* to perfuse the coronary arteries. The heart and lung were excised in block, mounted on a Langendorff apparatus, and then perfused sequentially with the following buffers: (1) normal Tyrode solution for approximately 3 minutes, to wash blood out from heart and lung, (2) nominally \( \text{Ca}^{2+} \)-free Tyrode solution for 5 min, and (3) \( \text{Ca}^{2+} \)-free Tyrode solution containing 0.05% (w/v) collagenase and 0.005% (w/v) elastase (Wako Pure Chemicals, Osaka, Japan) for 30 min. The composition of the normal Tyrode's solution (mM) was: \( \text{NaCl}, 136.9; \text{KCl}, 5.4; \text{CaCl}_2, 1.8; \text{MgCl}_2, 0.5; \text{NaH}_2\text{PO}_4, 0.33; \text{HEPES}, 5.0; \text{and glucose}, 5.5 \) (pH 7.4 adjusted with NaOH). After digestion, the heart was perfused with 50 mL of a high-\( \text{K}^+ \), low-\( \text{Cl}^- \) solution composed of (mM): L-glutamic acid, 70; KOH, 70; KCl, 30; \( \text{KH}_2\text{PO}_4 \), 10; \( \text{MgCl}_2 \), 2; taurine, 20; glucose, 10; EGTA, 0.3; and HEPES, 10 (pH 7.4 adjusted with KOH). We then trimmed off the soft tissue containing the vagus nerves, adipose tissue, and the pulmonary artery under a stereomicroscope. The LV was dissected and the left PV was excised from the digested block. The LV and left PV were then minced gently in the high-\( \text{K}^+ \), low-\( \text{Cl}^- \) solution before being gently agitated to dissociate the cells. The two cell suspensions were stored at 4 °C for later use.

PV preparation for molecular cloning of \( \text{Clcn}2 \) was obtained by a similar procedure to that described above, except that the enzymatic digestion was not performed. In the current study, samples of PV were collected from regions including PV ostia and the left PV trunk.

*Molecular cloning of Clcn2 and Hspa8 from rat PV cardiomyocytes*

Total RNA was extracted from rat PV using a RNeasy mini kit (QIAGEN, Hilden, Germany). cDNA was transcribed using a
PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Kusatsu, Japan). The Clcn2 and Hspa8 complete coding sequences were then amplified from the extracted cDNA using specific primers (see Table 1) containing HindIII and EcoRI and EcoRI and XbaI restriction sites, respectively. The following thermal cycler protocol was used to amplify the cDNA regions: 35 cycles of 98 °C for 10 s, 55 °C for 5 s, and 72 °C for 3 min, followed by a final extension at 72 °C for 7 min. All reactions were performed in a Dice thermal cycler (Takara). Amplicons were gel-purified and subcloned into the pcDNA3.1 vector (Thermo Fisher Scientific, Waltham, MA, USA) using the appropriate restriction enzymes and a DNA Ligation Kit, Long (Takara). Recombinant plasmids were purified, and their DNA sequences were confirmed by Sanger sequencing (Model 310; Applied Biosystems, Foster City, CA, USA).

Transfection of Clcn2 and Hspa8 into HEK293 and PC12 cells

For functional analysis, cloned Clcn2 was transiently transfected with or without subcloned Hspa8 into HEK293 and PC12 cells using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Enhanced green fluorescence protein (eGFP) was co-transfected to label transfected cells. The transfection efficiency was 40–50%. Cells were settled on poly-D-lysine-coated coverslips and subcultured for 48 h prior to electrophysiological recording.

Electrophysiological analysis by patch-clamping

The whole-cell patch clamp method was used to record membrane currents (patch-clamp amplifier Axopatch 200B; Molecular Devices, Chicago, IL, USA). Borosilicate glass electrodes had tip resistances between 2.0 and 5.0 MΩ when filled with internal solution. For Cl− current recording, KCl of Tyrode’s solution was substituted by equimolar CsCl. Membrane currents were recorded under voltage-clamp conditions at 37 ± 1 °C. Pulse protocols and data acquisition and storage were managed using CLAMPEX (Molecular Devices). The sampling frequency was 1 kHz and low-pass filtering was performed at 500 Hz. The cell membrane capacitance was determined by applying a 30-ms hyperpolarizing voltage-clamp step from a holding potential of 0 mV to −5 mV and integrating the area under the capacitive transient. All patch-clamp data were analyzed using IGOR software (Wavemetrics, Portland, OR, USA). The junction potential between Cs-Tyrode’s solution and the intracellular solution were corrected numerically using previously measured values (15), because solutions used in the current study were identical. The compositions of internal solutions for recording Cl− currents are presented in Table 2. To evaluate the voltage-dependent current availability, the current was activated by applying 2 s hyperpolarizing pulses to various test potentials from the holding potential of 0 mV, followed by depolarization to +40 mV. The relationship
between the test potentials and the relative amplitude of the tail current was fitted using the Boltzmann equation:

\[ \frac{I}{I_{\text{Max}}} = \frac{1}{1 + \exp \left( \frac{V_m - V_{1/2}}{S} \right)} \]

where \( I_{\text{Max}} \) is the predicted maximum current amplitude, \( V_m \) is the membrane voltage, \( S \) is the scaling factor, and \( V_{1/2} \) is the \( V_m \) needed to reach half the \( I/I_{\text{Max}} \). Summations of two Boltzmann functions were applied to analyze the activation curves of two-component currents in HSPA8-expressing cells. Time-dependent kinetics of the \( I_{\text{Cl,h}} \) of LV cardiomyocytes was analyzed by fitting the current with a first-order biexponential function:

\[ I(t) = y_1 \left\{ 1 - \exp \left( -\frac{t}{\tau_{\text{fast}}} \right) \right\} + y_2 \left\{ 1 - \exp \left( -\frac{t}{\tau_{\text{slow}}} \right) \right\} + y_0 , \]

where \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) are fast and slow time constants, respectively.

**Immunocytochemistry and confocal imaging**

Isolated PV and LV cardiomyocytes settled on poly-D-lysine-coated coverslips were incubated for 30 min at room temperature and subsequently fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were then permeabilized using PBS containing 0.2% (w/v) Triton X-100 for 15 min and subsequently incubated with blocking buffer (PBS containing 5% bovine serum albumin). Cells were then stained with antibodies against ryanodine receptor (RyR; Abcam, Cambridge, UK; cat. no. ab2827), Na/K pump (Abcam, cat. no. ab76020), CLCN2 (Santa Cruz Biotechnology, Dallas, TX, cat. no. sc-377284; Alomone Labs, Jerusalem, Israel; cat. no. ACL-002), and HSPA8 (Abcam; cat. no. ab2788). All primary antibodies were diluted 1:500 in antibody buffer-PBS containing 1% (w/v) BSA. Appropriate secondary antibodies were then used (either anti-mouse or anti-rabbit, conjugated with either Alexa Fluor 488 or Alexa Fluor 594) at a 1:100 dilution. Cells were mounted on glass slides using Permoun (Fisher Scientific, Hampton, NH, USA). Single slice and/or image stacks were obtained using a Zeiss 700 confocal microscope (×63, pinhole size of an arbitrary unit). Captured images were processed using ZEN Image software (Zeiss).

**Co-immunoprecipitation and mass spectrometry**

To prepare crude membrane proteins fraction from rat hearts, the LV and PV were dissected from rats as described above in Ca\(^{2+}\)-free Tyrode solution at 4 °C and then flash frozen in liquid nitrogen before being stored at −80 °C for later use. To extract membrane proteins, the heart samples were resuspended in a solution containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, and protease inhibitor cocktail (cOmplete Mini, Thermo Fisher Scientific) and then homogenized. The homogenates were centrifuged at 800 × g for 10 min to remove nucleus proteins and then recentrifuged at 15,000 × g for 15 min to remove lysosomes and mitochondria. Supernatants were pelleted at 100,000 × g for 1 h at 4 °C. The resultant pellets were used as the
plasma membrane-enriched fraction. For solubilization, the pellets were incubated for 1 h at 4 °C in cell lysis buffer composed of 10 mM Tris (pH 7.5), 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 10% glycerol, and protease inhibitor cocktail. Aliquots (0.5 ml) of the solubilized samples containing membrane proteins (500 or 1000 µg quantified by the Bradford assay) were incubated for 1 h with 2 µg of mouse anti-CLCN2 (Santa Cruz Biotechnology, cat. no. sc-377284) or rabbit anti-HSPA8 (Proteintech, Chicago, IL; cat. no. 10654-1-AP) coupled with Dynabeads Protein G superparamagnetic beads (Thermo Fisher Scientific). Precipitants with 10 µg normal mouse or rabbit IgG were used as negative controls. After being washed three times, bound proteins were eluted in Laemmli buffer and run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. For liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS), Coomassie brilliant blue-stained gel lanes were excised, and proteins were digested with high-grade trypsin (Promega, Fitchburg, WI, USA). Trypsinized peptides were resolved in 0.5% trifluoroacetic acid and then loaded onto a pre-column (C18 PepMap100, 5 µm; Dionex, Idstein, Germany). After demineralization of the loaded sample, liquid chromatography was performed on an EASY-nLC1000 liquid chromatography system (Thermo Fisher Scientific) and directly electrosprayed into a Q-Exactive mass spectrometer (Thermo Fisher Scientific). MS/MS spectra were analyzed using Proteome Discoverer 1.3 (Thermo Fisher Scientific).

**SDS-PAGE and western blotting**

Proteins extracted from PV, LV, and PC12 cells were separated by electrophoresis on 10% SDS-PAGE gels using the Mini-PROTEAN system (BioRad, Hercules, CA, USA) and then transferred onto polyvinylidene fluoride membranes. The membranes were blotted using mouse anti-CLCN2 (Santa Cruz Biotechnology, cat. no. sc-377284) or rabbit anti-HSPA8 (Proteintech, cat. no. 10654-1-AP) primary antibodies at a 1:1000 dilution. Appropriate secondary antibodies were then used (either anti-mouse or anti-rabbit, conjugated with horseradish peroxidase (HRP), at 1:10000 dilution). Housekeeping protein GAPDH was blotted using anti-GAPDH antibody (Cell Signaling, cat. no. 2118), if necessary. The HRP reaction was carried out using ECL Prime Detection Reagent (GE Healthcare). Results were visualized using a ChemiDoc XRS 255 imaging system (BioRad) or ImageStation 2000R (Kodak).

**Homology modeling of protein structure and protein-protein docking simulation**

Template-based homology modeling of rat CLCN2 and HSPA8 was performed using the SWISS-MODEL bioinformatic modeling server (https://swissmodel.expasy.org/). Human CLC-1 (26) and HSP90 (29) and bovine HSPA8 (28) were used as templates. Protein models were simulated as both dimers and monomers, downloaded as PDB files, and uploaded to Cluspro 2.0 (https://cluspro.org/).
This computer server calculates interaction energies of billions of conformation models and outputs 10 protein-protein docking states. The top-ranked docking state was used as the CLCN2/HSPA8 docking model. The model was visualized using PyMol software (Schrödinger, LLC).

Statistical analysis

Results are expressed as the mean ± standard error. The number of cells (n) used in each experiment is indicated in the figures and text. Statistical significance was determined using Mann-Whitney’s U-tests, one-way ANOVA, Kruskal-Wallis test or two-way ANOVA appropriately using Prism 8.1.0 (GraphPad Software, San Diego, CA, USA). A p value less than 0.05 was considered statistically significant and indicated with asterisk in figures.

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Declarations of interest: none.

Contributions

Y. Okamoto and K. Ono conceptualized the study and wrote the manuscript. Experimental procedures were designed by Y. Okamoto, Y. Nagasawa, Y. Obara and K. Ishii. Experiments and data analysis were performed by Y. Okamoto, Y. Nagasawa, and D. Takagi.
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Footnotes

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### Table 1

| Primer's Name           | Sequences (5' - 3')                                                                 |
|-------------------------|-----------------------------------------------------------------------------------|
| Full_rClcn2_F           | 5'-ACAaagcttAGAAGCAAGGAGGAGGAGGAGG-3'                                             |
| Full_rClcn2_R           | 5'-ACACAGaattCTTCTGGTCACTGCTGCTGGCTTGCTGC-3'                                    |
| EcoRI-Hspa8-Forward     | 5'-AAgaattcTCTGTTGGTCTCAGTCATCAGC-3'                                             |
| XbaI-Hspa8-Reverse      | 5'-AAtctagaGGGCCCTGTGGAAACATGC-3'                                                |

**Table 1.** List of cloning primers. The top pair of primers was used in the cloning of full-length rat *Clcn2*. The lower pair was used in the subcloning of rat *Hspa8*. The restriction enzyme cutting sites are indicated in lower-case characters.
Table 2. Composition of internal solutions used in Cl⁻ current recording. (1) [Cl⁻]ᵢ = 40 mM, (2) [Cl⁻]ᵢ = 100 mM, (3) [Cl⁻]ᵢ = 150 mM. The pH of all internal solution was adjusted with CsOH.

| Internal Solutions | (1)  | (2)  | (3)  |
|--------------------|------|------|------|
| CsCl               | 30.0 | 90.0 | 140.0|
| CsOH               | 110.0| 50.0 |-    |
| Aspartic acid      | 110.0| 50.0 |-    |
| Na₂-ATP            | 5.0  | 5.0  | 5.0  |
| MgCl₂              | 5.0  | 5.0  | 5.0  |
| EGTA               | 5.0  | 5.0  | 5.0  |
| HEPES              | 5.0  | 5.0  | 5.0  |
| GTP-Na₂            | 0.1  | 0.1  | 0.1  |
Figure legends

**Figure 1.** Functional cloning of *Clcn2* from rat pulmonary vein (PV). (A) Detection of full-length 2811 bp rat *Clcn2* in the small intestine (SI), used as a positive control, and in PV. The DNA sizes are indicated on the left. The red arrow indicates the exact size of CLCN2. Each pair of adjacent lanes is derived from the same rat (i.e. the PV of four individual rats is shown on the gel). The two most intense bands in the middle of the gel were extracted for gene cloning. (B) Hyperpolarization-activated Cl⁻ currents of cloned *Clcn2* were recorded under chloride ion concentrations ([Cl⁻]) of 40, 100, and 150 mM, with [Cl⁻]₀ = 148.9 mM. The pulse protocol is indicated at the bottom of the figure. Dashed lines indicate current levels of 0. (C) Steady-state activation curves under different [Cl⁻], evaluated by current amplitudes of tail currents at 40 mV. Magenta, marine blue, and green indicate data obtained under [Cl⁻] of 40, 100, and 150 mM, respectively. n = 7 for each [Cl⁻]. *V*₁/₂ and *S* values determined by Boltzmann fitting are plotted against [Cl⁻] in (D) and (E), respectively. Statistical difference was calculated from Kruskal-Wallis test. (F) Slow (τ_slow) and fast (τ_fast) time constants at −120 mV obtained by fitting the raw data with a biexponential function are plotted against [Cl⁻].

**Figure 2.** Immunocytochemical analysis of cardiomyocytes isolated from pulmonary vein (PV) (left, middle) and left ventricle (LV) (right). PV myocyte is labeled with a pair of CLCN2 (green) and Na/K pump (red), and a pair of ryanodine receptor (RyR) (green) and CLCN2 (red). Immunostaining of Na/K pump performed as a plasma membrane maker, and colocalization between CLCN2 and Na/K pump is indicated by yellow color in the overlay image. RyR indicates striated myocardial pattern. LV myocytes is stained with antibodies against RyR and CLCN2.

**Figure 3.** The effect of the chloride ion concentration ([Cl⁻]₀) on the voltage dependency of the hyperpolarization-activated Cl⁻ current (*I*₉₅₆₉) in left ventricle cardiomyocytes. (A) Raw traces recorded at [Cl⁻]₀ of 40, 100, and 150 mM [Cl⁻]₀, with [Cl⁻]₀ = 148.9 mM. Horizontal bars (500 ms) and vertical bars (5 pA/pF) are shown for all traces. The pulse protocol is shown. (B) Steady-state activation curves evaluated by tail currents under different [Cl⁻] at 40 mV. The color-coding is the same as in Figure 1. *V*₁/₂ and *S* values are plotted against [Cl⁻] in (C) and (D), respectively. (E) Slow (τ_slow, open circles) and fast (τ_fast, closed circles) time constants at −120 mV obtained by fitting the raw data with a biexponential function are plotted against [Cl⁻].
Figure 4. Identification of HSPA8 as a CLCN2-interacting protein. (A) Detection of CLCN2-interacting proteins in the membrane fraction by Coomassie brilliant blue staining. The membrane protein fractions from lysed pulmonary vein (PV) and left ventricle (LV) cardiomyocytes were immunologically purified by co-immunoprecipitation using an anti-CLCN2 antibody and separated by SDS-PAGE. Protein size is indicated on the left. CLCN2 and immunoglobulin heavy and light chain proteins were detected at approximately 90, 50, and 25 kDa, respectively. The protein at ~70 kDa (indicated with arrows) is an unidentified molecule. Precipitant with 10 µg mouse IgG was loaded in as a negative control. (B) Determination of the amino acid sequence of the unknown molecule by liquid chromatography-tandem mass spectrometry analysis. Mass spectrometry analysis identified eight peptides of rat origin, all of which corresponded to a single protein, HSPA8. The complete 646-amino acid sequence of HSPA8 is displayed. The 90 amino acids used in the identification of the eight peptides are indicated in bold, underlined, italic characters. Coverage was calculated as the ratio of the identified 90 amino acids to the 646 total amino acids. (C) Confirmation of the protein-protein interaction. Precipitants obtained from the crude membrane fractions of PV or LV using either CLCN2 (left panel) or HSPA8 (right panel) antibody are blotted with the other antibody. Origins of loaded proteins (i.e. PV or LV) are indicated above corresponding gel lanes. Precipitant with 10 µg rabbit IgG was also loaded in as a negative control on the right panel.

Figure 5. Expression and localization of Hspa8 in cardiomyocytes. (A) RT-PCR detects full-length 2027 bp Hspa8 (SI; small intestine). The cDNA is subcloned. (B) Immunocytochemistry of PV cardiomyocytes against HSPA8 (green) and Na/K pump (red). Immunostaining of Na/K pump indicates the location of cell surface. The overlay image (bottom) emphasizes colocalization of HSPA8 with the plasma membrane marker. Scale bar is 10 µm. (C) Immunocytochemistry of LV cardiomyocytes against HSPA8 (green) and Na/K pump (red). HSPA8 locate on the cell surfaces. Of note, Immunostaining of Na/K pump detects enriched transverse tubule. Scale bar is 10 µm. (D) Immunoprecipitants in PC12 cells reconfirm the interaction between cloned CLCN2 and HSPA8 again.

Figure 6. Effect of Hspa8 expression on the chloride ion concentration ([Cl−]) -dependent voltage-gating in HEK293 cells. Hspa8 was co-transfected with Clcn2. (A) The [Cl−]i, was 40 mM (left) and 150 mM (right). The pulse protocol is shown in the inset. Activation curves were evaluated based on the amplitude of tail currents at 40 mV. The initial portion of the tail current is shown in expanded-scale in (B). Experimental results are summarized by Boltzmann fitting in (C). $V_{1/2}$ and $S$ values are plotted against [Cl−]i in (D) and (E), respectively. Of note, the activation curve at [Cl−]i is divided into two components, and the low voltage-activated component is statistically different from the activation curve at 40 mM [Cl−].
**Figure 7.** Effect of Hspa8 overexpression on the [Cl−]-dependent voltage-gating in PC12 cells. (A) the chloride ion concentration ([Cl−])-dependent voltage-gating CLCN2 current in PC12 cells without co-transfection of Hspa8. Current traces were obtained using the pulse protocol shown in the inset. The [Cl−] was 40 mM (left) and 150 mM (right). Activation curves were evaluated based on the amplitude of tail currents at 40 mV. The tail current is shown in expanded-scale in (B). The color-coding of the results is the same as in Figure 1. (C) The activation curves are gained by Boltzmann fittings. (D) CLCN2 current in PC12 cells with co-transfection of Hspa8. The pulse protocol is same as (A). The tail current and activation curve are shown in (E). The activation curves are fitted with double Boltzmann function in (F), and contributions of low voltage-activated component are overviewed in (G). (H) time constants of the Cl− currents activated -160 - -80 mV. The currents were evoked in PC12 cells with or without overexpression of Hspa8 in the both condition of [Cl−] = 40 and 150 mM. two-way ANOVA undetected impact of Hspa8 on the value. (I) τfast (closed circles) and τslow (open circles) of tail currents at 40 mV following to activation by hyperpolarization of -160 mV are measured in PC12 cells with or without overexpression of Hspa8 in the both condition of [Cl−] = 40 and 150 mM.

**Figure 8.** Other electrophysiological parameters for HEK293 and PC12 cells. The amplitudes of the CLCN2 currents were evaluated by tail currents at 40 mV following the hyperpolarization to −120 mV. The data obtained using HEK293 cells are summarized in (A) and PC12 data are summarized in (B). The data are classified by experimental conditions, i.e. [Cl−], cell-type and co-expression of Hspa8, as indicated. (C) and (D) summarize cell capacitance (Cm) of cells. According to Mann-Whitney test for each experimental condition (cell type and [Cl−]), Hspa8 expression affects current amplitude at 40 mM [Cl−] in HEK293 cells, otherwise no statistical significance was detected.

**Figure 9.** High-throughput simulation of protein-protein docking between rat CLCN2 and human HSP90. Protein structures of CLCN2 (cyan and green) and HSP90 (olive and wheat) were homologically modeled by SWISS-MODEL. Docking states were calculated using ClusPro 2.0. The highest priority model is visualized as a cartoon ribbon structure (A) and with protein surface (B). The upper panels in (A) and (B) are side views of the model with a tentative plasma membrane (gray lines). The corresponding lower panels are bottom views, which illustrate the intracellular side of the subunit assembly. Anion-selective protopore gates are indicated in red. (C) Surface electrostatic potential is color-coded by the kT/e unit. One of the HSP90 proteins (olive color in the top panel) is subtracted in the middle, displaying an electrostatically strong docking site (dotted line). The cartoon structure (bottom) indicates that the docking site does not affect the homodimeric interface.
**Figure 10.** Protein-protein docking between rat CLCN2 and HSPA8. Protein structures of CLCN2 (cyan and green) and HSPA8 (olive and wheat) were homologically modeled and docking states were calculated using ClusPro 2.0. The highest priority model is visualized with a tentative plasma membrane (gray lines). The upper and lower panels in (A) and (B) display side and bottom views, respectively. The anion-selective protopore gates are colored red. (C) Surface electrostatic potential is color-coded by the kT/e unit. One of the HSPA8 proteins (olive color in the top panel) is subtracted in the middle, displaying an electrostatically strong docking site (dotted line). The cartoon structure (bottom) indicates that the docking site interacts with the homodimeric interface.
Figure 2

PV

CLCN2

Na/K pump

Overlay

LV

CLCN2

RyR

Overlay

10 μm

10 μm

10 μm
Figure 3

Panel A: Shows voltage traces at different concentrations of chloride ([Cl]i).

Panel B: Depicts time constant (ms) against chloride concentration.

Panel C: Illustrates voltage traces with concentration labels.

Panel D: Displays slope factor against chloride concentration.

Panel E: Graphs fractional conductance against chloride concentration.
Figure 4

**A**

- IP: IgG
- PV
- LV
- IP: CLCN2
- 75
- 75
- 63
- 48
- 35
- 25

**B**

- IB: anti-HSPA8
- 100
- 75
- 75
- 63

**C**

- IB: anti-CLCN2
- 100
- 75

**Coverage = 13.9%**

**Sequence:**

- MSKGPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNR
- TTPSYVAFTDTER
- LIGDAAK
- NQVAMNPTNTVFDAKRLIGRRFDDAVVQSDMKHWVFVVNDAGRPKVQVEY
- KGETKSFYPEEVSSMVLTKMKEIAEAYLGKTVTNAVVTVPAYFNDSQR
- QATK
- DAG
- TIAGLNVLRIINEPTAAAIAYGLDKK
- VEGARNVLIFDLGGGTFDVSILTIEDGIFEVKS
- TAGDTHLGGEDFDNRMVNHFIAEFKRMHISNPRFAVAAVAGAALISGDSENVODLL
- TOASISLSTVGEIDTEFISTTRARRQFEELANIDLRTDPEVAKERQDDASGDHIL
- TAGDTHLGEQDDPNRMVHNIAEFKRMHISNPRFAVAAVAGAALISGDSENVODLL
- TAILGVIVEN Egyptian Ludwig GPPAAYGDLKSDLRNNUVRDLGGDDFVSTLIDEFEVRK
- KGETRSPFEVESSMNLRKELKAEATLCKPVTAVAYPNDSDGRRQDAK
- NGQVMNPFTYDARKLRILGFPDDAVAAVAGAALISGDSEPMDWDYNDAGPQIVER
- MSKGPAVGIDTLYSCVGVFQHGKVEIIANDDNRRNPYVATTHERLIEGDPAK
Figure 6

(A) [Cl\textsubscript{\textit{i}}] = 40 mM
(B) [Cl\textsubscript{\textit{i}}] = 150 mM

Fractional conductance

[Cl\textsubscript{\textit{i}}] (mM)
40
150

V\textsubscript{1/2} (mV)
0
-150
-100
-50
0
50
100
150
200

Low voltage-activated
High voltage-activated

Scale factor
-35
-30
-25
-20
-15
-10
-5
0

p = 0.012
p = 0.06
p = 0.40
p = 0.02

Cl\textsubscript{\textit{i}} = 40 mM
Cl\textsubscript{\textit{i}} = 150 mM

(C) Cl\textsubscript{\textit{i}} = 40 mM

D

E

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z
**Figure 7**

- **A**: Fractional conductance in PC12 cells with different concentrations of [Cl]-
- **B**: Fractional conductance in PC12 cells with [Cl]- concentrations of 40 mM and 150 mM.
- **C**: Fraction of low voltage-activated component.
- **D**: Fractional conductance with and without HSPA8.
- **E**: Comparison of tail currents at different [Cl]- concentrations.
- **F**: Comparison of conductance at different [Cl]- concentrations.

**Data**

- **Vm (mV)**
  - 200
  - 100
  - 50
  - 0
  - -50
  - -100
  - -150

**HSP48**

- Clcn2 only
- Clcn2 + Hspa8

**p Values**

- p = 0.190
- p = 0.738
- p = 0.126
- p = 0.178

**Fractional Conductance**

- 0.0
- 0.2
- 0.4
- 0.6
- 0.8
- 1.0

**τ of Activation (ms)**

- 200 ms
- 100 ms

**Fraction of Low Voltage-Activated Component**

- 1.0
- 0.8
- 0.6
- 0.4
- 0.2
- 0.0

**Fractional Conductance**

- 0.0
- 0.5
- 1.0
- 1.5

**τ of Tail Current (ms)**

- 200 ms
- 100 ms

**[Cl]- (mM)**

- 40
- 150
Molecular identification of HSPA8 as an accessory protein of a hyperpolarization-activated chloride channel from rat pulmonary vein cardiomyocytes

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