ASSOCIATION BETWEEN HSP90 AND THE CIC-2 CHLORIDE CHANNEL UPREGULATES CHANNEL FUNCTION

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Running title: Functional interaction between Hsp90 and CIC-2

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ABSTRACT

The voltage-dependent ClC-2 chloride channel has been implicated in a variety of physiological functions including fluid transport across specific epithelia. ClC-2 is activated by hyperpolarization, weakly acidic external pH, intracellular Cl\(^-\) and cell swelling. To add more insight into the mechanisms involved in ClC-2 regulation, we searched for associated proteins which may influence ClC-2 activity. Using immunoprecipitation of ClC-2 from HEK 293 cells stably expressing the channel, followed by electrophoretic separation of co-immunoprecipitated proteins and mass spectrometry identification, Hsp70 and Hsp90 were unmasked as possible ClC-2 interacting partners. Association of Hsp90 with ClC-2 was confirmed in mouse brain. Inhibition of Hsp90 by two specific inhibitors, geldanamycin (GA) or radicicol, did not affect total amounts of ClC-2, but did reduce plasma membrane channel abundance. Functional experiments using the whole-cell configuration of the patch-clamp technique showed that inhibition of Hsp90 reduced ClC-2 current amplitude and impaired the [Cl\(^-\)]\(_i\) dependent rightward shift of the fractional conductance. GA and radicicol increased both the slow and fast activation time constants in a chloride-dependent manner. Heat shock treatment had opposite effects. These results indicate that association of Hsp90 with ClC-2 results in greater channel activity due to increased cell surface channel expression, facilitation of channel opening, and enhanced channel sensitivity to [Cl\(^-\)]\(_i\). This association may have important patho-physiological consequences, enabling increased ClC-2 activity in response to cellular stresses such as elevated temperature, ischemia, or oxidative reagents.
KEYWORDS

Heat-shock, geldanamycin, cellular stress, channel trafficking, transepithelial chloride transport.
INTRODUCTION

ClC-2 is one of the nine mammalian members of the CIC chloride channel family. It is slowly activated by hyperpolarization and can be further activated by extracellular acidification or hypotonic cell swelling (25, 32, 61). ClC-2 is expressed in many tissues, such as brain, intestine, kidney, stomach, salivary glands (for review see (31)) and heart (16). Although, a clear understanding of its physiological function remains to be determined, a growing body of evidence suggests that ClC-2 can play different roles depending on the tissue in which it is expressed. Disruption of the ClC-2 encoding gene in mouse leads to degeneration of male germ cells and photoreceptors, probably resulting from a defect in transepithelial transport across Sertoli cells and the retinal pigment epithelium (6). ClC-2 may participate in fluid secretion in the murine small intestine (26) or in fluid absorption in the colon as suggested by immunolocalization (7, 35) and functional data (8). Based on studies performed with hippocampus cells (56) or transfected DRG neurons (57), ClC-2 has been implicated in the regulation of the effects of GABA\(_A\) receptor action by controlling intracellular chloride concentration. In humans, mutations in the gene CICN2 that can be predicted to cause hyperexcitability of GABAergic synapses have recently been found to be associated with idiopathic generalised epilepsies (27).

Most of the knowledge about the gating properties of CIC channels comes from biophysical and mutational analysis of ClC-0 and ClC-1 (for review see (31)). These studies suggested a homodimeric structure of the channels with two independent protopores, recently confirmed by crystal structures of bacterial CIC homologues (18). ClC-0 gating is controlled by two interdependent processes: a fast gate, activated by depolarization, that acts on each protopore, and a slow or common gate, activated by hyperpolarization, that controls both pores simultaneously (10, 48). It has been proposed that the side chain of a glutamate residue
within the pore serves as the protopore gate (19), but less is known about the molecular nature of the slow gate that might involve a large conformational change as indicated by its strong temperature dependence (49). The fast gate strongly depends on voltage and external Cl$^-$ concentration. This particular feature results from the effect of chloride ions on channel gating coupled to translocation of Cl$^-$ in the membrane electric field (11, 50). Recent studies performed on ClC-2 transiently expressed in HEK 293 (62, 64) have shown that ClC-2 current relaxation could be described by two exponentials, probably reflecting the presence of the two gates (protopore and common gates, by analogy to ClC-0). The ClC-2 conductance (8, 15, 22, 39, 56) and the hyperpolarization-activated slow gates of ClC-0 and mutant ClC-1 (48) depend on intracellular chloride concentration ([Cl$^-$]$_i$), an increase in [Cl$^-$]$_i$ favouring channel opening. It has been suggested that the [Cl$^-$]$_i$ and voltage-dependence and ClC-2 might arise from a voltage-dependent movement of intracellular chloride in the outward direction.

Studies on endogenous ClC-2 channels or in heterologous expression systems have shown important differences in the voltage sensitivity and activation kinetics of the current. For example, ClC-2 activation kinetics are slower when ClC-2 is expressed in ovocytes (61) as compared to HEK 293 cells (12, 42). Activation kinetics of endogenous currents recorded in neurons (13, 56), T84 cells (22), salivary glands (42), or colonocytes (7) also differ, requiring from less than one to several seconds for steady-state activation. These observations suggest that splice variants or unknown protein partners differentiably expressed according to the cell type may modulate channel kinetics and gating parameters.

Little is known about the existence of ClC channels partners modulating channel gating. The renal human ClC-K channel is the unique example among the ClC channel family that requires a β subunit for proper function (20). It is reasonable to assume that additional proteins are also involved in the regulation of channel trafficking and function. For example,
interaction between ClC-5 and cofilin seems essential for the role of ClC-5 in albumin uptake in the proximal tubule (29). Using the two-hybrid technology to probe the carboxy termini of ClC-2, Furukawa et al detected a direct interaction with protein phosphatase 1 (23). By immobilization of purified intact ClC-2 on a solid phase, Dhani et al identified an interaction between ClC-2 and the retrograde motor dynein, important for regulation of ClC-2 cell surface expression (14).

The aim of the present work was to extend the search for possible interacting partners that may modulate ClC-2 function. By means of mild lysis conditions, ClC-2 immunoprecipitation, and mass spectrometry, we found an association between ClC-2 and the two protein chaperones Hsp90 and Hsp70 in HEK 293 cells stably expressing ClC-2. Interaction of native ClC-2 with Hsp90 was also detected in mouse brain. Hsp90 inhibition reduced plasma membrane channel expression without affecting protein stability. Electrophysiological studies demonstrated that Hsp90 inhibition decreased ClC-2 current amplitude and decreased the sensitivity of the channel to intracellular chloride.
MATERIALS AND METHODS

**Constructs** - The cDNA of rat ClC-2 was kindly provided by T.J Jentsch (Hamburg, Germany). Mutagenesis was performed using recombinant PCR and sequencing. To facilitate immunoprecipitation of a large amount of ClC-2, we decided to engineer a construct containing a Flag sequence (gac tac aag gat gac gac gac aag) encoding for the Flag epitope (DYKDDDDKD). Preliminary experiments were conducted to assess whether the epitope could affect channel expression or activity. Insertion of the epitope was tested in each of the four extracellular loops. Insertion was made between amino acids L132 and N133 (Loop1), K210 and E211 (Loop2), K313 and T314 (Loop3) or K400 and E401 (Loop4). Insertion of the Flag epitope in Loop1 did not significantly affect expression, localization, conductance, or voltage-dependence of the channel. On the other hand, insertion in Loop3 and Loop4 markedly decreased current amplitude, and insertion in Loop2 even abolished ClC-2 current, reflecting modifications of channel activity and/or absence of surface expression. Sequences coding for WT ClC-2 or for ClC-2Flag (insertion in loop1) were subcloned in the pIRES-EGFP plasmid (Clontech, Palo Alto, USA), allowing independent expression of the two proteins EGFP and ClC-2. This expression system thus enabled us to visualize transfected cells while keeping the terminal parts of ClC-2 free.

**Cells** - HEK 293 cells were cultured in DMEM media supplemented with 10% serum (Gibco, Paisley, UK) at 37°C, 5% CO₂ atmosphere. Cells were transfected with linearized pIRES ClC-2, pIRES ClC-2Flag, or with empty plasmid using Lipofectamine (Invitrogen, Carlsbad, USA). Stably expressing cells were selected by adding 500µg/mL of G418 (Invitrogen, Carlsbad, USA) in the media. Isolated clones were tested for fluorescence, protein expression and ClC-2 current.
**Antibodies and Drugs** - Two polyclonal anti-ClC-2 antibodies were used: one raised against an N-terminal peptide (residues 1-90 of human ClC-2, H-90) purchased from Santa Cruz Biotechnology, Inc. (California, USA), and the second, homemade (35), named pAb137, raised against a carboxy-terminal peptide (residues 847-862). The anti-Flag M2 antibodies coupled to agarose beads were purchased from Sigma-Aldrich (St. Louis, USA). Anti-GFP antibodies were from Santa Cruz Biotechnology and anti-Hsp90 (Spa-835) from Stressgen (Victoria, Canada). Geldanamycin (GA) was bought from InVivogen (San Diego, USA) and radicicol from Sigma-Aldrich (St. Louis, USA).

**Immunoprecipitation and silver staining** - To identify co-immunoprecipitated proteins, cells stably expressing WTClC-2 or ClC-2Flag were lysed on ice with lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% Glycerol, 1% Triton X-100 and 0.1% IGEPAL (buffer A) complemented with anti-proteases (Roche mini tablets, Mannheim, Germany) and centrifuged at 24 000g for 60min at 4°C. Soluble protein concentration was measured using the RCDC assay from BioRad (Hercules, USA). Equal amounts of protein (200 mg) were precleared with 50 µg of a non-relevant antibody coupled to agarose beads for 60 min before adding 50 µg of anti-Flag antibodies and were incubated over-night. Beads were pelleted at 3000 g, washed 6 times with lysis buffer, and resuspended in 2x Laemli before SDS-PAGE electrophoresis separation. The mass spectrometry (30)-compatible silver staining was used for MS experiments (53). Briefly, gels were fixed in a 45% ethanol, 5% acetic acid solution, sensitized with 0.02% sodium thiosulfate and impregnated with 0.1% AgNO₃. Staining was developed with 0.04% formaldehyde, 2% Na₂CO₃ and the reaction was stopped with 1% acetic acid.

**Mass spectrometry** - Differentially expressed protein bands were excised, and proteins of interest were digested with trypsin (sequencing grade; Promega, Madison, USA) while still in the gel (5). Peptides eluted from protein bands after tryptic digestion were
separated by HPLC (RP-18 column, 75 mM, Promega, Madison, USA) and then analysed by ESI-MS on a Q-TOF (Micromass, USA) mass spectrometer working in the regime of data dependent MS to MS/MS switch. Proteins were identified using Mascot and PeptIdent software, available online at http://www.matrixscience.com and http://www.expasy.org/tools/peptident.html, respectively.

**Hsp90/ClC-2 co-immunoprecipitation** - Co-immunoprecipitation experiments were performed using cells stably expressing WTClC-2, lysed with buffer A. Lysis buffer was complemented with 10mM sodium molybdate, since it has been described to stabilize Hsp90/client protein complexes (59). Protein samples were precleared with agarose beads coupled to a non-relevant antibody and concentration was measured using the RCDC BioRad assay.

ClC-2 was immunoprecipitated from 1 mg of total extracts with 10 µg of anti-ClC-2 antibody (pAb137). Hsp90 was immunoprecipitated from 1 mg of total proteins with 5 µg of anti-Hsp90 antibodies. Non-immunized IgG’s were used as a negative control. Immunoprecipitates were resuspended in 2x Laemli, heated for 1 min at 95°C, complemented with 2 volumes of 1x Laemli containing 3.5M urea and incubated at 37°C for 1 hour. Samples were separated by SDS-PAGE (7%) in the presence of 5M urea before being transferred onto nitrocellulose membranes. Membranes were blocked for one hour in a solution containing 5% non-fat milk, 0.1% Tween-20 in TBS and then incubated overnight at 4°C with anti-ClC-2 or anti-Hsp90 antibodies in the blocking solution. Protein bands were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfont, UK).

Normal 57BL6 mice (Charles River, France) were briefly anaesthetized with halothane and decapitated. Experiments were carried out under licence n° 7514 of the Veterinary Department of the French Ministry of Agriculture (Decret 87-848, oct 19th 1987). Brains were
rapidly removed, disrupted in a Potter and lysed in buffer A complemented with 10 mM sodium molybdate at 4°C during 3 hours. Samples were centrifuged twice for 1 hour at 25,000 g to remove the non-soluble fraction. Agarose beads were used to pre-clear the sample, and the protein concentration was measured using the RCDC assay. 6 µg of anti-Hsp90 or non-specific rat IgG were added to equal amounts of proteins (10 mg) to precipitate Hsp90. Precipitates were washed five times in lysis buffer, and detection of ClC-2 was performed as described above.

Pretreatment of the cells - Two types of treatment were used to investigate the possible functional consequences of Hsp90/ClC-2 interaction before protein analysis, immunolabeling or patch-clamp measurements: (1) a 3 to 24 hours incubation of the cells with 10 µM GA or 200 nM radicicol, and (2) a one hour incubation of the cells at 43°C, followed by a 6-hour recovery period at 37°C.

Whole cell and membrane protein analysis - For whole-cell protein analysis, 40 µg of total protein extracts were solubilized in RIPA buffer and re-suspended in 2x Laemli containing 7M urea. Samples were heated at 37°C for 1 hour before being separated by SDS-PAGE as above. The amount of ClC-2 was estimated by Western blot, and GFP detection was used to verify equal protein loading.

To follow surface expression of ClC-2, membrane proteins were biotinylated with sulfo-NHS-LC-Biotin prior to lysis. Samples were then precleared with sepharose beads for 1 hour at 4°C. Biotinylated proteins were purified with monomeric avidin beads (Pierce, Rockford, USA), washed 5 times in lysis buffer and re-suspended in 2x Laemli as above. ClC-2 expression was analysed by Western blot and GFP was probed in the same membranes to assess the absence of intracellular proteins in the sample.

Immunofluorescence and Confocal Microscopy - ClC-2 immunostaining was performed on HEK 293 cells stably expressing WTC1C-2. Cells were grown on poly-D-lysine
precoated coverslips, fixed with cold methanol and permeabilized with 0.1% Triton in PBS. Coverslips were blocked with 1% bovine serum albumin in PBS before incubation with the primary ClC-2 antibody (Santa-Cruz; final dilution 1:125 in PBS-Triton). After washing and blockage with 5% goat serum in PBS, coverslips were incubated with the Alexa-fluor 594 secondary antibody (Molecular probes, USA) at a dilution of 1:1000. Coverslips were subsequently mounted on glass slides with the Vectashield mounting medium (Vector Laboratories, CA) and micrographs were taken with a Zeiss confocal microscope.

**Whole cell patch-clamp recordings** - Stably transfected cells were plated in 35 mm cell culture plastic Petri dishes that were mounted on the stage of an inverted microscope. Patch-clamp experiments were performed at room temperature with an Axopatch 200A amplifier controlled by a computer via a CED 1401 interface (CED, Cambridge, UK). The bath was grounded via an agar bridge. Pipettes were pulled from hard glass (Kimax 51) using a Setter micropipette puller. Current recordings were performed using the nystatin-perforated patch-clamp configuration that gave stable recordings over long time courses. Nystatin stock solution (50 mg ml⁻¹) was prepared daily in DMSO. Aliquots were diluted (1:250) in the internal solutions, which were then sonicated for at least 30 s. The bath solution contained (in mM): 150 NaCl, 2 CaCl₂, 1 MgCl₂, 10 Heps-Na, 35 sucrose, pH 7.4 adjusted with NaOH. The pipette solution (135 mM Cl⁻) contained (in mM): 131 NaCl, 2 MgCl₂, 10 Heps, pH 7.3 adjusted with NaOH. Lower Cl⁻ solutions were prepared by equimolar replacement of NaCl with Na gluconate (for the 100 mM Cl⁻ solution) or by replacement of NaCl with 35 mM Na₂SO₄ and the appropriate concentration of Na gluconate (for the 64, 35 and 15 mM Cl⁻ solutions). Access resistance (Ra) gradually declined after the formation of an on-cell patch, and recordings were started when Ra decreased to < 20 MΩ, usually 15-20 minutes after patch formation. Mean Ra was 14.2 ± 3.6 MΩ. Accurate control of internal chloride
concentration was verified by measuring the reversal potentials of the currents elicited by voltage ramps applied at the end of a -120 mV hyperpolarizing voltage jump.

Changes in liquid junction potential were calculated (4) and taken into account when necessary.

Currents were recorded by applying regular voltage pulses of desired length and amplitude from a holding potential of 0 mV with an interval of at least 60 s to allow current deactivation.

Time courses for activation and deactivation were described by fitting a mono- or a double-exponential plus a constant term in equations of the form: $I(t) = A \exp(-t/\tau_1) + B \exp(-t/\tau_2) + C$ where $I(t)$ is current as a function of time, $A$, $B$, and $C$ are constants, and $\tau_1$ and $\tau_2$ are time constants.

The relative open probability as a function of voltage was estimated from measurements of the initial currents at 40 mV following each test negative voltage jump. The conductance values were adjusted by a Boltzmann distribution of the form $G = G_o + G_{\text{max}}/[1+\exp(V-V_{0.5})/k]$, where $G$, $G_o$ and $G_{\text{max}}$ are conductance as a function of voltage, residual conductance, and maximal conductance (extrapolated), respectively. $V_{0.5}$ is the voltage at which 50% activation occurs, and $k$ is the slope factor.

All measured values are presented as means ± S.E. Significance of differences between means was determined by unpaired t-test.
RESULTS

Identification of CIC-2 interacting proteins - To facilitate immunoprecipitation of a large amount of CIC-2, we inserted a Flag tag sequence in the first extracellular loop of the protein. The Flag tag sequence has previously been shown to bind with high affinity to the anti-Flag M2 monoclonal antibody (28). Insertion of this epitope did not modify either the expression or the activity of the channel (not shown). This epitope also presented the advantage of keeping free the long cytoplasmic regions of the protein, which are possible targets for interacting proteins.

CIC-2 was immunoprecipitated from 200 mg of proteins obtained from $5 \times 10^7$ HEK 293 cells stably expressing CIC-2Flag or WTClC-2 as a control. As shown in Figure 1A (lane 1), four prominent bands with respective molecular weights of 90 kDa, 200 kDa and two greater than 250 kDa were specifically (with respect to lane 2) immunoprecipitated with the anti-Flag antibody. Another less abundant band with an apparent weight of 70 kDa was also differentially precipitated from the CIC-2 expressing cells. Experiments were repeated 3 times, giving similar protein profiles.

The four bands indicated by arrows in Figure 1 were identified by mass spectrometry. Samples at the same position in the control line were also analysed. CIC-2 was present in the bands at 70 kDa (68 peptides; score 2103), 90 kDa (74 peptides; score 2283), 200 kDa (77 peptides; score 2278), and in both bands above 250 kDa (56 peptides; score 2092 and 68 peptides; total score 2215), suggesting that the quaternary structure of the protein was maintained. The presence of CIC-2 at 70 kDa could be due to partial proteolysis of the protein. In addition, Hsp70 (12 peptides; score 767), Hsp90α (6 peptides; score 320) and Hsp90β (6 peptides, score 234) were identified as co-immunoprecipitated proteins in samples at 70 kDa and 90 kDa. The access numbers were gi|228578 for CIC-2, gi|12653415 for
Hsp70, gi|123678 for Hsp90α, and gi|72222 for Hsp90β. All identified proteins were absent in the control lane.

**Co-immunoprecipitation of Hsp90 with ClC-2** - In this study, we focused on the interaction between ClC-2 and Hsp90. Hsp90 is an abundant cytosolic protein (accounting for 1 to 2 % of cytosolic proteins in unstressed eukaryotic cells), that functions as a molecular chaperone facilitating the folding of a variety of proteins (43).

To rule out the possibility that the interaction of Hsp90 with ClC-2 detected by mass spectrometry could be caused by the presence of the Flag epitope, we performed immunoprecipitation of WTCIC-2 using the carboxy-terminal ClC-2 antibody. Interaction between the two proteins was also tested by reverse immunoprecipitation using the anti-Hsp90 antibody. HEK 293 cells stably expressing WTCIC-2 or the empty plasmid as a control were lysed using buffer A containing 10 mM sodium molybdate. Samples were thoroughly precleared with non-relevant antibodies coupled to agarose beads and the immunoprecipitated partner was probed using the appropriate antibody. As shown in the left panel of Figure 1B, ClC-2 was detected in anti-Hsp90 immunoprecipitates (lane 1, n=3) but not in immunoprecipitates obtained with non-specific IgG (lane 2). Conversely, Hsp90 could be detected in the anti-ClC-2 immunoprecipitates from WTCIC-2 expressing cells (Figure 1B, right panel, lane 1, n=3) but not from cells transfected with the empty plasmid (lane 2).

To determine whether this interaction existed in vivo, the same experiments were repeated using mouse brain homogenates known to express high levels of ClC-2. Protein extracts were incubated with the anti-Hsp90 antibody, and immunoprecipitated proteins were analysed by Western blotting using anti-ClC-2 IgGs. ClC-2 was detected as a band migrating at around 85 kDa (Figure 1C, lane 1) in this tissue, whereas the signal was absent when protein extracts were incubated with a non-relevant antibody (lane 2). Similar electrophoretic
migration was observed by Western Blot analysis of total protein extracts (lane 3). These results thus suggest that Hsp90 interacts with endogenous CIC-2 in mouse brain.

**Inhibition of Hsp90 decreases channel cell surface abundance without affecting protein expression.** - Hsp90 activity depends on binding and hydrolysis of ATP to the N-terminal domain coupled to a conformational cycle involving opening and closing of a dimeric molecular clamp (46). The two structurally unrelated antibiotics, geldanamycin and radicicol, specifically inhibit Hsp90 activity by competing with ATP on its binding site (52, 58). This inhibition most often increases ubiquitination and proteasomal degradation of Hsp90 client proteins. To investigate the possible effect of Hsp90 inhibition on CIC-2 expression, cells were treated during 24 hours with 10 µM GA or 200 nM radicicol. Total proteins were analysed by immunoblot using antiCIC-2 antibodies while the GFP protein amount was used as an index of protein loading (Figure 2A). Inhibition of Hsp90 activity did not significantly affect the amount of CIC-2 in the total cell extracts (n=3).

Since interaction of Hsp90 with client proteins may influence protein trafficking (44), we further investigated whether Hsp90 inhibition could modify the distribution of CIC-2 using an immunolabeling approach. As shown in Figure 2B, the pattern of CIC-2 staining was quite similar between untreated and treated cells except that membrane staining was less visible. However, the faint and non-uniform membrane staining in control cells did not allow an accurate comparison of the amount of CIC-2 at the membrane. A more quantitative analysis was performed using cell surface proteins isolated with the biotinylation procedure. Western blotting of membrane proteins showed that treatment by GA significantly decreased the overall amount of CIC-2 present in the bands at 200 kDa and 90 kDa (Figure 2C), by 39.1 ± 9 %, n=4, with respect to control cells (Figure 2D).

**Functional assay for CIC-2/Hsp 90 interaction: effect of geldanamycin and radicicol on CIC-2 current** - We performed whole-cell patch-clamp studies to investigate
whether there was any functional evidence for ClC-2/Hsp90 interaction in HEK 293 cells stably expressing ClC-2. As previously described (12, 42), HEK 293 cells transfected with EGFP alone exhibited no voltage-dependent current with the solutions used. In ClC-2 expressing cells, hyperpolarizing voltage jumps elicited typical time-dependent inward currents whose amplitude remained stable in control conditions during tens of minutes. Application of 10 µM GA resulted in a gradual decrease of ClC-2 amplitude which reached steady state within 30 minutes. Shown in Figure 3A are example of current traces at -120 mV recorded before and after 30 min application of GA using a pipette solution containing 64 mM Cl\textsuperscript{−}. The current decrease was often preceded (in about 80% of the cells studied) by a small transient current increase (by 1.29 ± 0.08 fold). The same effects were observed during application of 200 nM radicicol. The rapid transient current increase was not further investigated in the present study. At -120 mV, with 64 mM internal Cl\textsuperscript{−}, current amplitude was decreased by 45.3 ± 3.6% (n=10) and by 40.0 ± 1.2% (n=7) in response to GA and radicicol, respectively. Results from dose-response experiments (Figure 3B) showed that maximal inhibitory effects of the two inhibitors were obtained within the same range of concentrations needed for inhibition of ATP hydrolysis (51). We observed that the extent of current inhibition varied according to the chloride concentration in the pipette solution. Current recordings before and after GA treatment, at 15 and 135 mM internal Cl\textsuperscript{−} are shown in Figure 3C and 3D, respectively. At 15 mM internal Cl\textsuperscript{−}, current amplitude decreased by 36.9 ± 4.3% (n=8) and by 72.22 ± 3.8% (n=7) at 135 mM internal Cl\textsuperscript{−}.

**Effect of geldanamycin on the chloride dependence of ClC-2 gating** - Current inhibition could result from a decrease of the channel conductance, a decrease of its open probability or a decrease of the amount of ClC-2 channels at the plasma membrane. Since inhibition of Hsp90 by GA diminished membrane expression of ClC-2 (Figure 2C,D), part of the current inhibition was probably linked to a decrease in the amount of ClC-2 channels at
the plasma membrane. However, the differential current inhibition according to the value of $[\text{Cl}^-]_i$ suggested that GA could additionally modulate $[\text{Cl}^-]_i$-dependent gating parameters. Activation of ClC-2 channels is known to depend on both the membrane potential and intracellular chloride concentration (8, 15, 22, 27, 39, 56). Figure 4A shows families of current traces recorded in untreated or GA-treated cells at various internal $[\text{Cl}^-]$. The threshold of current relaxation became less negative when $[\text{Cl}^-]_i$ increased. At 135mM internal Cl\(^-\), sizeable current relaxations were recorded at -40 mV and a fraction of the channels was already activated at the resting potential, as evidenced by the presence of instantaneous currents when stepping to the test voltages. This time-independent component could not be ascribed to the activation of a different class of channels, since it could be inhibited by 100 µM ZnCl\(_2\) (a known blocker of ClC channels, (9, 13, 34)) in the bath solution (by 82.3 ± 2.3 %, n=4). Activation by changes in cell volume could also been ruled out since the same profile of current recording was obtained when the osmolarity of the extracellular medium was increased with sucrose (not shown).

The relative open probability ($P_{\text{open}}$) of the channels as a function of voltage was estimated from the single Boltzmann fit of the initial currents at the +40 mV post pulse (Figure 4B and C). Quite similar to previous reports, (27, 39), increasing $[\text{Cl}^-]_i$ in untreated cells resulted in a shift of the activation curve to more depolarised potentials (Figure 4B). $V_{0.5}$ at 135mM internal Cl\(^-\) was shifted by about 46 mV in the rightward direction as compared to that at 15 mM. Treatment of the cells with GA did not significantly affect the $P_{\text{open}}/V$ relationship at the lowest $[\text{Cl}^-]_i$, but attenuated the rightward shift induced by increasing $[\text{Cl}^-]_i$ without major changes in the slope factors (Figure 4C). The values for $V_{0.5}$ at different $[\text{Cl}^-]_i$ are plotted in Figure 4D. The effect of GA was chloride-dependent so that the slope of $V_{0.5}$ vs. $[\text{Cl}^-]_i$ became less steep with respect to that for untreated cells (Figure 5G). Thus, in the presence of GA, opening of the channels required more negative potentials for $[\text{Cl}^-]_i$, higher
than 15 mM. The change in the slope of $V_{0.5}$ vs. $[\text{Cl}^-]_i$ first suggests that GA did not simply increase the intrinsic energy difference between the open and closed states but also altered parameters involved in the chloride sensitivity of current activation. In addition, this change could explain why current inhibition in the presence of GA was greater for the highest internal Cl$^-$ concentrations (Figure 3D).

**Effect of geldanamycin on current activation and deactivation kinetics** - In previous studies, ClC-2 current relaxations have been described by two exponentials, probably reflecting the presence of two gates (12, 62). We thus examined whether GA affected both or preferentially one of the two components of current activation.

Activation time constants measured at $-120\text{mV}$ as a function of $[\text{Cl}^-]_i$, are plotted in Figure 5. In control conditions, the time dependence of current activation at 15 and 35 mM internal Cl$^-$ was well fit by a single-exponential time function, whereas at higher $[\text{Cl}^-]_i$, current relaxation was better described by a double-exponential model. As previously reported (12, 62), the two time constants differed by around ten fold (named fast and slow hereafter). Increasing $[\text{Cl}^-]$ resulted in a decrease of the two time constants values, (Figure 5A). The weight of the fast component (Figure 5B, white bars) did not significantly change between 64 and 135 mM Cl$^-$, whereas the fractional amplitude of the instantaneous component ($A_0$) increased at the expense of the proportion of the slow phase, (As) (Figure 5C and D, white bars). GA treatment attenuated the chloride-dependent decrease of the slow time constant of current activation and completely prevented the decrease of the fast one (Figure 5A). The relative proportions of the two components were not affected up to 100 mM Cl$^-$ (Figure 5B and C, black bars), but at 135mM GA decreased by half the fractional amplitude of $A_0$ while increasing the weight of As. (compare Figure 5C and D).

Deactivation at positive potentials was well described by two exponential time courses even at low chloride concentrations (Figure 5E), implying that current deactivation was not
strictly the reverse process of current activation. Increasing [Cl\textsuperscript{i}]. did not markedly change the
time constants of deactivation between 15 and 100 mM Cl\textsuperscript{i}, whereas at 135 mM Cl\textsuperscript{i}, the slow
time constant was slightly but significantly increased (Figure 5E, p<0.05) and deactivation
was not complete as revealed by the presence of a sizeable non-closing fraction (A\textsubscript{0}) (Figure
5H, white bar). Deactivation was less affected than activation by GA. The slow time constant
of deactivation was slightly decreased (Figure 5E), whereas the fast component remained
unchanged. In agreement with the results described above, the time independent fraction of
the current at 135 mM internal Cl\textsuperscript{i} was markedly decreased by GA application (Figure 5H,
black bar). Since P\textsubscript{open} is a function of the transitions rates between open and closed states
these data indicate that GA reduced the [Cl\textsuperscript{i}]. dependent shift of the P\textsubscript{open}/V curves mainly by
slowing the two gating processes at negative voltages in a Cl\textsuperscript{i}-dependent manner.

Effect of a thermal stress on ClC-2 currents - Thermal stress that increases Hsp90
synthesis often enhances immunodetection of Hsp90 partners and induces an effect opposite
to that of Hsp90 inhibitors. The effect of a one hour heat treatment to 43°C was first
investigated by immunoblot analysis of the amount of Hsp90 in whole-cell extracts during
recovery at 37°C. As shown in Figure 6A, the Hsp90 signal increased progressively during the
first hours of recovery, and a two fold increase (n=2) was detected after 6 hours recovery at
37°C, (n=2). This increase was not accompanied by significant changes of ClC-2 in whole-
cell extracts nor in the amount of ClC-2 at the membrane (not shown).

To determine whether heat shock treatment could affect ClC-2 gating parameters, we
measured ClC-2 using a 35 mM Cl\textsuperscript{i} pipette solution. Figure 6B illustrates currents recorded
during a hyperpolarizing jump to -120 mV in basal conditions or after a one-hour thermal
stress followed by 6 hours recovery. As visible on the current traces, heat shock treatment
increased the macroscopic rate of current activation by decreasing by around two fold the
slow time constant (Figure 6C) without significantly changing the rate of current deactivation
(Figure 6D). These effects were accompanied by a shift of $V_{0.5}$ from $-116.5 \pm 2.7$ mV ($n=12$) to $-106.8 \pm 2.7$ mV ($n=7$) that reached statistical significance ($p=0.038$).
DISCUSSION

In the present study, we have identified two heat shock proteins, Hsp70 and Hsp90, that co-immunoprecipitate with ClC-2 stably expressed in HEK 293 cells, and we provide evidence for a similar association in mouse brain. Pharmacological experiments designed to disrupt the association between Hsp90 and the channel protein showed that Hsp90 does not affect ClC-2 stability but favours cell surface expression. Patch-clamp experiments demonstrated that interaction between both proteins up-regulates the chloride conductance by shifting the voltage dependence of channel opening to less negative potentials and by increasing the sensitivity of the channel to $[\text{Cl}]$. Hsp70 is described to play an important role in protein folding, quality control, and in membrane translocation processes (55). Hsp90 may also have a role in nascent chain folding but is best known to control the conformational maturation and the activity of a variety of proteins within multichaperone complexes comprising Hsp70, immunophilins, p23 and Hop (43). Hsp90 client proteins include steroid hormone receptors, transcription factors, various protein kinases and phosphatases (43). Apart from signalling proteins, Hsp90 has also been shown to interact with a number of plasma membrane receptors (1, 60, 63) and ion channels (21, 36).

Co-immunoprecipitation of Hsp70 along with Hsp90, suggests an interaction with ClC-2 in a large complex which probably involves co-chaperone partners. We have not yet performed more investigations to test for the presence of such partners. Since the expression of co-chaperones may differ among cells and tissues, it would be interesting to analyse whether differentially represented co-chaperones could contribute to the variability of the gating properties of ClC-2 between endogenous channels and channels expressed in ovocytes or cell lines.
The molecular basis underlying the interaction between Hsp90 and ClC-2, either direct or indirect, remains to be determined. Some chaperone client proteins contain a tetratricopeptide motif (TPR) which binds to an acceptor site in the C-terminus of Hsp90 (47) but such a motif is absent from the ClC-2 sequence. In any case, little is known regarding the mechanism by which Hsp90 binds to substrates in the absence of TPR motifs (45). It must be recognized that an indirect interaction through an unknown ClC-2 partner cannot be excluded.

Among others, a possible candidate is dynein which has already been demonstrated to associate with ClC-2 (14) and is also known as an Hsp90 client protein in other systems (24, 38). However, we could not identify dynein in any band size analysed. The contribution of dynein could also be questioned from the inhibitory effects of GA and radicicol on cell surface ClC-2 expression. The role of Hsp90 and immunophilins in protein trafficking has been particularly well examined for the glucocorticoid receptor and the tumour suppressor p53 (44), requiring assembly of these proteins with the motor protein dynein. Dhani et al reported that pharmacological inhibition of dynein increased the expression of ClC-2 at the plasma membrane (14). Hence, the possible disruption of Hsp90/dynein interaction by GA might produce the same effect which can be in agreement with the transient increase of current amplitude induced by the two Hsp90 inhibitors but not with the reduced amount of ClC-2 at the plasma membrane following prolonged exposure. ClC-2 trafficking to the plasma membrane has also been shown to be modulated by anterograde or retrograde vesicular transport involving PI3kinase (2), the glucocorticoid inducible kinases SGKs and the ubiquitin ligase Nedd4-2 (41). Since Hsp90 has been shown to be involved in eNOS translocation to the apical membrane in a PI3kinase dependent manner (40), it would be interesting to investigate whether a similar mechanism exists for ClC-2 and Hsp90.

We found no evidence of ClC-2 degradation following Hsp90 inhibition, implying that association between the two proteins does not involve the well established role of Hsp90 as a
chaperone implicated in client protein maturation. The functional consequences of pharmacological inhibition of Hsp90 or heat shock treatment are rather reminiscent of the role for tyrosine phosphorylated Hsp90 as a repressor of the purinergic P2x7 receptor function (1) or as a modulator of the NKCC transporter (54) without modifications of the expression level of these two proteins.

Functional experiments demonstrated that GA and radicicol decreased current amplitude. ClC-2 activity can be modulated by phosphorylation or dephosphorylation events (22) and more directly by p34^{cyc2}/cyclinB and the protein phosphatase PP1 (23). The question thus arose whether the reduction of current amplitude involved changes in the channel phosphorylation state through a modulation of kinases and phosphatases activities by Hsp90 inhibitors. However, none of the treatments tested here (heat shock, GA or radicicol) significantly altered channel phosphorylation level (data not shown), thus excluding overlapping effects via altered channel phosphorylation.

GA treatment shifted the P_{open}/V curve to more hyperpolarizing potentials in a [Cl^-]-dependent manner, leading to a V_{0.5}/[Cl^-] relation less steep in the presence of GA as compared to that under control conditions. Both the slow and fast activation time constants were affected by GA and became less Cl^- dependent above 35 mM [Cl^-]. Heat shock treatment exerted an opposite effect, shifting the voltage dependence of gating towards less negative potentials and increasing the macroscopic rate of channel opening. It must be noted that the Cl^- dependence of the time constant for the slow component shown here is not in complete agreement with previous results which suggested independence of the slow gate to [Cl^-]; (39, 64). Another difference concerns the presence of only one (our results) versus two current activation components at low chloride concentrations (15 and 35 mM), described by others (12). We checked whether the latter difference could be linked to the experimental protocols, conventional (62) or nystatin perforated whole cell patch-clamp recordings (this
work). Recordings performed with the two methods indicated that current activation displayed a mono-exponential time course when using pipette solutions with low \([\text{Cl}^-]_i\). Since at the same \([\text{Cl}^-]_i\), we could consistently extract two gating processes from deactivation currents, it is reasonable to assume that the weight of the fast process was too weak during activation to be detected in our experiments. The reason for this discrepancy is unclear. It is possible that the mode of ClC-2 expression, transient in the previous works (12, 62) or stable in the present study, may influence basal regulation of channel gating. Since GA treatment did not affect the voltage-dependence of current activation at low chloride concentrations nor the apparent gating charge whatever the \([\text{Cl}^-]_i\), the most likely explanation for the decreased sensitivity to \([\text{Cl}^-]_i\) was a change in the apparent affinity for chloride binding. Considering that gating of the channel probably involves conformational changes of the protein (64) and that Hsp90 usually helps conformational changes of its partners, it is tempting to speculate that the chaperone favours an optimal channel conformation for ion binding and/or movement through the pore. It is evident that such an hypothesis can be raised only if a direct interaction exists between the two proteins. Despite this concern, our observations have brought to light a new regulatory element which may be important for the \([\text{Cl}^-]_i\) sensitivity of channel gating thought to provide a mechanism for coupling of Cl⁻ fluxes across apical and basolateral membranes in epithelia (8). Within this context, it is interesting to note that the recently reported interaction between Hsp90 and the co-transporter NKCC may play an important role for the \([\text{Cl}^-]_i\) dependence of the transporter (54). Modulation of ClC-2 function by Hsp90 may be of pathophysiological importance linking Hsp90 stimulators such as elevated temperature, oxidative stress or ischemia to channel activation. It is noteworthy that recovery of barrier function in ischemia-injured ileum requires chloride secretion through ClC-2 channels (37). It has also been suggested that in the heart, an increase in ClC-2 conductance could be pro-arrhythmic under some pathological conditions such as ischemia and hypoxia (17, 33). Moreover,
oxidation has been shown to potentiate the activation of CIC-2 channels by cell swelling in
*Plasmodium Falciparum* infected erythrocytes (30). Even though oxidants have been shown
to exert a rapid and possible direct effect on CIC-2 expressed in Xenopus ovocytes, it cannot
be excluded that CIC-2 activation in infected erythrocytes may additionally involve
recruitment of the host chaperones to the plasma membrane (3). Along with CFTR and
NKCC1, CIC-2 thus represents the third class of chloride transport systems modulated by
Hsp90, suggesting a key role for the chaperone in the regulation of cell volume and epithelial
secretory or absorptive functions.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Identification of proteins co-immunoprecipitated with ClC-2. *A*, ClC-2Flag was immunoprecipitated with anti-Flag antibodies from stably transfected HEK 293 cells (*lane 1*). As a control, the same procedure was performed using cells stably transfected with the WT channel (*lane 2*). The specific bands in *lane 1* and their counterparts in *lane 2* were excised and identified by MS-MS mass spectrometry. Their identities are given on the left. *B*, left panel: Western blot of ClC-2 after immunoprecipitation of Hsp90 from HEK 293 cells stably expressing ClC-2 WT (*lane 1*) or the empty plasmid (*lane 2*). Precipitated proteins were probed with the H-90 anti-ClC-2 (1:1000); right panel: Western blot of Hsp90 after immunoprecipitation of ClC-2 from the same cells with pAb137 (*lane 1*) and from control immunoprecipitates obtained with non-relevant rabbit IgGs (*lane 2*). *C*, Western blot of ClC-2 after immunoprecipitation of Hsp90 from 10 mg mouse brain homogenates (*lane 1*). Control experiments were performed using non-relevant rat IgGs (*lane 2*). Total cell extracts (40 µg) from mouse brain proteins were probed with ClC-2 antibody pAb137 (*lane 3*).

**Figure 2.** Geldanamycin and radicicol do not modify total ClC-2 expression but decrease the abundance of ClC-2 at the cell surface. *A*, HEK 293 cells stably expressing WTClC-2 were treated for 24 hours with 10µM GA or 200nM radicicol. 40 µg of whole cell extracts were probed with the H-90 antibody. Blots were also probed with anti-GFP to assess protein loading. *B*, ClC-2 staining with H-90 anti ClC-2 in HEK 293 cells stably expressing WTCIC-2, before or after a 5-hour incubation with GA. Arrows indicate cell surface ClC-2. Scale bar represents 10 µm. *C*, ClC-2 expression at the cell surface was estimated after biotinylation of membrane proteins. ClC-2 was absent from cells transfected with the empty plasmid and its expression decreased when cells were treated by GA. Blots were probed with anti-GFP to
verify that cytoplasmic proteins were absent in the membrane fractions. D, densitometry analysis of the bands at 200 and 90 kDa shows that GA treatment decreases the amount of ClC-2 at the cell surface (n=4) by 39± 9%.

**Figure 3.** Effect of Hsp90 inhibitors on ClC-2 current. A, typical slow activating ClC-2 currents recorded before and 30 minutes after addition of 10 µM GA in the bath solution. Currents were recorded with a pipette solution containing 64 mM Cl\(^{-}\) during a 5s voltage jump from a holding potential of 0 mV to -120 mV followed by a +40 mV step. B, percentages of current inhibition at various concentrations of Hsp90 inhibitors. GA and radicicol reduced ClC-2 currents by 45.3 ± 3.6 % and 40.0 ± 1.2 % at the maximal doses tested, respectively. Error bars indicate S.E. from a minimum of 7 independent experiments. C and D, current traces recorded at -120mV before and after 30 min application of GA, with pipette solutions containing 15 and 135 mM Cl\(^{-}\), respectively.

**Figure 4.** Intracellular chloride dependence of ClC-2 current. A, representative families of current traces recorded with internal solutions containing 15, 64 and 135 mM Cl\(^{-}\) in untreated cells and in cells preincubated for 3 hours with 10 µM GA. Currents were measured (as shown in the left panel) in response to voltage jumps elicited from 0 mV to -160 mV in 20 mV steps followed by a +40 mV step. The duration of the pulses at 135 mM [Cl\(^{-}\)]\(\text{i}\) was decreased at the most negative voltages to prevent changes in [Cl\(^{-}\)]\(\text{i}\) and consequently in the reversal potential of the current. For illustration purposes, the beginning of the tail currents at +40 mV was set at the same time. B, \(P_{\text{open}}/V\) curves at 15 mM and 135 mM [Cl\(^{-}\)]\(\text{i}\) in control conditions (solid circles and triangles, respectively). C, \(P_{\text{open}}/V\) curves at 15 mM and 135 mM [Cl\(^{-}\)]\(\text{i}\) after a 3-hour incubation with 10 µM GA (open circles and triangles, respectively). For B and C, solid curves were drawn according to a Bolzmann equation and show fits to all
points on the graph. The apparent $P_{\text{open}}$ as a function of voltage was calculated by measuring the current at the beginning of the pulse at $+40$ mV given after the various conditioning prepulses. GA did not affect the $P_{\text{open}}/V$ curve at 15 mM internal Cl\textsuperscript- but decreased the rightward shift observed when internal Cl\textsuperscript- was increased. The fits obtained gave mean values ± S.E. of the slope factors (k, mV) of: -21.6 ± 1.1 (at 15 mM [Cl\textsuperscript-], n=7) and -30.38 ± 1.2 (at 135mM [Cl\textsuperscript-], n=11) for control cells and -24.6 ± 1.0 (at 15 mM [Cl\textsuperscript-], n=13) and -29.98 ± 1.22 (at 135 mM [Cl\textsuperscript-], n=13) for GA treated cells. $D$, dependence of $V_{0.5}$ on internal Cl\textsuperscript-. $V_{0.5}$ values in order of increasing [Cl\textsuperscript-], for untreated or (treated cells) were: -124.9 ± 1.8 mV, (-128.2 ± 2.4 mV); -116.5 ± 2.7 mV, (-121.8 ± 3.2 mV); -104.0 ± 2.1 mV, (-116.0 ± 2.7 mV); -90.6 ± 2.1 mV, (-109.2 ± 2.9 mV); -78.1 ± 4.1 mV, (-104.8 ± 4.9 mV). Results are means ± S.E. of 7 to 14 cells for each concentration tested.

**Figure 5.** Effect of GA on the gating properties of ClC-2 at various [Cl\textsuperscript-]. $A$, time constants of the fast (filled triangles) and slow (filled circles) components of current activation measured at -120 mV as a function of [Cl\textsuperscript-] for untreated cells or GA treated cells (open triangles and circles). $B, C and D$, fractional amplitudes of the fast ($A_f$), slow ($A_s$) and time independent ($A_0$) components of current activation at -120 mV, with internal solutions containing 64, 100 and 135 mM Cl\textsuperscript-. *White columns*: untreated cells, *grey columns*: GA treated cells. $E$, time constants for the fit of current deactivation at 40 mV from a conditioning test pulse at-120 mV as a function of [Cl\textsuperscript-], (same symbols as in $A$). $F, G and H$, fractional amplitudes of the fast ($A_f$), slow ($A_s$) and time independent ($A_0$) components of current deactivation at 15, 64, and 135 mM [Cl\textsuperscript-]. *White columns*: untreated cells, *grey columns*: GA treated cells. All data are means ± S.E. of at least 7 cells. *, data significantly different (p<0.0001, Student’s $t$ test).
**Figure 6.** Effect of a thermal stress on ClC-2 current gating. *A*, Western analysis of Hsp90 in whole cell lysates as a function of recovery time at 37°C after a 1-hour heat shock treatment at 43°C. *B*, typical current traces elicited by a voltage step from 0 to -120mV and then returning to +40 mV with a pipette solution containing 35 mM Cl⁻, in control conditions and after a 1-hour heat shock at 43°C followed by a 6-hour recovery period at 37°C. Activation currents were best fit by a mono-exponential equation. *C*, time constants of current activation for untreated (*white bars*, n=10) or heat-shock treated cells (*grey bars*, n=7). *D*, time constants of current deactivation at +40 mV. Deactivation currents were best fit by a bi-exponential equation with two time constants. *White bars* represent data for untreated cells (n=13) and *grey bars* represent data for stressed cells (n=7). *, data significantly different (p<0.0001, Student’s *t* test).
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