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Leiter: Prof. Dr. Christoph Fahlke

**Funktionelle Untersuchung von Epilepsie-assoziierten Punktmutationen im
CLCN2 Gen**

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Gabriel Stölting

aus Köln

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Betreuer der Arbeit: Prof. Dr. med. Christoph Fahlke

Referentin/Referent: Prof.'in Dr. med. Ursula Seidler

Korreferent: Prof. Dr. med. Alexi Alekov

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Prüfungsausschussmitglieder: Prof. Dr. med. Evgeni Ponimaskin

Prof.'in Dr. rer. nat. Rita Gerardy-Schahn

Prof. Dr. rer. nat. Reinhard Schwinzer

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Für

Andrea, Leah und Simeon

Regulation of ClC-2 gating by intracellular ATP

Gabriel Stölting · Georgeta Teodorescu ·
Birgit Begemann · Julian Schubert · Rima Nabbout ·
Mohammad Reza Toliat · Thomas Sander ·
Peter Nürnberg · Holger Lerche · Christoph Fahlke

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Abstract ClC-2 is a voltage-dependent chloride channel that activates slowly at voltages negative to the chloride reversal potential. Adenosine triphosphate (ATP) and other nucleotides have been shown to bind to carboxy-terminal cystathionine- β -synthase (CBS) domains of ClC-2, but the functional consequences of binding are not sufficiently understood. We here studied the effect of nucleotides on channel gating using single-channel and whole-cell patch clamp recordings on transfected mammalian cells. ATP slowed down macroscopic activation and deactivation time courses in a dose-dependent manner. Removal of the complete carboxy-terminus abolishes the effect of ATP, suggesting that CBS domains are necessary for ATP regulation of ClC-2 gating. Single-channel recordings identified long-lasting closed states of ATP-bound channels as basis of this gating deceleration. ClC-2 channel dimers exhibit two

largely independent protopores that are opened and closed individually as well as by a common gating process. A seven-state model of common gating with altered voltage dependencies of opening and closing transitions for ATP-bound states correctly describes the effects of ATP on macroscopic and microscopic ClC-2 currents. To test for a potential pathophysiological impact of ClC-2 regulation by ATP, we studied ClC-2 channels carrying naturally occurring sequence variants found in patients with idiopathic generalized epilepsy, G715E, R577Q, and R653T. All naturally occurring sequence variants accelerate common gating in the presence but not in the absence of ATP. We propose that ClC-2 uses ATP as a co-factor to slow down common gating for sufficient electrical stability of neurons under physiological conditions.

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G. Stölting (✉) · C. Fahlke (✉)
Institute of Complex Systems-Zelluläre Biophysik (ICS-4),
Forschungszentrum Jülich, 52425 Jülich, Germany
e-mail: g.stoelting@fz-juelich.de
e-mail: c.fahlke@fz-juelich.de

B. Begemann
Institut für Neurophysiologie, Medizinische Hochschule
Hannover, 30625 Hannover, Germany

G. Teodorescu · J. Schubert · H. Lerche
Abteilung Neurologie mit Schwerpunkt Epileptologie, Hertie-
Institut für Klinische Hirnforschung, Universität Tübingen, 72076
Tübingen, Germany

R. Nabbout
Department of Pediatric Neurology, Centre de Reference des
Epilepsies Rares, Inserm U663, Hopital Necker Enfants Malades,
APHP, Paris Descartes University, 75743 Paris Cedex 15,
France

M. R. Toliat · T. Sander · P. Nürnberg
Cologne Center for Genomics, Universität zu Köln, 50931 Köln,
Germany

P. Nürnberg
Center for Molecular Medicine Cologne (CMMC),
University of Cologne, 50931 Köln, Germany

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Introduction

CIC-2 is a member of the CLC family of anion channels and transporters broadly expressed in neuronal and non-neuronal tissues [37]. It is an anion channel with two separate conductance pathways, the so-called protopores [12, 26]. This double-barreled architecture is the basis of two different gating mechanisms, one acting on individual protopores and another common process jointly acting on both protopores. Protopore as well as common gating is stimulated by membrane hyperpolarization resulting in CIC-2 being closed at positive potentials and slowly activating upon hyperpolarizing voltage steps [10, 46].

Each CIC-2 monomer contains 18 α -helices and a long carboxy-terminus containing two cystathionine- β -synthase (CBS) domains [12, 14, 32]. Adenosine triphosphate (ATP) was shown to bind to CBS-domains of various CIC channels and transporters [23, 25, 36] and to modify their functional properties. The effects of ATP on CIC-1 are pH-dependent, resulting in CIC-1 inhibition by intracellular ATP at acidic pH [2, 3, 39, 40]. CIC-4 undergoes rapid downregulation in cells dialyzed with ATP-free solution [1, 41], indicating that CIC-4 requires intracellular ATP to be active. Experiments with CIC-5 demonstrated that AMP, ADP, and ATP increase the probability of CIC-5 to be in an active state [45]. For CIC-2, ATP depletion was reported to change sub-cellular trafficking [11] and to modify gating [19]. Furthermore, several sequence variants were identified in association with idiopathic generalized epilepsies (IGE) [21, 22, 33] that were localized within the putative ATP binding region at the carboxy-terminus of CIC-2.

We hypothesized that naturally occurring *CLCN2* mutations might modify ATP-dependent gating processes and decided to perform a detailed investigation of CIC-2 gating at various internal ATP concentrations. We observed significant effects on gating kinetics but only slight changes in steady-state open probabilities. The effects of ATP on gating can be described by a kinetic model in which protopore gating is unaffected by ATP while common gating transitions are distinct for ATP-bound channels. In addition, we investigated three sequence variants associated with IGE and found that these mutations alter channel function in response to ATP.

Methods

Heterologous expression of WT and mutant hCIC-2

IGE-associated sequence variants R577Q, R653T, and G715E were introduced into the plasmid pcDNA5/FRT/TO hCIC-2

[16] using QuikChange (Agilent Technologies). Polymorphisms R688Q and E718D were inserted into the plasmid pSVL hCIC-2 [16]. All mutations were verified by DNA sequencing, and two independent mutant clones were tested by transient expression in HEK293T cells or by stable expression in Flp-In T-Rex 293 cells. Stable inducible cell lines expressing wild-type (WT) or mutant CIC-2 channels were generated by selection of Flp-In T-Rex 293 cells (Invitrogen) transfected with pcDNA5/FRT/TO-hCIC-2 [16] and used either with or without induction with tetracycline.

Electrophysiology and data analysis

Standard whole-cell or single-channel patch clamp recordings were performed using an EPC10 amplifier (HEKA, Lambrecht/Pfalz, Germany) as described [18]. Pipettes were pulled from borosilicate glass and had resistances between 1.3 and 2.5 M Ω for whole-cell recordings and 7–50 M Ω for single-channel recordings. For noise analyses and single-channel experiments, pipettes were covered with SigmaCote (Sigma-Aldrich Chemie, Munich, Germany) for capacitance reduction. More than 85 % of the series resistance was compensated by an analog procedure, resulting in calculated voltage errors <5 mV. Cells/patches were clamped to 0 mV for at least 45 s between test sweeps. The extracellular solution for whole-cell recordings contained (in millimolars): NaCl or NMDG-Cl (140), KCl (4, not in NMDG containing solutions), CaCl₂ (2), MgCl₂ (1), HEPES (5), pH 7.4; the intracellular solution contained (in millimolars): NaCl or NMDG-Cl (95), MgCl₂ (5), EGTA (5), HEPES (10), pH 7.4. ATP, ADP, AMP, or AMP-PNP was added to the final solutions at given concentrations followed by pH readjustment. Solutions containing ATP were stored at –20 °C for a maximum of 24 h or at –80 °C for a maximum of 3 days before use. To prevent osmotic gradients, we included mannitol in the extracellular solution at internal nucleotide concentrations higher than 1 mM. Liquid potentials were calculated and subtracted a priori. To permit equilibration between the patch pipette and the cytoplasm, we normally recorded currents starting 5 min after breaking into the whole-cell mode.

Inside-out patch recordings were made in symmetrical solutions containing (in millimolars): NMDG-Cl (130), MgCl₂ (5), EGTA (5), HEPES (5), pH 7.4. Only few patches contained channels with two equally spaced conductance levels and common closures and conductances around 2 pS. Channels that did not show these characteristics were discarded. We usually observed background noise levels around 55–65 fA RMS in single-channel experiments before applying software filtering. Single-channel recordings were sampled at 50 kHz and filtered with a 1-kHz Bessel filter. Traces were digitally filtered at 200 Hz prior to dwell time analysis.

Data were analyzed by PatchMaster (HEKA, Lambrecht/Pfalz, Germany), QuB (SUNY; Buffalo, NY; USA) and Origin 8 (OriginLab; Northampton, MA, USA). To obtain the voltage dependence of the relative open probability, instantaneous current amplitudes were determined 200 μ s after a voltage step to 60 mV following prepulses to various voltages, normalized by their maximum value and plotted against the preceding potential. Prepulse durations were adjusted to allow steady-state activation. For the voltage dependences of absolute open probabilities (P_{abs}), relative open probabilities at different voltages were normalized to the absolute open probability at -160 mV obtained by noise analysis and single-channel analysis at -100 mV. Noise analysis was performed as recently described [16]. Dead time (T_d) for dwell time analysis was initially calculated according to [8] ($T_d=0.179/f_c$ with f_c being the cut-off frequency of 200 Hz) to be 0.9 ms and later increased to 1 ms after careful inspection of recordings. Single-channel amplitudes were determined from amplitude histograms and averaged over several recordings. Dwell times were measured by accumulating all recordings while omitting first and last events.

Unpaired two-sided student's *t* test was used for statistical analysis unless stated otherwise. Statistical significance is either denoted in figures with a single asterisk for $p < 0.05$ and double asterisk for $p < 0.01$ or explicitly stated in the text or corresponding tables.

Computational modeling of CIC-2 gating

Modeling was based on Q-Matrix theories by Colquhoun and Hawkes ([7]) and implemented using custom-written Python scripts incorporating Q-Matrix implementations (C. Schmidt-Hieber; version 2010-08-23; <http://code.google.com/p/pyqmatrix>), a directed evolution Algorithm (P. Sederberg; <https://github.com/compmem/desolver>) running on ParallelPython (Vitalii Vanovschi; <http://www.parallelpython.com>).

Rate constants (k) were constructed from Eq. 1, with k_1 or k_{-1} being the voltage-independent component, $z\delta$ the apparent gating charge, v the voltage, F the Faraday constant, R the gas constant, and T the assumed constant temperature of 296 k.

$$k = k_1 e^{z\delta v F / RT}$$

Starting values of rate constants were manually adjusted and compared with experimentally obtained data by eye. Detailed values composing rate constants were obtained using directed evolution on a least-square deviation from 100–250 logarithmically spaced data points from normalized recorded currents. Time-dependent open probabilities for fast and slow gating were calculated from the rate

constant Q-Matrix as described [7]. Finally, open probabilities were calculated as product of probabilities of the fast gate being open and the sum of all open slow gate probabilities (Fig. 4b). Rate constants from the Q-Matrix were used to simulate single-channel behavior of the common gating process in QuB Express (SUNY; Buffalo, NY, USA) to check for the occurrence of a long-lasting closed state.

Fitting was repeated until the least-square deviation did not change by more than 0.1 % over more than 50 cycles. Rate constants obtained by this algorithm were then used to plot the ATP concentrations and the voltage dependences of time constants (Fig. 4c and d). Data sets that differed greatly from experimentally obtained values were discarded. Finally, a ranking of multiple runs of the fitting algorithm by the least-squares criterion was used to select the data presented in Table 3.

Patients and gene screening

We analyzed a cohort of 95 multiplex families, each including at least (1) one proband with a defined IGE phenotype, (2) one additional individual with IGE, and (3) a third one with generalized epileptic discharges on electroencephalography. Families and normal controls were recruited through the EPICURE consortium (<http://www.epicureproject.eu/home.aspx>) (Supplementary Text 2; Supplementary Table 1). All analyzed family members gave written informed consent, and all procedures were in accordance with the declaration of Helsinki and approved by the local Ethical Committees.

The entire coding region of the *CLCN2* gene was analyzed by direct Sanger sequencing. Polymerase chain reaction (PCR) products were purified by Exo/SAP digestion with Exonuclease I (New England Biolabs, Beverly, MA) and shrimp alkaline phosphatase (Promega, San Diego, USA) and directly sequenced using the ABI PRISM BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), and the ABI 3730 sequencing instrument, as described by the manufacturer. All primer sequences are given in Supplementary Material (Supplementary Tables 2 and 3). Ninety-five Caucasian controls were sequenced using the PSQ HS96A pyrosequencing system (Qiagen, Hilden, Germany), according to manufacturers' instructions using the PSQ 96 SNP Reagent Kit (Biotage AB) on a PSQ HS96A instrument (Qiagen, Hilden, Germany) and analyzed by pyrosequencing software (PSQ HS96A1.2). Segregation analysis was done by Sanger sequencing. Further genotyping was performed in a control cohort consisting of 751 individuals of European descent (94 Spanish, 94 Finnish, 94 Turkish, 94 Dutch, 188 Italian, and 188 German) using the ABI TaqMan® 7900 Real-Time PCR

System. TaqMan probes and primers were obtained from the custom TaqMan SNP genotyping products provided by Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Data were analyzed with the TaqMan[®] Genotyper Software 1.0.1 and SDS 2.1 provided by ABI.

Results

ATP slows CIC-2 channel kinetics

Figure 1 shows representative whole-cell patch clamp recordings from Flp-In T-Rex cells expressing WT human CIC-2 channels. Cells were bathed in physiological external solutions and dialyzed internally with pipette solutions containing either 0 (Fig. 1a) or 5 mM ATP (Fig. 1b). For both ATP concentrations, WT channels were closed at positive potentials and activated on a bi-exponential time course upon hyperpolarizing voltages. Consecutive membrane depolarization resulted in a slow bi-exponential channel deactivation. Internal dialysis with 5 mM ATP resulted in CIC-2 currents with slower activation and deactivation time courses than under ATP-free conditions.

CIC currents usually activate and deactivate on bi-exponential time courses [10, 13, 15, 18, 30, 31], and two time constants are also sufficient to describe macroscopic gating of CIC-2 [10, 42]. To quantify ATP-dependent alterations of CIC-2 gating, we determined fast and slow activation/deactivation time constants from cells dialyzed with ATP-free or ATP-containing solutions. Fast time constants for activation were determined by using a separate short (<250 ms) protocol using P/4 leak subtraction to remove linear capacitive artifacts (Supplemental Fig. 1a). Afterward, bi-exponential functions were fitted to long recordings such as in Fig. 1a with the fast time constant fixed to this value. Relative contributions of fast and slow time constants to the observed currents were not different at low or high concentrations of ATP and similar to values previously reported for mouse CIC-2 (Supplemental Fig. 1c) [10].

Whereas fast activation time constants were identical for both conditions (Supplemental Fig 1b), we observed a significant increase of slow activation time constants in the presence of ATP (Fig. 1c, Table 1). Deactivation time constants showed a twofold increase for both—fast and slow—time constants with intracellular ATP (Fig. 1c, Table 1).

ATP does not alter maximal open probability of CIC-2

To test for a possible ATP dependence of steady-state open probabilities, we determined relative open probabilities by plotting the normalized instantaneous current amplitudes at

a fixed tail pulse of +60 mV versus the preceding voltage. The voltage dependence of CIC-2 open probabilities could be well described by a single Boltzmann distribution with half-maximal activation ($V_{1/2}$) at -81.1 ± 2.2 mV ($n=7$; Fig. 2a, grey squares) in the absence of ATP. Increasing the concentration of cytosolic ATP to 5 mM caused a shift of the $V_{1/2}$ to -89.8 ± 3.2 mV ($n=5$; $p=0.03$; Fig. 2a, black squares). Absolute open probabilities were determined at -160 mV by noise analysis yielding only insignificant differences in the maximum absolute open probability between ATP-free conditions and 5 mM ATP (0 mM ATP, 0.81 ± 0.03 ; 5 mM ATP, 0.75 ± 0.07 ; $p=0.35$). The voltage dependence of absolute open probabilities was then calculated by normalizing relative open probabilities to these values (Fig. 2a).

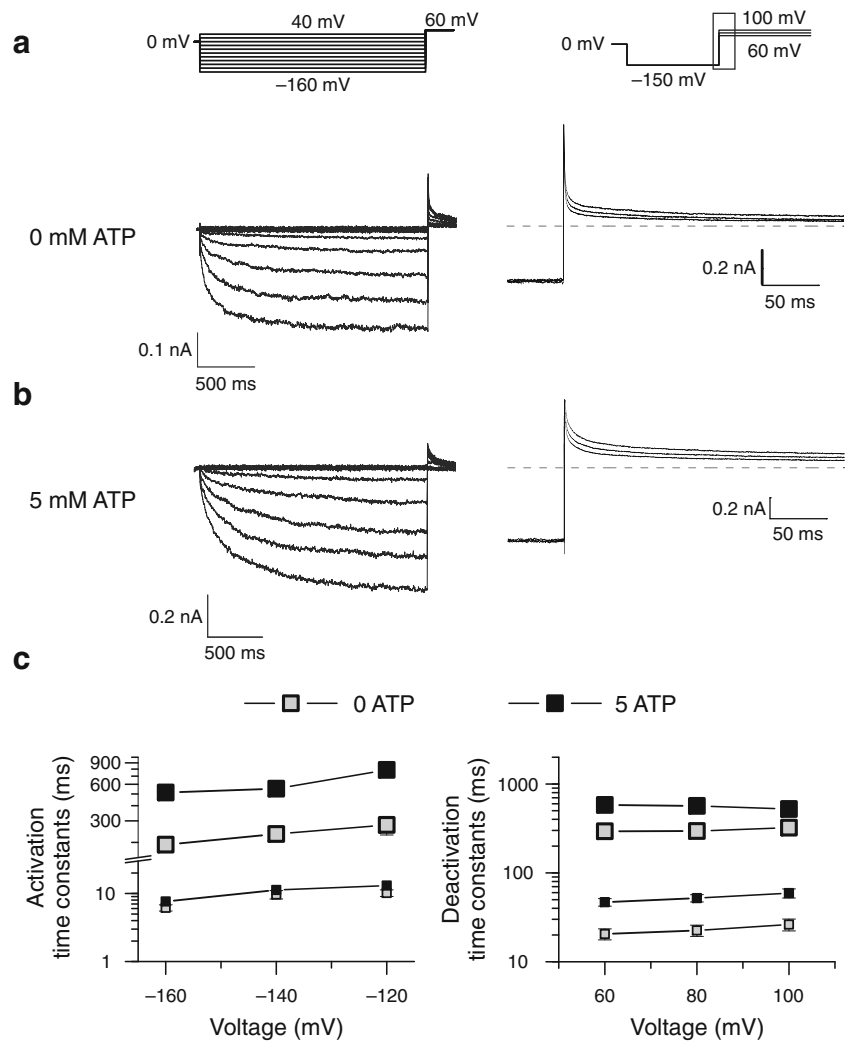
Figure 2b illustrates the consequences of ATP-dependent gating alterations on the time dependence of CIC-2 currents. We compared activation and deactivation time courses upon a voltage step to -160 mV followed by a depolarization to $+100$ mV for two representative cells dialyzed with ATP-containing or ATP-free solutions. To correct for differences in expression levels of the two cells, currents were normalized to the steady-state absolute open probability at -160 mV (Fig. 2a). This comparison demonstrates that deceleration of slow activation by ATP together with almost unaltered steady-state open probabilities results in about 20 % smaller CIC-2 currents at the begin of the hyperpolarizing voltage step (Fig. 2c). The opposite effect was observed during deactivation, where ATP increased currents by about 30 % very soon after the voltage step (Fig. 2c). These robust changes in time-dependent open probabilities during activation or deactivation gradually disappeared after several hundreds of milliseconds and finally reached similar values for both ATP concentrations at steady-state conditions.

ATP modifies CIC-2 gating in a dose-dependent manner

Figure 3 shows the ATP concentration dependence of fast and slow activation and deactivation time constants. Whereas fast activation constants were independent of the ATP concentration, fast deactivation as well as slow activation and deactivation time constants increased in a concentration-dependent manner. Concentration dependences could be well fit with Michaelis–Menten relationships (lines in Fig 3a and b), providing apparent Michaelis–Menten constants (K_M) in the low millimolar range (Table 2). K_M values are only slightly voltage-dependent but differed for activation and deactivation.

Under metabolic stress, ATP is degraded to ADP so that the ADP concentration rises quickly at the expense of ATP maintaining a stable [ATP]/[ADP] ratio. ATP regulation will thus only modify CIC-2 function under metabolic stress if

Fig. 1 ATP slows CIC-2 activation and deactivation at physiological concentrations. **a**, **b** Representative whole-cell current responses of cells expressing WT CIC-2 to the indicated voltage steps at 0 or 5 mM intracellular ATP. Cells were held at 0 mV, and voltage steps from -160 to 40 mV were applied to study activation kinetics (*left side*). Steps from 60 to 100 mV following a preconditioning pulse to -150 mV were used to analyze channel deactivation (*right side*). For improved illustration, only a section of the deactivation current responses are shown (depicted as *black box* in voltage protocol). Dashed lines indicate 0 nA. **c** Voltage dependence of fast and slow activation (*left side*) or deactivation (*right side*) time constants at zero (*grey squares*) or physiological ATP (*black squares*). Slow time constants are given by *large symbols* and connected for easier identification



the binding site can separate between ATP and its metabolites. We additionally tested the ADP dependence (Supplemental Fig. 2) and observed similar effects on gating

and comparable apparent Michaelis–Menten constants for ADP and ATP. Obviously, CIC-2 is not capable of distinguishing between these two compounds.

Table 1 Fast and slow time constants of wild type CIC-2 at various voltages

| Voltage (mV) | | 0 mM ATP | | 5 mM ATP | | Pvalue |
|--------------|---------------|----------|--------------|----------|--------------|---------|
| | | n | (ms) | n | (ms) | |
| 100 | τ_{fast} | 5 | 26.23±3.96 | 6 | 65.97±8.89 | 0.027 |
| | τ_{slow} | | 322.58±28.42 | | 597.88±75.68 | 0.044 |
| 80 | τ_{fast} | 6 | 22.53±3.25 | 11 | 55.58±5.69 | 0.004 |
| | τ_{slow} | | 296.00±34.52 | | 599.15±46.08 | 0.009 |
| 60 | τ_{fast} | 6 | 20.55±2.93 | 10 | 49.40±4.92 | 0.007 |
| | τ_{slow} | | 294.23±35.09 | | 620.08±44.90 | 0.006 |
| -120 | τ_{fast} | 5 | 13.09±1.62 | 5 | 10.18±1.14 | 0.178 |
| | τ_{slow} | | 278.26±48.14 | | 786.50±17.35 | < 0.001 |
| -140 | τ_{fast} | 5 | 11.26±1.21 | 5 | 9.67±1.37 | 0.409 |
| | τ_{slow} | | 233.98±7.25 | | 552.14±36.38 | 0.001 |
| -160 | τ_{fast} | 5 | 7.62±0.74 | 5 | 6.16±0.65 | 0.178 |
| | τ_{slow} | | 191.32±8.64 | | 513.38±34.72 | 0.002 |

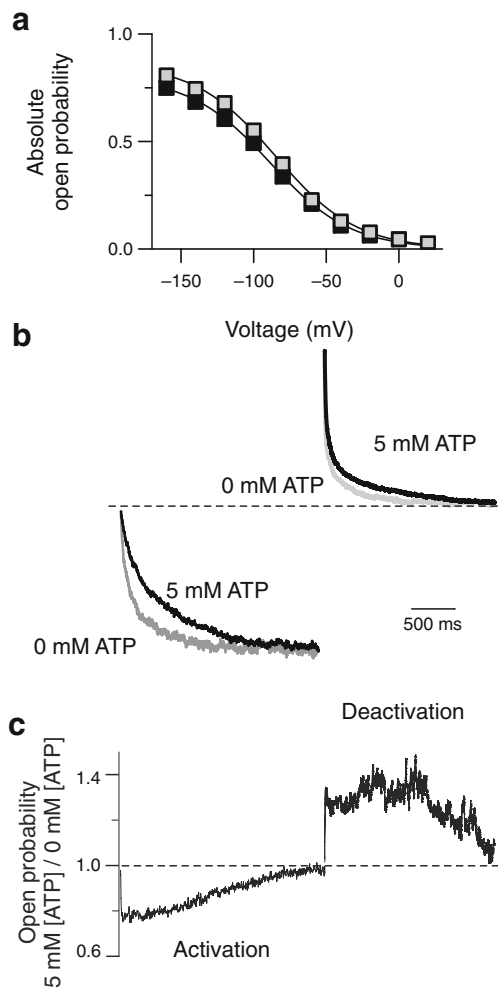


Fig. 2 ATP modifies the time-dependent of CIC-2 open probabilities. **a** Voltage dependence of absolute open probabilities at 0 mM (grey squares) or 5 mM ATP (black squares) **b**, Superimposed current traces at -160 or at $+100$ mV to illustrate ATP-dependent changes in activation and deactivation. Recordings at 5 mM ATP are given as black lines whereas grey lines indicate ATP-free conditions. Mean current amplitudes for the last 50 ms of each voltage step were normalized to the steady-state absolute open probability at -160 or $+100$ mV. **c**, Ratio of current amplitudes at 5 versus 0 mM of internal ATP from the recordings shown in **b** to quantify the differences in open probabilities during hyper- and depolarizing voltage steps

We did not find any evidence for a deceleration of gating kinetics in the presence of 5 mM AMP (Fig. 3c and d). This might be due to greatly impaired binding of AMP as compared with ATP or to an absent effect of AMP binding on CIC-2 gating. We reasoned that AMP—if it can bind with high affinity to the ATP binding site without modifying gating—would shift the concentration dependence of the ATP effects on gating—resembling a competitive blocker. We therefore performed experiments with 1 mM of ATP and 5 mM AMP combined in the intracellular solution. Under these conditions, fast and slow time constants were similar to 1 mM ATP alone (at -140 mV— $\tau_{\text{slow}}=552.0\pm 87.0$ ms,

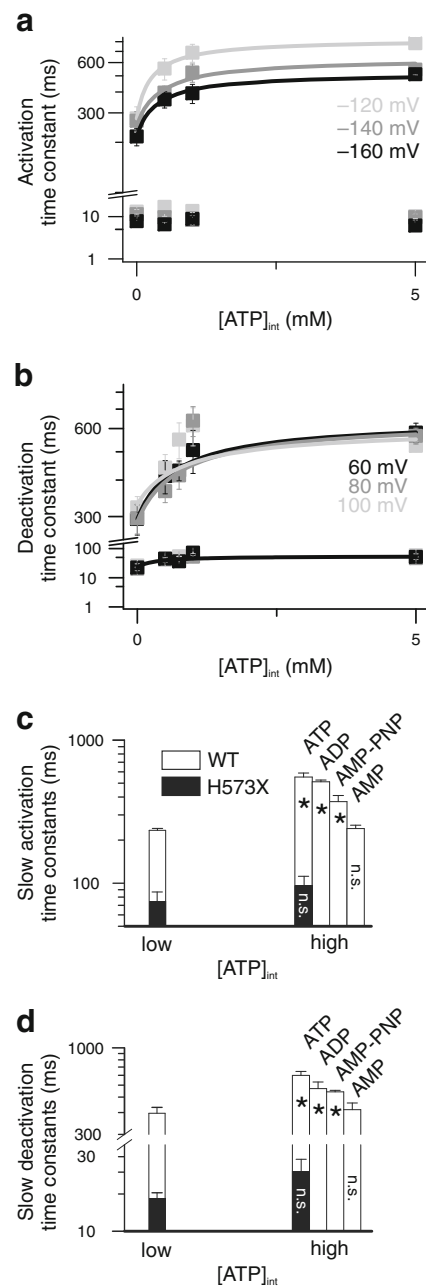


Fig. 3 ATP causes concentration-dependent changes in activation and deactivation time constants. **a**, **b** ATP concentration dependences of fast and slow activation (**a**) or deactivation (**b**) time constants for WT CIC-2 at indicated voltages. Solid lines provide fits with a Michaelis–Menten equation. **c**, **d** Comparison of activation time constants at -140 mV (**c**) or deactivation time constants at 80 mV (**d**). Cells expressing WT CIC-2 (open bars) were studied without (low) or with 5 mM (high) of the indicated nucleotide inside the patch pipette. Recordings from the H573X mutant were done without (low) or with 10 mM (high) of intracellular ATP. Values for ATP, ADP, and AMP-PNP were significantly slower at high concentrations (asterisk denotes $p<0.05$, $n=3-6$) while AMP and the H573X mutant did not show concentration dependence (n.s.)

$n=6$, $p=0.99$; at $+80$ mV— $\tau_{\text{slow}}=510.4\pm 33.0$ ms, $p=0.89$; $\tau_{\text{fast}}=51.6\pm 8.4$ ms, $n=3$, $p=0.64$). We conclude that AMP

Table 2 Michaelis–Menten constants (K_m) for ATP on CIC-2

| Voltage [mV] | Fast [mM] | | Slow [mM] | |
|--------------|-----------|-------|-----------|-------|
| | Wild-type | G715E | Wild-type | G715E |
| 100 | 0.456 | – | 0.87 | 0.71 |
| 80 | | | 2.04 | |
| 60 | | | 1.74 | |
| –120 | – | – | 0.44 | 0.37 |
| –140 | – | – | 0.67 | 0.57 |
| –160 | – | – | 0.55 | 0.44 |

binds only with low affinity and does not act as competitive inhibitor of the ATP effect.

ATP might exert its effect on gating via direct association to CIC-2, by stimulating direct phosphorylation of CIC-2 or by interaction with—yet unknown—phosphorylation-dependent proteins. To test whether nucleotide binding alone is necessary to change gating kinetics of CIC-2, we used the non-hydrolyzable ATP analog adenosine 5'-(β,γ -imido)triphosphate (AMP-PNP) that has been used to study ATP-dependent gating of CIC-4 [41]. Cells containing 5 mM of AMP-PNP indeed showed a significant deceleration compared with cells without nucleotides (Fig. 3c and d). The degree of deceleration was found to be slightly smaller than for ATP ($p < 0.05$ for slow activation as well as fast and slow deactivation; $n = 3–7$). This small difference to ATP suggests that the conformational change of the CIC-2 carboxy-termini depends on the molecular identity of the nucleotide used. However, we cannot exclude additional minor effects of ATP hydrolysis on CIC-2 gating.

ATP has been shown to bind to isolated CIC-2 carboxy-termini via CBS domains [36]. To test whether the observed effects on CIC-2 gating are mediated by association of nucleotides with the CBS domains, we tested the effects of ATP on gating of H573X CIC-2 [16]. H573X CIC-2 lacks the complete carboxy-terminus, including both CBS-domains, and exhibits faster activation and deactivation kinetics [16]. We observed activation and deactivation time constants that were independent on ATP concentrations even at concentrations as high as 1 mM (Fig. 3c and d), indicating binding of nucleotides to carboxy-terminal CBS domains as the basis of kinetic changes.

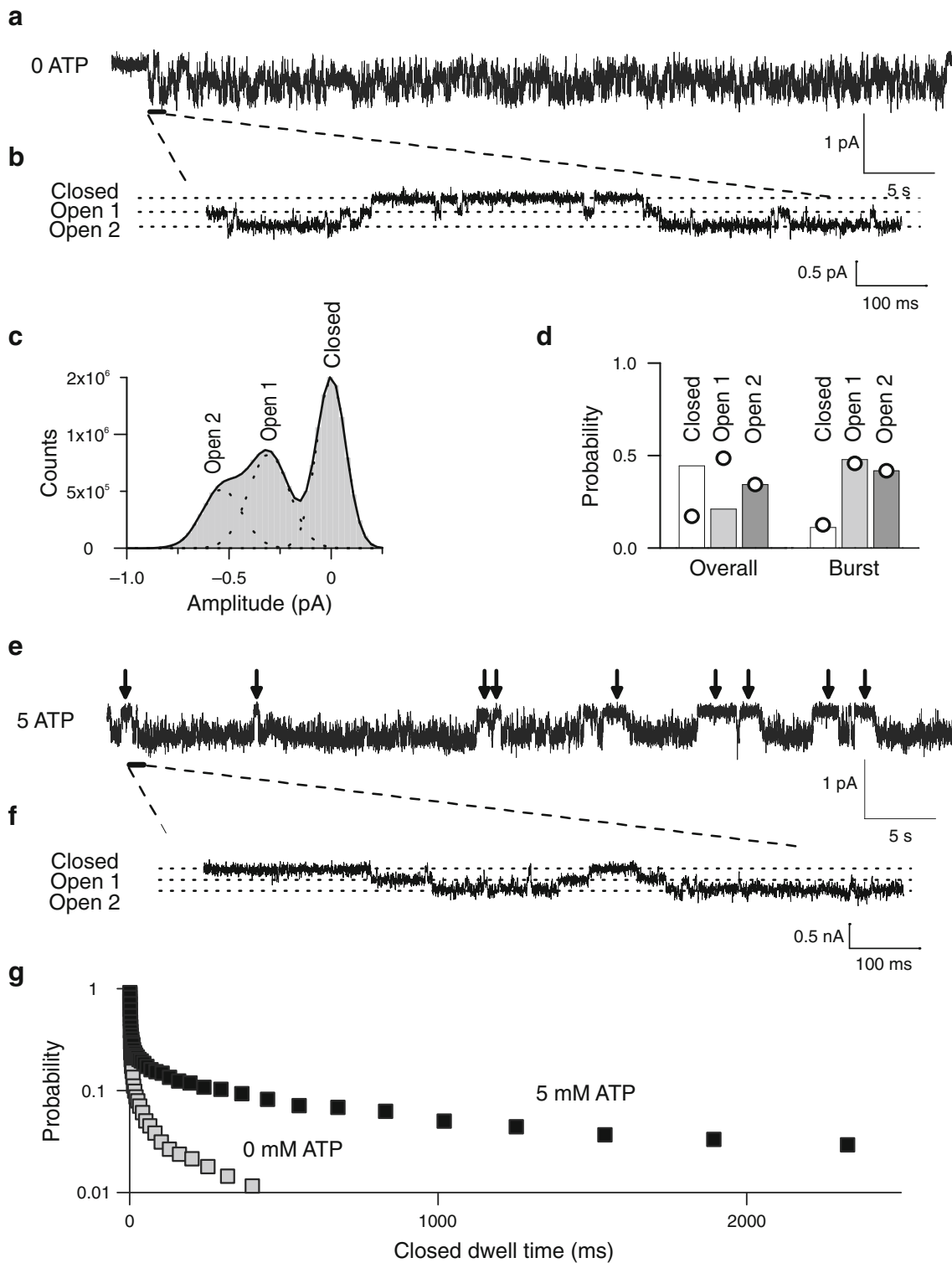
Single-channel analysis of CIC-2 reveals long-lasting closed states in the presence of ATP

To test whether ATP modifies individual or common gating processes, we performed single-channel recordings with and without ATP at the cytoplasmic membrane side. Figure 4a shows a representative recording from an inside-out patch

containing a single CIC-2 channel subjected to 0 mM ATP at the cytoplasmic side at -100 mV. CIC-2-mediated single channel currents showed two equally spaced conductance states (Fig. 4b, c; unitary current amplitude— 0.23 ± 0.02 pA; $n = 6$). Bursts of activity with transitions between closed and both conductance states were interrupted by longer closed states (Fig. 4b), similar to previously published single channel recordings of CIC-2 [38] or of other CLC channels [15, 26, 35]. This characteristic gating arises from the dimeric double-barreled architecture of CLC channels [12, 26] that permits individual opening and closing transitions of the protopores, as well as common gating of both protopores together.

Figure 4c depicts a representative amplitude histogram from which the probability to assume the closed or one of the two conducting states (open 1: one open and one closed protopore, open 2: both protopores open) could be calculated [26, 38]. Amplitude histograms (Fig. 4c) obtained only during bursts of activity were binomially distributed (open circles, Fig. 4d, right). Such a behavior was expected if the two conduction pathways open and close independently of each other. When amplitude histograms were constructed from recording intervals that also included long closed states, open probabilities deviated from a binomial distribution (Fig. 4d, left) as expected if slow gating acts on both protopores together. These findings suggests slow common gating processes that jointly open and close both protopores as well as fast protopore gating that occurs independently of the adjacent protopore.

Five millimolar ATP neither modified single-channel amplitudes nor binomial bursting behavior (Fig. 4e and f). However, under these conditions, long closed states could be frequently observed (arrows in Fig. 4e). To quantify the kinetics of single-channel gating, we collected large numbers of transitions between closed and open states from multiple patches. Time intervals spent in one state were then plotted as dwell time histograms for the closed as well as for the two open states (Supplementary Fig. 3). This analysis could only be performed at -100 mV since less negative voltages resulted in unitary currents too small for a reliable analysis whereas more negative potentials did not allow sufficiently long recordings. Plotting the probability of finding a closed event longer than a certain value at either 0 or 5 mM cytosolic ATP (Fig. 4f) showed a significant increase of long-lasting closed states with 16.2 ± 7.4 % ($n = 5$) of closed events being longer than 100 ms in the presence of ATP versus only 4.4 ± 5.0 % ($n = 3$; $p = 0.05$) for ATP-free conditions. The closed dwell time distribution at high ATP could be described by at least one additional exponential function with a time constant of more than 1.6 s (Supplemental Fig. 3, Supplemental Table 4). Longer closed times without significant changes in open dwell times (Supplemental Fig. 3) predict lower open probabilities at



this potential (-100 mV)—in good agreement with our experimental results. The slight shift of the activation curves (Fig. 2a) and the insignificant reduction of the maximum absolute open probability indeed resulted in a slight reduction of the absolute open probability at -100 mV by ATP. We observed similar absolute open probabilities by single

channel analysis (0 mM ATP—0.59; 5 mM ATP—0.43) and by noise analysis (Fig. 2a; 0 mM ATP— 0.56 ± 0.02 , $n=7$; 5 mM ATP— 0.49 ± 0.01 , $n=5$; $p=0.02$).

ATP-dependent time constants determined in macroscopic and microscopic current recordings are very different. This is not surprising since changes in single-channel dwell times

Fig. 4 ATP modifies common gating of CIC-2. **a** Representative single-channel recording of WT CIC-2 at -100 mV and an internal ATP concentration of 0 mM. **b** Segment of **a** showing three separate conductance levels (closed, open 1, and open 2). **c** Histogram plot from the WT CIC-2 single-channel recordings shown in **a**. *Solid line* shows fit with a sum (*black line*) of three Gaussian (*dotted lines*) functions corresponding to the three separate conductance levels at approximately 0 , 0.24 , and 0.48 pA. **d** Probabilities of the three conductance states during bursts or from complete recordings. Values expected from binomial distributions were calculated assuming that the probability of the conductance state open 2 is the square of the protopore open probability and are shown as *superimposed circles*. **e** Representative recordings of WT CIC-2 channels at -100 mV and an internal ATP concentration of 5 mM. *Arrows* indicate long closed durations absent from the recording in **a**. **f** Segment of **e** showing three separate conductance levels (closed, open 1, and open 2). **g** Cumulative closed time distribution for WT CIC-2 at 0 mM (*grey squares*) or at 5 mM (*black squares*) of cytosolic ATP. For each abscissa value, the probability of encountering longer closed dwell times were taken from binned dwell time distributions

result only in comparable changes of whole-cell current time constants for very simple microscopic schemes. Time constants from single-channel dwell time distributions directly relate to one rate constant leaving one particular state, whereas macroscopic relaxations result from gating transition of multiple channels that will—individually—shift through various states and thus depends on the majority of rate constants within a kinetic scheme of an ion channel [7].

ATP association changes the voltage dependence of common gating

To develop a kinetic scheme of voltage- and ATP-dependent gating of CIC-2, we assumed that the two structurally distinct gating processes, protopore and common gating, occur independently of each other. This assumption has been successfully used to describe gating mechanisms of various CIC channels, including CIC-2 [10, 16, 46]. We developed independent schemes for fast and slow gating and calculated the apparent open probability as the product of fast and slow gate open probabilities.

Since the fast gate is fully closed at positive potentials [16], we described fast gating by a two-state model with one voltage-dependent transition (α). The common gate of CIC-2 does not close completely at positive potentials [16]. We therefore inserted another open state (O_{min} ; Fig. 5a) with voltage-independent opening and closing rate constants to account for this feature. The balance between closed and the voltage-independent open state (transitions α) determines the minimum open probability of the slow gate at very positive potentials while transitions (transitions β) to another open state (O_v) account for voltage-dependent activation and deactivation. A linear model separating the two open states by the closed states ($O-C-O$) was necessary to correctly predict the effects of ATP on current kinetics. A

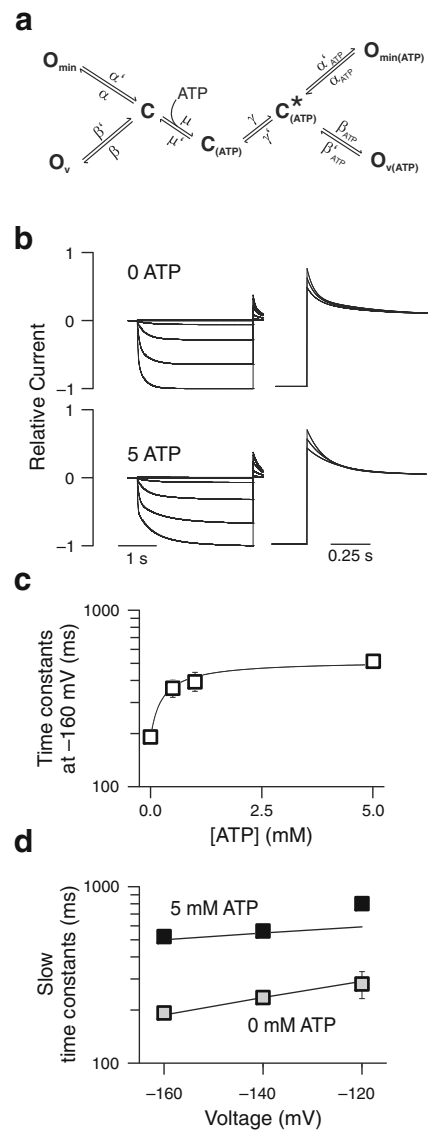


Fig. 5 A seven-state kinetic scheme describes the voltage and ATP dependence of CIC-2 common gating. **a** Seven-state diagram representing slow gating of CIC-2. In the absence of ATP (*left half*), the slow gate cycle consists only of one closed (C) and two open (O_{min} , O_v) states with voltage-dependent opening transitions to O_v and voltage-independent transitions to O_{min} . ATP binding causes transition into a cycle with altered rate constants that result in slower gating kinetics. **b** Predicted current responses to the voltage steps seen in Fig. 1. **c** Predicted ATP dependence for wild type channels (*solid line*) follow measured values (*open squares*). **d** Predicted voltage dependence of time constants of WT (*continuous lines*, either at 0 or 5 mM ATP internally) correspond to measured values (*grey and black squares*)

model with direct connection of the two open states ($C-O-O$) resulted in pronounced ATP-dependent changes of the minimal open probability of the common gate, in clear contrast to the experimental results.

Since activation and deactivation time constants exhibited distinct ATP dependencies (Fig. 1c), addition of a single ATP-bound closed state corresponding to the long-lasting closed

Table 3 Rate constant components of whole-cell current models

| Fast gate | Forward | | Backward (') | |
|---------------------------------|---------------------------|-----------|-----------------------------|-----------|
| | k_1 (s ⁻¹) | $z\delta$ | k_{-1} (s ⁻¹) | $z\delta$ |
| α | 0.3 | -0.9 | 1.9 | 0.1 |
| Slow gate | Forward | | Backward (') | |
| | k_1 (s ⁻¹) | $z\delta$ | k_{-1} (s ⁻¹) | $z\delta$ |
| α | 7 | - | 11 | - |
| α_{ATP} | 0.3 | - | 0.6 | - |
| β | 0.46 | -0.5 | 3 | 0.37 |
| Wild-type: β_{ATP} | 0.34 | -0.35 | 1.94 | 0.35 |
| G715E: β_{ATP} | | -0.44 | | 0.58 |
| R577Q: β_{ATP} | | -0.36 | | 0.54 |
| R653T: β_{ATP} | | -0.38 | | 0.63 |
| μ | 165,000 mol ⁻¹ | - | 13.7 | - |
| Wild-type: γ | 2.5 | - | 0.5 | - |
| G715E: γ | 4.2 | | | |
| R577Q: γ | 3.3 | | | |
| R653T: γ | 3.5 | | | |

state was not sufficient to describe ATP-dependent gating of CIC-2. We thus assumed that all steps could occur in ATP-bound and -unbound conditions leading to a duplication of all states. From our experimental data, it was impossible to deduce in which state ATP binding occurs. However, CIC-0 carboxy-termini have been shown to come closer during opening and to move away from each other during closing of the common gate [5]. Moreover, ATP association leads to a conformational change within isolated CIC-5 carboxy-termini [43]. ATP also binds to isolated CIC-2 carboxy-termini [36], and we thus assumed that ATP association occurred in the closed, spaced apart conformation of the carboxy-termini and inserted a transition (μ) between an ATP-unbound and ATP-bound closed state. A conformational change following the association of ATP (transition γ) to the state C_{ATP}^* then allows for opening of the ATP-bound channel.

The rate constants within this kinetic scheme were initially estimated by fitting the model simultaneously to experimentally determined time courses of WT hCIC-2 in response to voltage steps between -160 and +40 mV and to the absolute open probabilities at these voltages for ATP concentrations of 0 as well as for 5 mM. Repeated global fitting of current amplitudes at exponentially distributed time points yielded several sets of parameters that well reproduced the time and voltage dependences of CIC-2 currents (Fig. 5b). We then chose the final parameter set (Table 3) by testing the ability to reproduce the experimentally observed ATP-dependence of slow activation time constants (Fig. 5c). The thus obtained kinetic model predicts that ATP slightly reduces the apparent gating charge $z\delta$ of the voltage-dependent rate constants

leading to $O_{v(\text{ATP})}$ (β_{ATP}), and reduces the backward rate constant (δ') by ~75 % compared with the value of the equivalent rate constant (β') without bound ATP. This modification results in longer closed states of the common gate in the presence of ATP and thus accounts well for the experimentally observed ATP-dependent (Fig. 5c, d) and voltage-dependent deceleration of the slow gate (Fig. 5d). The predictions for the deactivation time courses were unexpected. Common gating was shown to have faster gating kinetics than protopore gating in the absence of ATP but to be similar in the presence of ATP. It was therefore not easily possible to attribute the fast and slow time constants of macroscopic deactivation to fast and slow time constants obtained for activation. This finding might explain why ATP-binding changes both, fast and slow macroscopic deactivation time constants, but only the slow activation time constant even though only the common gate is changed.

In qualitative agreement with experimental data (Supplemental Fig. 3), dwell time analysis of simulated single channels revealed an additional ATP-dependent closed dwell time of the common gate longer than 1 s. However, very long closed states are rare, and microscopic time constants are therefore not well defined. We hence decided not to use single-channel data for setting quantitative constraints on the fitting procedure, but to exclusively use whole-cell ensemble currents for determination of rate constants within this kinetic scheme.

Sequence variants found in patients with idiopathic generalized epilepsies accelerate ATP-dependent slow gating of CIC-2

In recent years, several *CLCN2* sequence variants have been described in patients suffering from IGE [21, 22, 33]. One cluster of such mutations is localized in the carboxy-terminus of the protein. Since it is already known that ATP binds to the CBS domains of CIC proteins [36], we investigated whether variants in the carboxy-terminus might affect ATP-dependent gating of CIC-2. We decided to re-evaluate known missense mutations in *CLCN2* using identical and improved experimental techniques. We studied two variants that have already been reported, G715E [22] and R577Q [33], and characterized a novel sequence variant R653T that we discovered in a genetic screening of 95 families with members suffering from IGE (Fig. 6a and b; Supplemental Fig 4 and Supplemental Text 1). As a control, we included a combination of sequence variants, R688Q and E718D, that was first found in a patient suffering from epilepsy [9] but later found to rather represent a common polymorphism [4, 22].

None of the sequence variants changed the general characteristics of CIC-2 gating (Fig. 6). However, at 5 mM cytosolic ATP, slow gating time constants were significantly

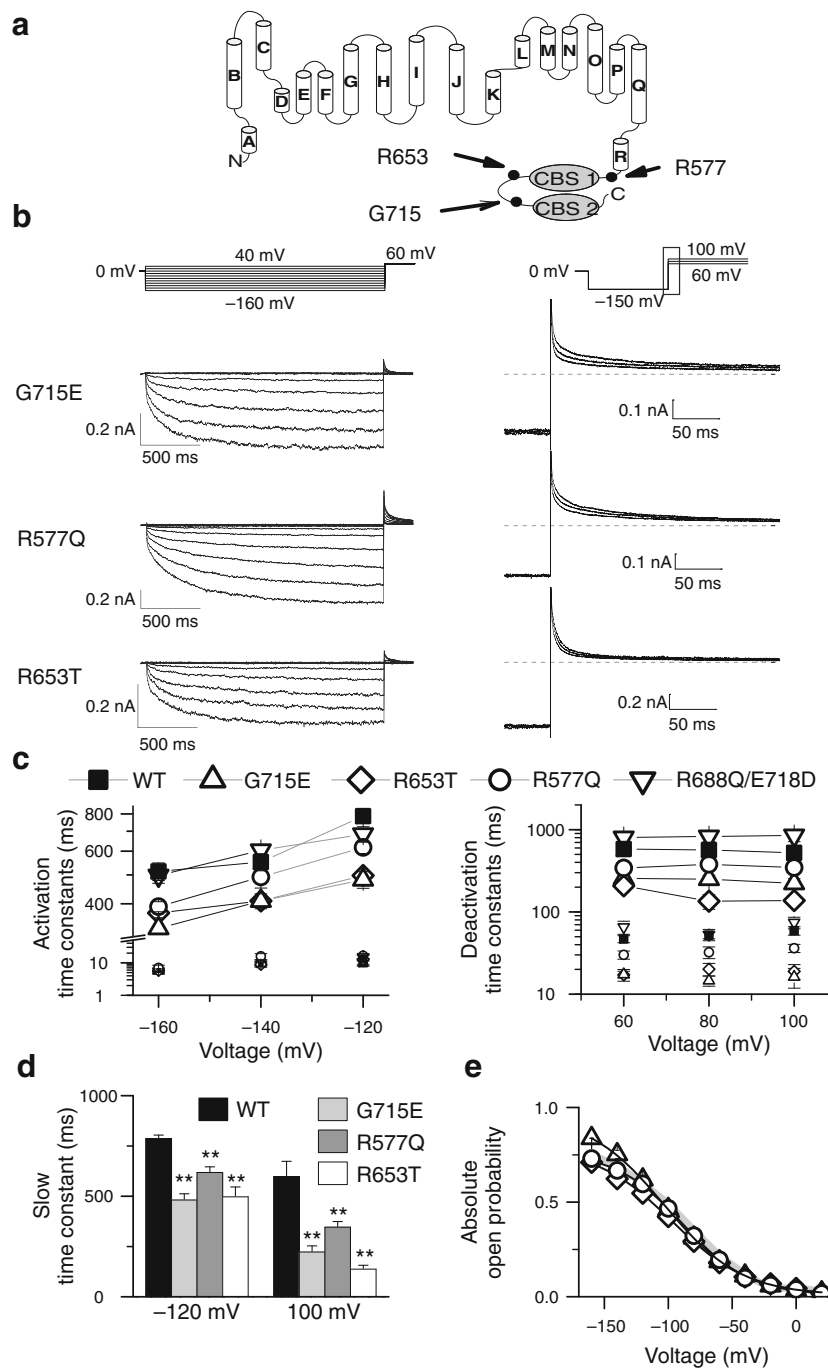


Fig. 6 Disease-associated sequence variants accelerate CIC-2 under physiological ATP concentrations. **a** Position of tested disease-associated sequence variations in a topology model of CIC-2. 18 transmembrane helices are shown, followed by a cytoplasmic carboxy-terminus with two cystathionine- β -synthase (CBS) domains. **b** Representative whole-cell current responses to indicated voltage steps. Cells were held at 0 mV, and voltage steps from -160 to 40 mV were applied to study activation kinetics (*left side*). Steps from 60 to 100 mV following a preconditioning pulse to -150 mV were used to analyze channel deactivation (*right side*). For improved illustration, only a section of the deactivation current responses are shown (depicted as *black box* in voltage protocol). *Dashed grey lines* are added to indicate a level of

0 nA. Cells either express indicated variants, and all cells were dialyzed with solution containing 5 mM ATP. **c** Voltage dependence of fast and slow activation (*left side*) or deactivation (*right side*) time constants for WT and CIC-2 variants at physiological ATP. Slow time constants are shown as *larger symbols* and connected for easier identification. **d** Exemplary statistical analysis of activation and deactivation time constants at indicated voltages. *Asterisks* denote $p < 0.01$ (**). **e** Absolute open probabilities are given as the instantaneous current at the tail-pulse of 60 mV versus the preceding voltage and normalized to the value at -160 mV obtained by stationary noise analysis or single channel recordings. A fit to a Boltzmann function is shown as *solid lines*. WT values are shown by a *gray line*

faster than WT for all tested disease-associated sequence variants (Fig. 6c and d) across all observed voltages. The acceleration was most pronounced for R653T and G715E, both being accelerated to values similar as the wild type in the absence of ATP. R688Q/E718D CIC-2 showed kinetics that is not significantly different from WT values. The change in kinetics for all three disease associated sequence variants did not significantly change steady-state open probabilities (Fig. 6e; G715E ($n=5$)— $V_{1/2}=-101.6\pm 4.6$ mV $p=0.1$; $P_{\text{open}(-160\text{ mV})}=0.84\pm 0.06$; $p=0.14$; R577Q ($n=5$)— $V_{1/2}=-89.7\pm 3.3$ mV; $p=0.85$; $P_{\text{open}(-160\text{ mV})}=0.73\pm 0.03$; $p=0.92$; R653T ($n=5$)— $V_{1/2}=-94.5\pm 7.0$ mV; $p=0.58$; $P_{\text{open}(-160\text{ mV})}=0.71\pm 0.06$; $p=0.71$).

Single-channel recordings from G715E, R577Q, and R653T CIC-2 revealed one closed and two open states with amplitudes similar to WT CIC-2 (Fig. 7a). However, the long-lasting closed states present in WT channels upon exposure to 5 mM ATP at the cytoplasmic side were missing for all three mutant channels. G715E, R577Q, and R653T CIC-2 displayed significantly reduced numbers of closed dwell times longer than 100 ms (Figs. 7b and 4f, WT— 16.2 ± 7.4 %; $n=5$; G715E— 1.9 ± 0.86 %; $n=3$; $p=0.02$; R577Q— 4.2 ± 0.6 ; $n=5$; $p=0.02$; R653T— 3.05 ± 1.7 %; $n=4$; $p=0.03$).

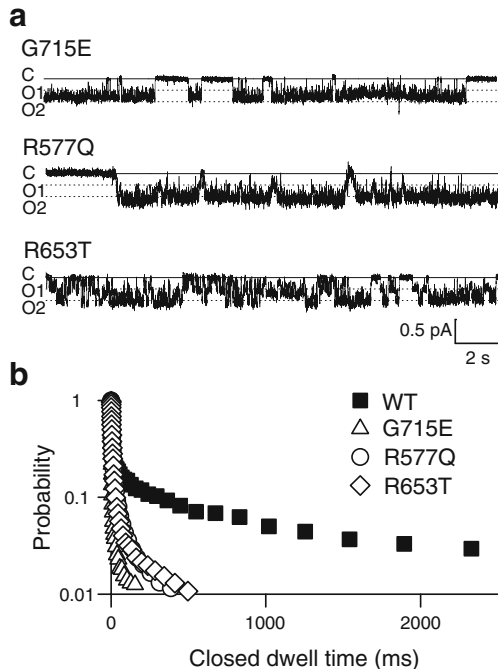


Fig. 7 ATP modification of common gating is changed by IGE-associated sequence variants. **a** Representative recordings of mutant CIC-2 channels at -100 mV and an internal ATP concentration of 5 mM. **b** Cumulative closed time distribution for G715E (open triangles), R577Q (open circles), and R653T (open diamonds) compared with WT channels (filled squares) at 5 mM ATP

To test which rates within the kinetic scheme developed for WT CIC-2 (Fig. 5) are affected by the disease-associated sequence variants, we performed global fitting of the scheme developed for WT CIC-2 (Fig. 5) to the time and voltage dependence of G715E CIC-2. This procedure revealed changed voltage dependencies of opening and closing rates for ATP-bound channels (β_{ATP}). Apparent gating charges $z\delta$ for opening (β_{ATP}) and closing (β_{ATP}') of ATP-bound channels were increased in comparison to WT channels resulting in faster activation and slower deactivation kinetics (Table 3). The resulting parameters were used to simulate the ATP-dependence (dashed line in Fig. 8a) as well as the voltage dependence of activation (dashed line in Fig. 8b). Both simulations were in good agreement with experimentally determined WT and the G715E values (symbols in Fig. 8a and b). The modifications determined by global fitting were sufficient to explain all observed G715E-mediated changes of CIC-2 gating without changing ATP association and dissociation. Similar results could be obtained for R577Q and R653T by varying the degree to which the apparent gating charges in transitions β_{ATP} , β_{ATP}' , and γ were altered (Table 3).

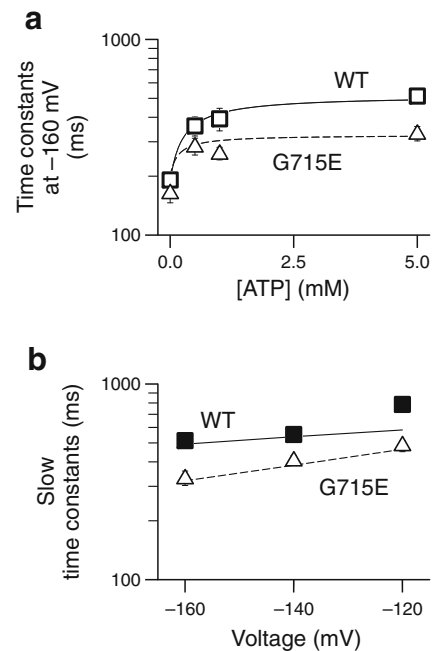


Fig. 8 Modification of voltage-dependent opening and closing of the common gate is sufficient to describe functional alterations by disease-associated sequence variants. **a** Dependence of slow activation time constants at -160 mV for WT and G715E channels. Experimental values are plotted as open squares or triangles, respectively. Lines are computed following the scheme with values stated in Table 3. **b** Slow time constants of activation across all voltages analyzed are well described by the seven-state model used and can be modified to represent the G715E mutant

Discussion

In native cells as well as in heterologous expression systems, CIC-2 is closed at positive potentials and slowly opens upon membrane hyperpolarization resulting in a strong rectification of chloride currents [6, 16, 27, 34, 37]. We here show that the kinetics of CIC-2 activation and deactivation are significantly altered by intracellular ATP (Figs. 1 and 3). Using single-channel analysis, we were able to link the ATP dependence of CIC-2 gating to the common gate (Fig. 4). Kinetic modeling identified changes in ATP-dependent rate constants that lead to the observed alterations in gating (Fig. 5).

Mammalian neurons contain ATP at concentrations between 1 and 5 mM [24, 28]. At these concentrations, CIC-2 assumes saturating values in activation and deactivation time constants indicating that under physiological conditions activation and deactivation time constants reach maximum values. Intracellular ATP concentrations vary under metabolic stress [44], and ATP regulation of CIC-2 might be part of the response to this condition. ATP degradation results in increasing ADP concentrations which modifies CIC-2 gating to a similar degree as ATP (Fig 3c and d, Supplemental Fig. 2). Only further degradation to AMP would change the kinetics of CIC-2 currents as AMP-bound channels lack the slow gating kinetics found with higher energy nucleotides. However, CIC-2 seems to have a much lower affinity toward AMP as indicated by experiments combining high concentrations of AMP with low concentrations of ATP. Nucleotide regulation thus most likely does not serve adaptation to metabolic stress but is rather necessary for the normal function of CIC-2.

When expressed in *Xenopus laevis* oocytes and studied by two-microelectrode voltage clamp, CIC-2 activates on a slower time course [37] than in patch-clamp transfected mammalian cells [10, 16, 42]. Since intact oocytes contain higher ATP concentrations than internally dialyzed cells, the here reported regulation by ATP provides a likely explanation for this difference.

ATP regulation has been studied in detail for the muscle-specific isoform CIC-1 [2, 3, 39, 40]. ATP binds to the cytoplasmic CBS domains and acts on the common gate of CIC-1. Its action is pH-dependent, resulting in CIC-1 inhibition by intracellular ATP at acidic pH. ATP thus exerts different effects on gating of CIC-1 and CIC-2 resulting in distinct alterations of electrical properties of muscle fibers and neuron. CIC-1 contributes to the resting conductance of skeletal muscle and regulates muscle excitability by changing threshold currents necessary for action potential formation. Downregulation of CIC-1 by pH and ATP reduces muscle excitability under metabolic stress [29]. The activation of CIC-2 at voltages negative to the chloride equilibrium potential leads to the efflux of negative charges

conferring a depolarizing effect on the cell. ATP regulation of CIC-2 thus modifies the time course of a potential excitatory anion outward current.

Genetic studies linked sequence variants in *CLCN2* to IGE. One cluster of sequence variants found in patients with these diseases is located within the region of the carboxy-terminus of CIC-2 that comprises parts of the ATP binding site in CIC-2. As there is an ongoing controversy about the role of *CLCN2* variants in the pathophysiology of IGE [21, 22, 33], we decided to investigate the effects of some of these variants, G715E, R577Q, and R653T, on ATP-dependent gating. Whereas the polymorphism combination R688Q/E718D did not change ATP-dependent kinetics, all three point mutations accelerated CIC-2 gating under physiological, high ATP conditions. These results suggest that ATP regulation might be physiologically important for excitability in central neurons. This notion is supported by another sequence variation associated to IGE, W570X [21]. W570X causes a significant acceleration of CIC-2 activation and deactivation with only slight changes to steady-state open probabilities even in the absence of ATP (our own unpublished observation), closely similar to H573X CIC-2 that also removes the entire carboxy-terminus (Fig 3c and d) [16]. Accelerated CIC-2 gating is thus a common functional consequence of IGE-associated sequence variants. IGE sequence variants do not alter steady-state parameters and cause only modest changes in time constants; however, these alterations can lead to a 20–40 % difference to wild-type channels in time-dependent open probability after few tens of milliseconds (Supplemental Fig. 5). However, the complex inheritance pattern suggests that changes in CIC-2 gating are only a contributing factor among others, yet unknown changes to cell excitability [21].

Our present findings are in contrast to an earlier study by Niemeyer et al. [27] who did not find differences in gating kinetics between WT and G715E CIC-2. A possible explanation for these differences might be the larger macroscopic current amplitudes in the earlier study. The slow activation time course makes CIC-2 very vulnerable for incorrect determination of activation time constants because of chloride depletion. Chloride efflux during long hyperpolarizing voltage steps reduces the driving force and thus reduces chloride currents. This reduction of chloride currents leads to incorrectly fast apparent activation time constants (Supplemental Fig. 6) [17]. Moreover, test pulses were often shortened to prevent significant chloride depletion in these early studies on CIC-2 gating [46]. Chloride depletion as well as different prepulse duration might have masked gating differences between WT and mutant CIC-2 in that earlier study. We used only cells with very low expression of CIC-2 to avoid these artifacts and are thus confident that our data represent accurate values for gating kinetics. Furthermore, the single-

channel recordings strongly support the results from macroscopic recordings. Open probabilities and amplitudes of single-channel recordings (Fig. 2a) perfectly matched those obtained from stationary noise analysis (Fig. 4c and d) at -100 mV. The strong shift in closed dwell times to longer durations (Fig. 4g) implies an involvement of a common ATP-dependent process that—within the context of CLC gating—we could fully attribute to the slow, common gate of CIC-2 (Fig. 5a).

A recent publication reported the identification of the accessory subunit GlialCAM that causes profound alterations of CIC-2 gating [20]. However, whereas CIC-2 is broadly expressed in various cells of the central nervous systems, GlialCAM and CIC-2 colocalize only in Bergmann glia, in astrocyte–astrocyte junctions at astrocytic endfeet around blood vessels, and in myelinated fiber tracts. We here expressed CIC-2 without GlialCAM to create conditions that resemble the most likely situation in neurons. Our work does not provide information about the small fraction of glial CIC-2 channels interacting with GlialCAM.

In conclusion, we have characterized the regulation of CIC-2 by intracellular ATP. Association of ATP decelerates the kinetics of activation and deactivation via changing voltage-dependent transitions within the common gating cycle. Certain disease-associated sequence variants modify CIC-2 regulation by ATP, suggesting that ATP-dependent deceleration might be important for the electrical stability of certain neurons.

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References

- Alekov A, Fahlke Ch (2009) Channel-like slippage modes in the human anion/proton exchanger CIC-4. *J Gen Physiol* 133:485–496
- Bennetts B, Parker MW, Cromer BA (2007) Inhibition of skeletal muscle CIC-1 chloride channels by low intracellular pH and ATP. *J Biol Chem* 282:32780–32791
- Bennetts B, Rychkov GY, Ng HL, Morton CJ, Stapleton D, Parker MW, Cromer BA (2005) Cytoplasmic ATP-sensing domains regulate gating of skeletal muscle CIC-1 chloride channels. *J Biol Chem* 280:32452–32458
- Blanz J, Schweizer M, Auberson M, Maier H, Muenscher A, Hübner CA, Jentsch TJ (2007) Leukoencephalopathy upon disruption of the chloride channel CIC-2. *J Neurosci* 27:6581–6589
- Bykova EA, Zhang XD, Chen TY, Zheng J (2006) Large movement in the C terminus of CLC-0 chloride channel during slow gating. *Nat Struct Mol Biol* 13:1115–1119
- Clark S, Jordt SE, Jentsch TJ, Mathie A (1998) Characterization of the hyperpolarization-activated chloride current in dissociated rat sympathetic neurons. *J Physiol* 506:665–678
- Colquhoun D, Hawkes AG (1995) The principles of the stochastic interpretation of ion-channel mechanisms. In: Sakmann B, Neher E (eds) *Single-channel recording*, 2nd edn. Plenum Press, New York and London, pp 397–482
- Colquhoun D, Sigworth FJ (1995) Fitting and statistical analysis of single-channel records. In: Sakmann B, Neher E (eds) *Single-channel recording*, 2nd edn. Plenum Press, New York and London, pp 483–588
- D'Agostino D, Bertelli M, Gallo S, Cecchin S, Albiero E, Garofalo PG, Gambardella A, St Hilaire JM, Kwiecinski H, Andermann E, Pandolfo M (2004) Mutations and polymorphisms of the CLCN2 gene in idiopathic epilepsy. *Neurology* 63:1500–1502
- de Santiago JA, Nehrke K, Arreola J (2005) Quantitative analysis of the voltage-dependent gating of mouse parotid CIC-2 chloride channel. *J Gen Physiol* 126:591–603
- Dhani SU, Kim CP, Huan LJ, Bear CE (2008) ATP depletion inhibits the endocytosis of CIC-2. *J Cell Physiol* 214:273–280
- Dutzler R, Campbell ED, Cadene M, Chait MB, MacKinnon R (2002) X-ray structure of a CIC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature* 415:287–294
- Fahlke Ch, Rosenbohm A, Mitrovic N, George AL Jr, Rüdell R (1996) Mechanism of voltage-dependent gating in skeletal muscle chloride channels. *Biophys J* 71:695–706
- Feng L, Campbell EB, Hsiung Y, MacKinnon R (2010) Structure of a eukaryotic CLC transporter defines an intermediate state in the transport cycle. *Science* 330(6004):635–641
- Fischer M, Janssen AG, Fahlke Ch (2010) Barttin activates CIC-K channel function by modulating gating. *J Am Soc Nephrol* 21:1281–1289
- Garcia-Olivares J, Alekov A, Boroumand MR, Begemann B, Hidalgo P, Fahlke C (2008) Gating of human CIC-2 chloride channels and regulation by carboxy-terminal domains. *J Physiol* 586:5325–5336
- Gonoi T, Hille B (1987) Gating of Na channels. Inactivation modifiers discriminate among models. *J Gen Physiol* 89:253–274
- Hebeisen S, Fahlke Ch (2005) Carboxy-terminal truncations modify the outer pore vestibule of muscle chloride channels. *Biophys J* 89:1710–1720
- Hinzpeter A, Fritsch J, Borot F, Trudel S, Vieu DL, Brouillard F, Baudouin-Legros M, Clain J, Edelman A, Ollero M (2007) Membrane cholesterol content modulates CIC-2 gating and sensitivity to oxidative stress. *J Biol Chem* 282:2423–2432
- Jeworutzki E, Lopez-Hernandez T, Capdevila-Nortes X, Sirisi S, Bengtsson L, Montolio M, Zifarelli G, Arnedo T, Muller CS, Schulte U, Nunes V, Martinez A, Jentsch TJ, Gasull X, Pusch M, Estevez R (2012) GlialCAM, a protein defective in a leukodystrophy, serves as a CIC-2 Cl⁻ channel auxiliary subunit. *Neuron* 73:951–961
- Klassen T, Davis C, Goldman A, Burgess D, Chen T, Wheeler D, McPherson J, Bourquin T, Lewis L, Villasana D, Morgan M, Muzny D, Gibbs R, Noebels J (2011) Exome sequencing of ion channel genes reveals complex profiles confounding personal risk assessment in epilepsy. *Cell* 145:1036–1048
- Kleefuss-Lie A, Friedl W, Cichon S, Haug K, Wamstedt M, Alekov A, Sander T, Ramirez A, Poser B, Maljevic S, Hebeisen S, Kubisch C, Rebstock J, Horvath S, Hallmann K, Dullinger JS, Rau B, Haverkamp F, Beyenburg S, Schulz H, Janz D, Giese B, Muller-Newen G, Propping P, Elger CE, Fahlke Ch, Lerche H (2009) *CLCN2* variants in idiopathic generalized epilepsy. *Nat Genet* 41:954–955
- Markovic S, Dutzler R (2007) The structure of the cytoplasmic domain of the chloride channel CIC-Ka reveals a conserved interaction interface. *Structure* 15:715–725

24. Merboldt KD, Chien D, Hänicke W, Gyngell ML, Bruhn H, Frahm J (1990) Localized ³¹P NMR spectroscopy of the amt human brain in vivo using stimulated-echo (STEAM) sequences. *J Magn Reson* 89(3):43–361, 1969
25. Meyer S, Savaresi S, Forster IC, Dutzler R (2007) Nucleotide recognition by the cytoplasmic domain of the human chloride transporter CIC-5. *Nat Struct Mol Biol* 14:60–67
26. Miller C (1982) Open-state substructure of single chloride channels from *Torpedo* electroplax. *Philos Trans R Soc Lond B Biol Sci* 299:401–411
27. Niemeyer MI, Cid LP, Yusef YR, Briones R, Sepulveda FV (2009) Voltage-dependent and -independent titration of specific residues accounts for complex gating of a CIC chloride channel by extracellular protons. *J Physiol* 587:1387–1400
28. Pan JW, Takahashi K (2005) Interdependence of *N*-acetyl aspartate and high-energy phosphates in healthy human brain. *Ann Neurol* 57:92–97
29. Pedersen TH, de Paoli FV, Flatman JA, Nielsen OB (2009) Regulation of CIC-1 and KATP channels in action potential-firing fast-twitch muscle fibers. *J Gen Physiol* 134:309–322
30. Pusch M, Jordt SE, Stein V, Jentsch TJ (1999) Chloride dependence of hyperpolarization-activated chloride channel gates. *J Physiol* 515:341–353
31. Pusch M, Ludewig U, Rehfeldt A, Jentsch TJ (1995) Gating of the voltage-dependent chloride channel CIC-0 by the permeant anion. *Nature* 373:527–530
32. Ramjeesingh M, Huan L-J, Garami E, Wang Y, Bear CE (2000) Quaternary structure of the chloride channel CIC-2. *Biochemistry* 39:13838–13847
33. Saint-Martin C, Gauvain G, Teodorescu G, Gourfinkel-An I, Fedirko E, Weber YG, Maljevic S, Ernst JP, Garcia-Olivares J, Fahlke Ch, Nabbout R, LeGuern E, Lerche H, Poncer JC, Depienne C (2009) Two novel *CLCN2* mutations accelerating chloride channel deactivation are associated with idiopathic generalized epilepsy. *Hum Mutat* 30:397–405
34. Sanchez-Rodriguez JE, De Santiago-Castillo JA, Contreras-Vite JA, Nieto-Delgado PG, Castro-Chong A, Arreola J (2012) Sequential interaction of chloride and proton ions with the fast gate steer the voltage-dependent gating in CIC-2 chloride channel. *J Physiol* 590:4239–4253
35. Saviane C, Conti F, Pusch M (1999) The muscle chloride channel CIC-1 has a double-barreled appearance that is differentially affected in dominant and recessive myotonia. *J Gen Physiol* 113:457–468
36. Scott JW, Hawley SA, Green KA, Anis M, Stewart G, Scullion GA, Norman DG, Hardie DG (2004) CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J Clin Invest* 113:274–284
37. Thiemann A, Gründer S, Pusch M, Jentsch TJ (1992) A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature* 356:57–60
38. Thompson CH, Olivetti PR, Fuller MD, Freeman CS, McMaster D, French RJ, Pohl J, Kubanek J, McCarty NA (2009) Isolation and characterization of a high affinity peptide inhibitor of CIC-2 chloride channels. *J Biol Chem* 284:26051–26062
39. Tseng PY, Bennetts B, Chen TY (2007) Cytoplasmic ATP inhibition of CLC-1 is enhanced by low pH. *J Gen Physiol* 130:217–221
40. Tseng PY, Yu WP, Liu HY, Zhang XD, Zou X, Chen TY (2011) Binding of ATP to the CBS domains in the C-terminal region of CLC-1. *J Gen Physiol* 137:357–368
41. Vanoye CG, George AL Jr (2002) Functional characterization of recombinant human CIC-4 chloride channels in cultured mammalian cells. *J Physiol* 539:373–383
42. Varela D, Niemeyer MI, Cid LP, Sepulveda FV (2002) Effect of an N-terminus deletion on voltage-dependent gating of the CIC-2 chloride channel. *J Physiol* 544:363–372
43. Wellhauser L, Luna-Chavez C, D'Antonio C, Tainer J, Bear CE (2011) ATP induces conformational changes in the carboxyl-terminal region of CIC-5. *J Biol Chem* 286:6733–6741
44. Younkin DP, Ivoria-Papadopoulos M, Maris J, Donlon E, Clancy R, Chance B (1986) Cerebral metabolic effects of neonatal seizures measured with in vivo ³¹P NMR spectroscopy. *Ann Neurol* 20:513–519
45. Zifarelli G, Pusch M (2009) Intracellular regulation of human CIC-5 by adenine nucleotides. *EMBO Rep* 10(10):1111–1116
46. Zuniga L, Niemeyer MI, Varela D, Catalan M, Cid LP, Sepulveda FV (2004) The voltage-dependent CIC-2 chloride channel has a dual gating mechanism. *J Physiol* 555:671–682

Supplementary Material

Regulation of ClC-2 gating by intracellular ATP

Gabriel Stölting, Georgeta Teodorescu, Birgit Begemann, Julian Schubert, Rima Nabbout, Mohammad Reza Toliat, Thomas Sander, Peter Nürnberg, Holger Lerche, Christoph Fahlke

Supplemental Text 1. Clinical description of family I

The father (I 1, Figure S1) developed typical absence seizures from age 3 with short 3Hz spike-waves on EEG recordings. He was free of seizures on valproate and stopped antiepileptic drug (AED) treatment at age 8 without recurrence of seizures. His son (II 1) developed typical absence seizures with eyelid myoclonia at 2.5 years of age. Absences partially responded to valproate and were mostly controlled by valproate and ethosuximide at age 3. He presented one grand mal with tonic clonic seizures on awakening at age 12 and was free of seizures since. His brother, index case II 2, presented with typical absence seizures at the age of 3 years, showing a 3Hz spike-wave pattern on EEG recordings. He was free of absence seizures under treatment with ethosuximide and has not developed seizures since. All three patients stopped their drug treatment around the age of 10 and showed normal development.

Supplemental Text 2. Epicure Consortium

This list contains all clinicians and scientists from the Epicure community who contributed to recruit the 95 IGE families in addition to the named co-authors of this paper: P. Striano, F. Zara (Muscular and Neurodegenerative Diseases Unit, "G. Gaslini" Institute, University of Genova, Genova, Italy); Y.G. Weber (Department of Neurology and Epileptology, University of Tübingen, Germany); R. Guerrero, J. Serratosa (Epilepsy Unit, Neurology Service, Fundación Jiménez Díaz, Instituto de Investigaciones Sanitarias FJD and CIBERER, Madrid, Spain); A. Polvi, A.-E. Lehesjoki (Folkhälsan Institute of Genetics, Department of Medical Genetics and Neuroscience Center, University of Helsinki, Finland); C. Marini, R. Guerrini (IRCCS Stella Maris Foundation, University of Pisa, Italy, Child

Neurology Unit, Children's Hospital A. Meyer, Florence, Italy); H. Hjalgrim, MD, J.M. Madsen, MD, R.S. Møller, MD (Department of Neurology, Danish Epilepsy Centre, Dianalund, Denmark); V. Gaus, D. Janz, MD, B. Schmitz, MD (Department of Neurology, Charité University Medicine, Campus Virchow Clinic, Humboldt University of Berlin, Berlin, Germany); C.E. Elger, MD, K. Hallmann, MD, A.A. Kleefuß-Lie, MD, W.S. Kunz, MD, A. Raabe, MD (Department of Epileptology, University of Bonn, Bonn, Germany); F. Rosenow, MD, P. Reich, MD (Department of Neurology at the University of Marburg, Germany); I. Helbig, MD, H. Muhle, MD, P. Ostertag, MD, T. Obermeier, MD, S. von Spiczak, MD, U. Stephani, MD (Department of Neuropediatrics University Medical Center Schleswig-Holstein, Kiel Campus, Kiel, Germany); E.H. Brilstra, MD, D.G.A. Kastelijjn-Nolst Trenité, MD, B.P.C. Koeleman, PhD, C.G.F. de Kovel, PhD, M.E.M. Swinkels, MD (Section Complex Genetics, Department of Medical Genetics, University Medical Center, Utrecht, The Netherlands); D. Lindhout, MD (Section Complex Genetics, Department of Medical Genetics, University Medical Center, Utrecht, The Netherlands and SEIN Epilepsy Institute in the Netherlands, Heemstede, The Netherlands); U. Ozbek, MD, B. Baykan, MD, N. Bebek, MD, O. Ozdemir, MD (Epilepsy Centre (EPIMER) and Experimental Medicine Research Institute (DETAE) Department of Genetics, Istanbul University, Istanbul, Turkey); G. Gul, MD (Bakirkoy Hospital for Psychiatric and Nervous System Diseases, Istanbul, Turkey); N. Calisir, MD, (Medicabil Hospital, Bursa, Turkey); G. Avanzini, MD (Istituto Neurologico Carlo Besta, University of Milano, Italy); S. Striano, MD, L. Bilo, MD, A. Coppola, MD, (Epilepsy Center, Federico II University, Napoli, Italy); M. Baulac, MD, PhD, I. An-Gourfinkel, MD (Epilepsy Center, Hôpital de la Pitié-Salpêtrière, Paris, France); S. Baulac, E. LeGuern (CRICM UPMC-Paris6 UMR-S975/Inserm U975, Paris, France); M.T. Giallonardo, C. Di Bonaventura, MD (Department of Neurological Sciences, Policlinico Umberto I, Sapienza University of Rome, Italy); P. Benna, MD (Centro Epilessia, A.O. O.I.R.M. Sant'Anna, Torino, Italy); P. Tinuper, MD, (Department of Neurological Sciences, University of Bologna, Italy); A. Vignoli, MD (Centro Epilessia, Azienda Ospedaliera San Paolo, University of Milan, Italy); G. Capovilla, MD (Epilepsy Center, Department of Child Neuropsychiatry, C. Poma Hospital, Mantova, Italy); G. Coppola, MD (Clinic of Child and Adolescent Neuropsychiatry, Second University of Naples, Italy); P. Vigliano, MD (Division of Child Neuropsychiatry, ASL2-Martini Hospital, Torino, Italy); E. Del Giudice,

MD (Division of Child Neuropsychiatry, Federico II University, Napoli, Italy); G. Stranci, MD (U.O. Neurophysiopathology, ASP 6, Palermo, Italy); M. Viri, MD (Epilepsy Center, Department of Neurosciences, Fatebenefratelli e Oftalmico Hospital, Milano, Italy); S. Franceschetti, MD (Department of Neurophysiopathology, IRCCS Foundation Carlo Besta Neurological Institute, Milano, Italy); O. Martinelli, MD (SC Child Neuropsychiatry, A.O. Ospedale di Lecco); A. Gambardella, MD (Institute of Neurological Science, National Research Council, Cosenza, Italy); A. Bianchi, MD (Epilepsy Center, Department of Neurology, San Donato Hospital, Arezzo, Italy)

Supplementary Table 1. Origin and distribution of the EPICURE sample of 95 nuclear and multiplex IGE families including Childhood Absence Epilepsy (CAE), Juvenile Myoclonic Epilepsy (JME), Juvenile Absence Epilepsy (JAE) and Epilepsy with Generalised Tonic-Clonic Seizures alone (EGTCS)

| Country | Number of families | Phenotype of index case |
|-----------------|---------------------------|---|
| France | 14 | CAE (4), JME (1), EGTCS (9) |
| Germany | 15 | CAE (7), JME (4), JAE (3), EGTCS (1) |
| Finland | 1 | JAE (1) |
| The Netherlands | 8 | CAE (2), JME (5), EGTCS (1) |
| Turkey | 5 | CAE (1), JME (3), EGTCS (1) |
| Spain | 2 | JME (1), EGTCS (1) |
| Italy | 47 | CAE (20), JME (17), JAE (6), EGTCS (4) |
| Denmark | 3 | CAE (1), JME (2) |
| Total | 95 | CAE (35), JME (33), EGTCS (17), JAE (10) |

Supplementary Table 2. Primers for Pyrosequencing

| | |
|-------------------|----------------------------|
| CLCN2-Ex17CG-Fwd | TCCATCGAGCGTTCACAG |
| CLCN2-Ex17CG-Rwd | BIOTIN-CCCTCACCTGGAAGCAGAC |
| CLCN2-Ex17CG-Seq. | CCGGCGGCAGCACAT |

Supplementary Table 3. CLCN2 Sanger Sequencing Primers

| PRIMER | SEQUENCE |
|------------------|-----------------------|
| CLCN2.Ex.1.F | AGCCGAGTCCAGGACAGAG |
| CLCN2.Ex.1.R | AGTTTCCCAGCTCAAATG |
| CLCN2.Ex.2.F | GGGAACTGTGCGTTCAAGAG |
| CLCN2.Ex.2.R | TGGCACCAAAGTGCTCCTAC |
| CLCN2.Ex.3.F | AGCAGAGGCCATTAGAGCTG |
| CLCN2.Ex.3.R | AGTGGTCAGTGGCCAAGAAG |
| CLCN2.Ex.4-5.F | ATACCCTCAGGGACCCTTTC |
| CLCN2.Ex.4-5.R | GCTTGCATATGCACAAAAG |
| CLCN2.Ex.6-7.F | CCGCTTGGCAAAGAGGTAAC |
| CLCN2.Ex.6-7.R | AAGGCCTCAGACCCAGATAAG |
| CLCN2.Ex.8-9.F | CCTTGGCCTCTCCTCCTTG |
| CLCN2.Ex.8-9.R | TTCCGGTTCAGGTAGACAAAG |
| CLCN2.Ex.10.F | CTTTGACCTGCAGGAGCTG |
| CLCN2.Ex.10.R | TTTGAAGTGGCCATTCTCTC |
| CLCN2.Ex.11-12.F | CAGCAAAGGAGACCTTCTGTC |
| CLCN2.Ex.11-12.R | GAGAGGCTTCGAGGAGTGAG |
| CLCN2.Ex.13.F | GGGTAGCTAGGAGGGTTACG |
| CLCN2.Ex.13.R | GTCAGGGTTCGGGCTAGG |
| CLCN2.Ex.14.F | TACACTGAATGACCCGTTGG |
| CLCN2.Ex.14.R | ACCTGTGCATCTGAGGGAAG |
| CLCN2.Ex.15.F | TTCCCTCAGATGCACAGGTC |
| CLCN2.Ex.15.R | CCTCCAGAGTGTGGACGAAG |
| CLCN2.Ex.16.F | CTGGGATGGCAATGCTCTG |
| CLCN2.Ex.16.R | CCAAGGAGACTGGTCCTGAG |
| CLCN2.Ex.17-18.F | GGCTTCCCTCTGGTGTGTG |
| CLCN2.Ex.17-18.R | TCTTCCAGCTGGGATGTACC |
| CLCN2.Ex.19.F | GGCTGGAACTGGGATACCTC |
| CLCN2.Ex.19.R | CGAAGGGCAGGGAGAATAG |
| CLCN2.Ex.20-21.F | CTCCCTGCTGTCAGCTCAG |
| CLCN2.Ex.20-21.R | CCTGCCCTTTTCCAACC |
| CLCN2.Ex.22.F | AATCATCATGTTGGCTGCTG |
| CLCN2.Ex.22.R | GAGCCAACTCCCTTCTCCTC |
| CLCN2.Ex.23-24.F | CAGCAGTCTGTCTCCTTCC |
| CLCN2.Ex.23-24.R | CAGCTGTAGCTGCCTCCTG |

Supplementary Table 4. Closed time constants

| | T1 (ms) | T2 (ms) | T3 (ms) | T4 (ms) | Recording time (min) |
|----------------|---------|---------|---------|---------|----------------------|
| WT 0 mM ATP | 1.76 | 15.98 | 131.72 | | 8 |
| WT 5 mM ATP | 1.51 | 9.79 | 142.25 | 1651.77 | 7 |
| G715E 5 mM ATP | 1.55 | 7.15 | 37.19 | | 7 |
| R577Q 5 mM ATP | 1.82 | 9.51 | 41.18 | | 19 |
| R653T 5 mM ATP | 2.1 | 6.34 | 29.58 | | 6 |

Supplementary Figure 1. Fast and slow gate contributions do not significantly change upon addition of ATP.

a, Representative recording of a cell subjected to 0 mM of ATP for determination of fast time constants of activation. P/4 leak subtraction was used to avoid capacitative artifacts. **b,** Representative recording of a cell with 5 mM of intracellular ATP and otherwise same conditions as in **(a)**. **c,** Relative contributions of fast and slow time constants to macroscopic currents at 0 mM of ATP (filled circles) or 5 mM of intracellular ATP (open circles). Differences are not statistically significant ($p > 0.05$; $n = 4 - 10$).

Supplementary Figure 2. ADP displays similar effects on CIC-2 gating as ATP.

a,b ADP concentration dependence of fast and slow activation **(a)** and deactivation **(b)** time constants at various voltages. Solid lines give fits with a Michaelis-Menten relationship with a K_M of 0.65 mM. Fast time constants do not depend on the ADP concentration.

Supplementary Figure 3. ATP modifies closed but not open dwell times.

a-e Dwell time distribution for WT, G715E, R577Q and R653T CIC-2 obtained from single channel recordings under conditions specified. Single channel recordings display two different open levels (named open 1 and 2) and one closed level due to the dimeric structure of CIC-2 with two separate conductance pathways.

Supplementary Figure 4. Genetic results

a, Pedigree of family I. Filled symbols represent affected and open symbols unaffected individuals. The index patient is shown by an arrow. +/m denotes the heterozygous state of the c.G1958C mutation, +/+ denotes two WT alleles. **b,** DNA sequence of patient II-2 revealing the point mutation

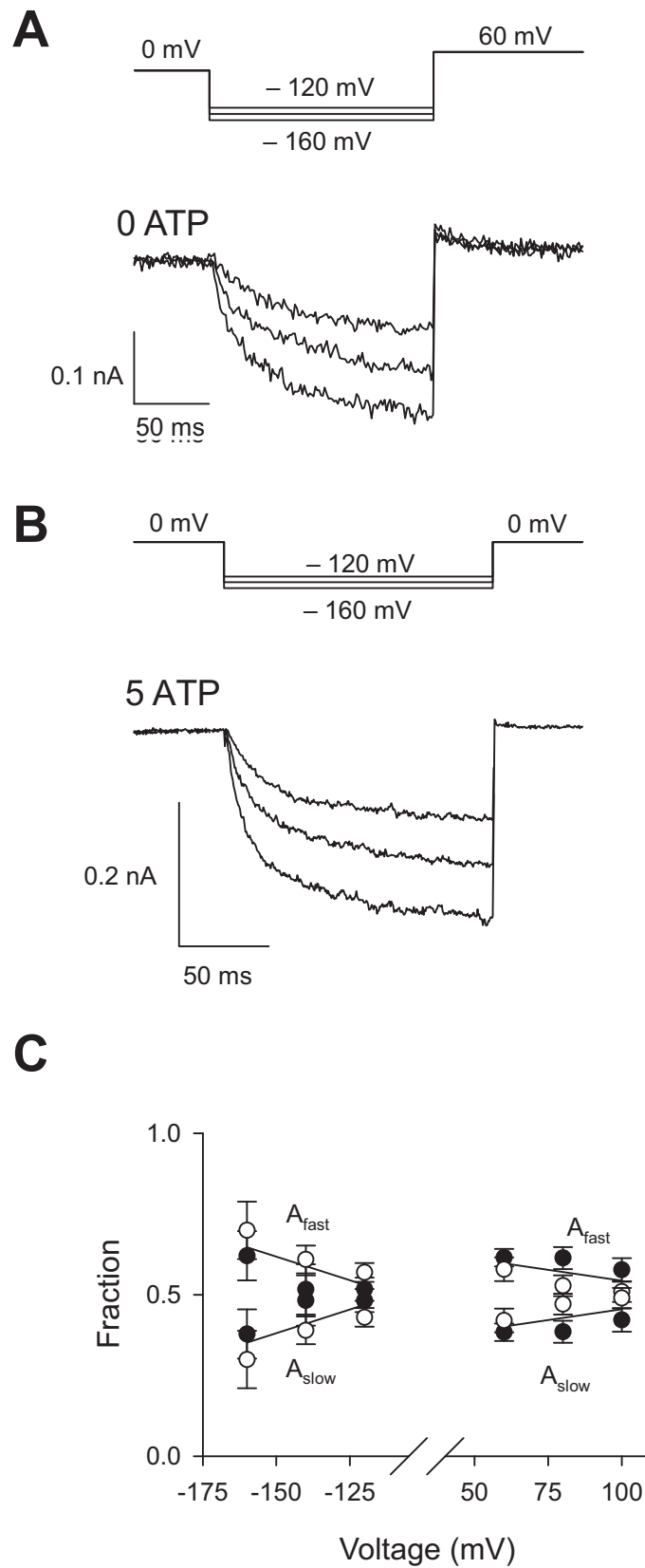
c.G1958C which predicts the amino acid exchange p.R653T. **c**, Evolutionary conservation of the region encompassing R653.

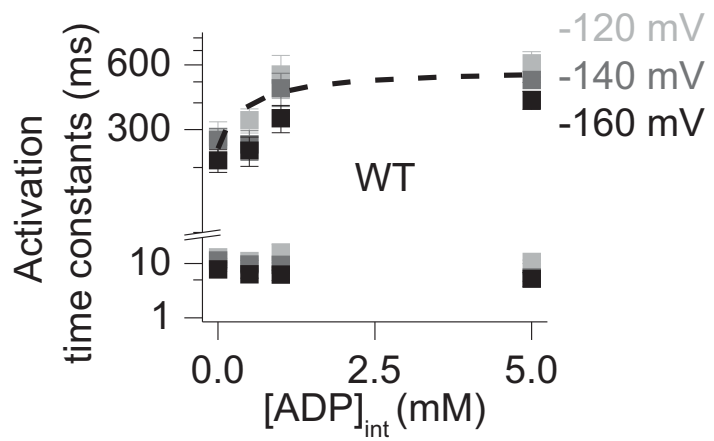
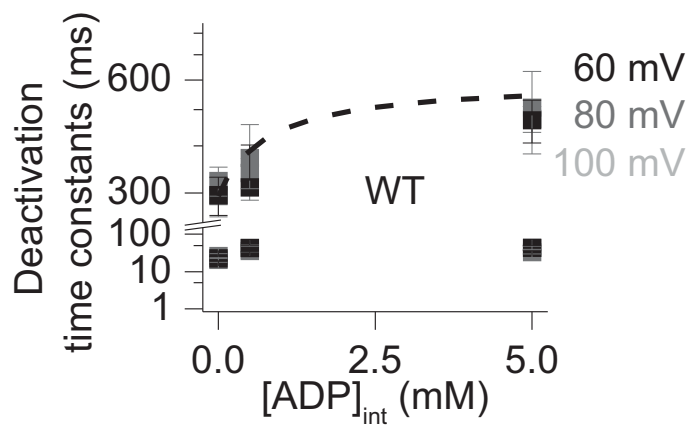
Supplemental Figure 5. G715E changes the time dependence of CIC-2 absolute open probabilities.

a, Superimposed current traces of G715E (black) and WT (grey) CIC-2 currents at 5 mM of internal ATP at -160 mV or at +100 mV from individual cells. To account for different number of functional channels in the surface membrane of the two cells mean current amplitudes during the last 50 ms of each voltage step were normalized to the steady-state absolute open probability at -160 mV. **b**, Ratio of current amplitudes of the G715E mutant to WT CIC-2 from the recordings shown in **a**.

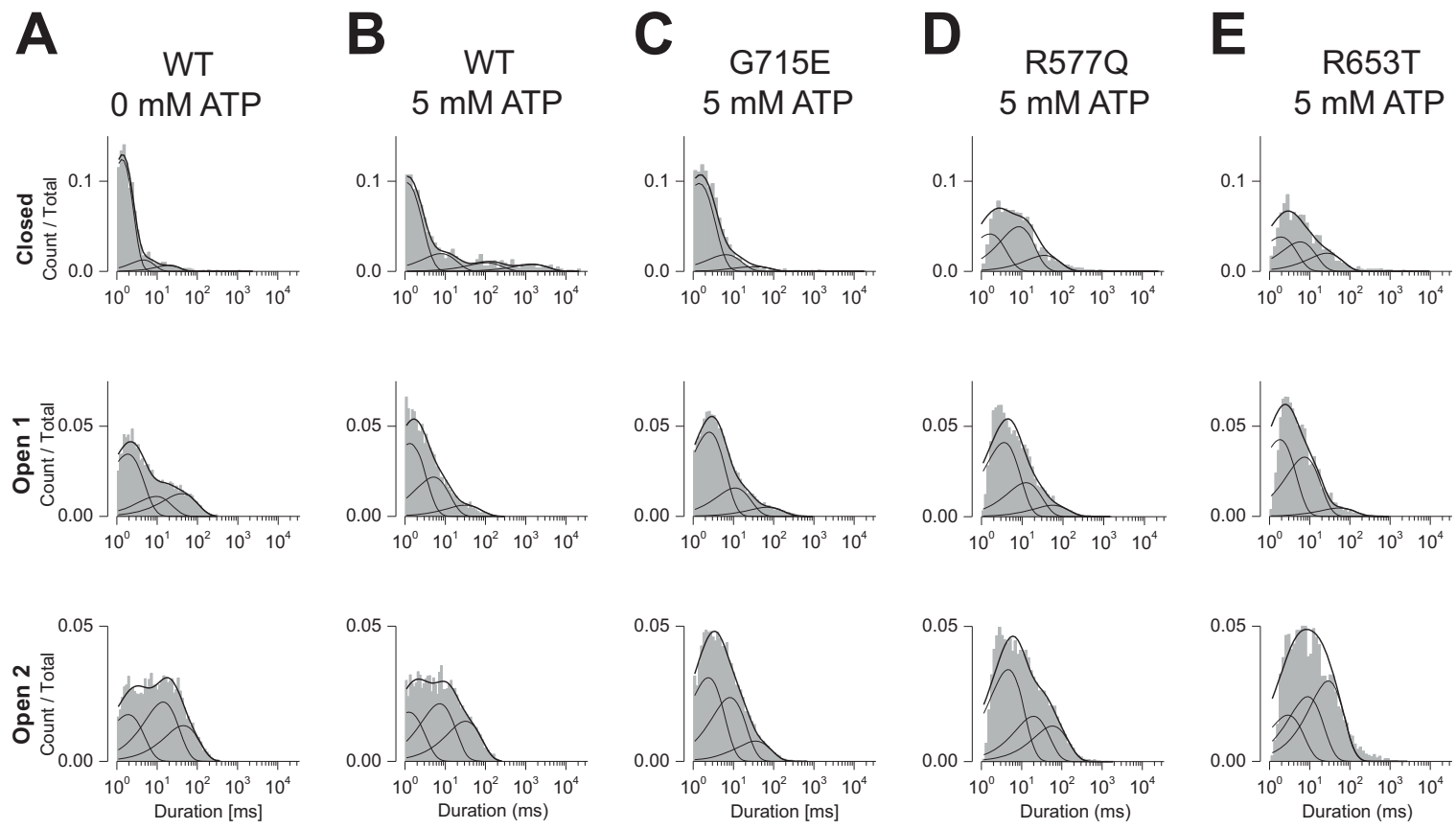
Supplementary Figure 6. Large currents cause Cl⁻ depletion that alters the appearance of time constants.

a, Currents at -140 mV from three separate cells demonstrate the occurrence of a deactivating component unlike recordings with lower amplitude as used for our study. Maximal currents are shown by dashed lines with the indicated amplitudes. Even the current shown with maximal amplitude of -1.6 nA showed a small deactivating component. **b**, Normalization of all traces to their maximal amplitude demonstrates the difficulty of accurate determination of time constants. In general, larger currents lead to an underestimation of time constants further suggesting the use of low expressing cells to study gating kinetics on CIC-2.

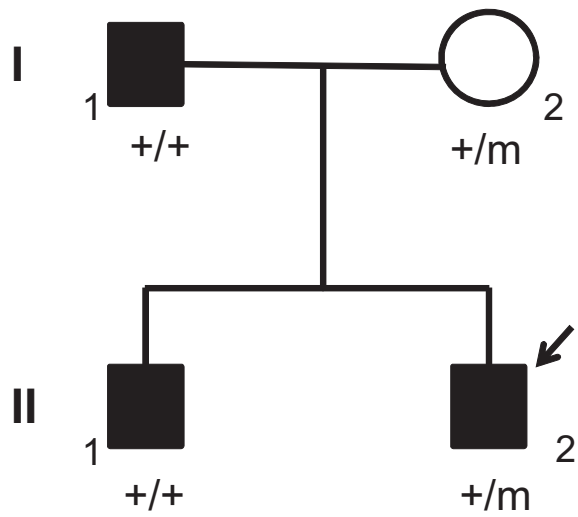
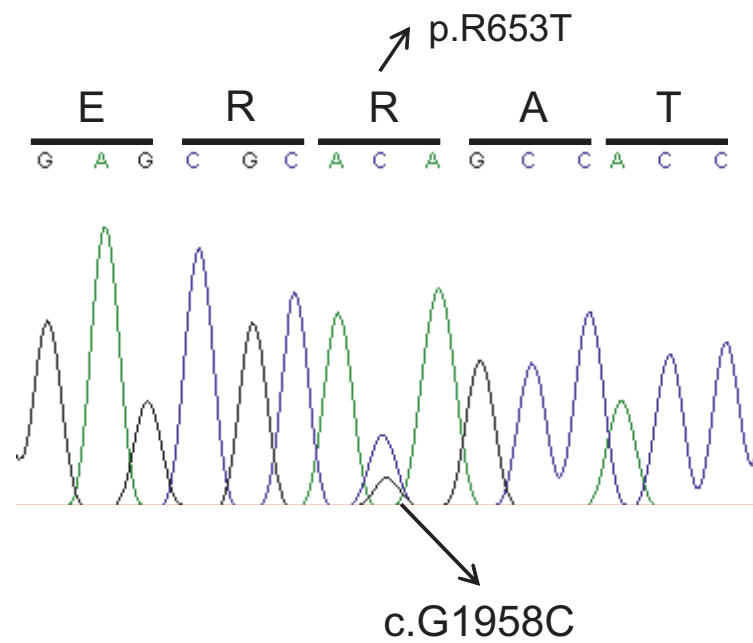


A**B**

Supplemental Figure 2 - Stölting et al.

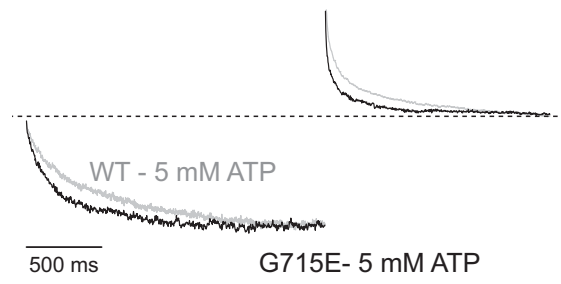


Supplemental Figure 3 - Stöling et al.

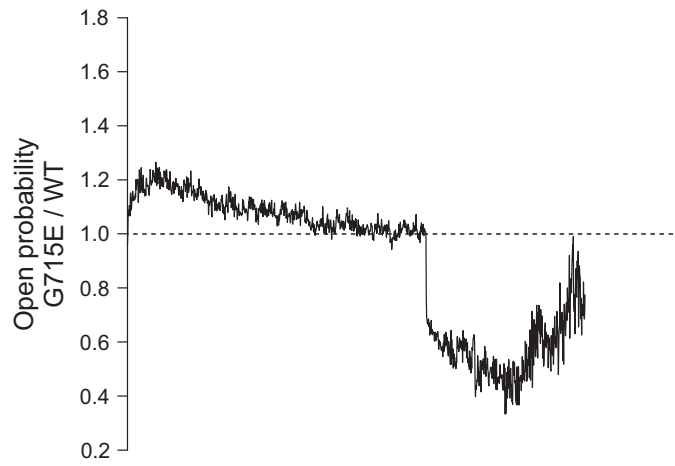
A**B****C**

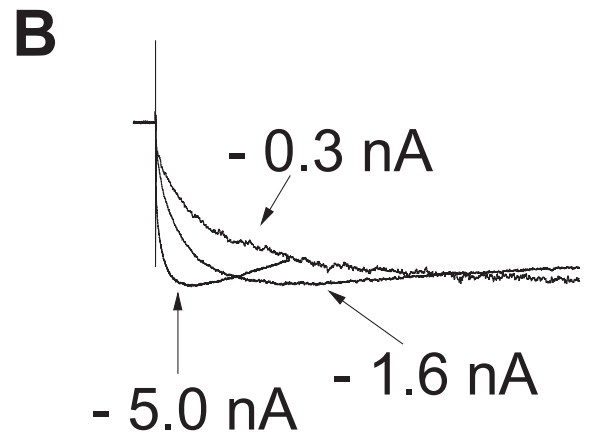
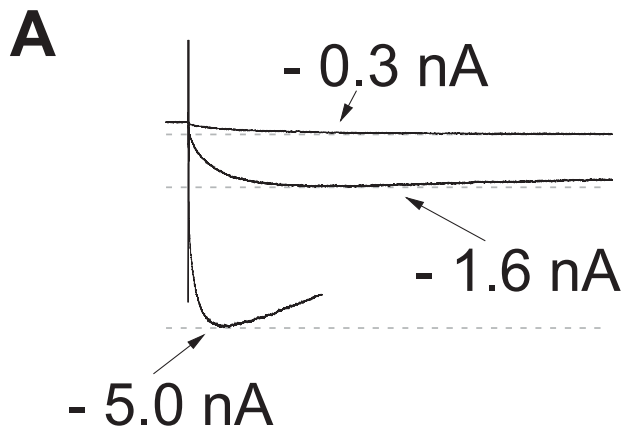
| | | | | | | | | | | | | | | | |
|------------------|---|---|---|---|---|---|---|----------|---|---|---|---|---|---|---|
| CIC-2 human | R | Q | H | M | Q | E | R | R | A | T | Q | T | S | P | L |
| CIC-2 rat | R | Q | H | M | Q | K | L | R | K | A | Q | M | S | P | P |
| CIC-2 mouse | R | Q | H | M | Q | K | L | R | K | A | Q | L | S | P | P |
| CIC-2 drosophila | Q | K | E | A | Q | K | L | A | L | E | E | E | K | K | K |

A



B





Supplemental Figure 6 - Stölting et al.

Einleitung

Einführung in die Familie der CLC-Transportproteine

Alle beschriebenen Mitglieder der CLC-Familie funktionieren entweder als Anionen-selektive Kanäle oder Anionen-Protonen Austauscher. Das physiologisch am häufigsten vorkommende Anion ist Chlorid, so dass vornehmlich von Chloridkanälen oder -Transportern gesprochen wird. Chloridkanäle wurden bereits seit längerer Zeit beschrieben, jedoch wurde das erste Mitglied aus der CLC-Familie (ClC-0) erst 1990 aus *Torpedo marmorata* kloniert [20]. Auf der Basis dieser Arbeit wurden in den folgenden Jahren weitere Mitglieder dieser Familie in verschiedensten Spezies von Pro- bis Eukaryoten gefunden.

Im Menschen sind neun Proteine aus der CLC-Familie bekannt. Es ist bekannt, dass sich ClC-3 bis -7 vornehmlich in intrazellulären Kompartimenten und ClC-1, -2, -Ka und -Kb in der Plasmamembran befinden [19]. Im Rahmen der Arbeiten an einem bakteriellen CLC-Transporter (ClC-ec1) wurde erstmals festgestellt, dass dieser nicht eine alleinige Diffusion von Chlorid-Ionen, sondern einen Austausch von Chlorid gegen Protonen ermöglicht [1]. Dieses Phänomen konnte anschließend auch in den intrazellulären ClC-3, -4, -5, -6 und -7 festgestellt werden [16, 26, 32, 36], wohingegen die plasmamembran-ständigen Mitglieder (ClC-1, -2, -Ka und -Kb) als reine Chloridkanäle fungieren.

Die humanen CLC-Transportproteine sind nicht nur in ihren Transporteigenschaften und der subzellulären Verteilung, sondern auch der Organexpression spezialisiert. So sind zum Beispiel ClC-1 im Skelettmuskel [25] und ClC-Ka und -Kb [22] in der Niere sehr spezifisch exprimiert, wohingegen die Transporter ClC-4 und ClC-5 in verschiedenen Geweben intrazellulär in Endo- und Lysosomen lokalisiert sind [19].

Alle bekannten CLC-Transportproteine zeigen einen ähnlichen Aufbau in ihrer Struktur: Es gibt einen kurzen, intrazellulären Amino-Terminus (N-Terminus) gefolgt von 18 Transmembranhelices und einem intrazellulären Carboxyl-Terminus (C-Terminus) [9]. Innerhalb dieses C-Terminus befinden sich bei den eukaryotischen Mitgliedern der CLC-Familie zwei Cystathionin- β -Synthase Domänen (CBS-Domänen) [18]. Diese Domänen sind in vielen anderen, auch nicht transportierenden, Proteinen konserviert und ermöglichen die Bindung von Nukleotiden wie ATP, ADP oder AMP [2]. Mutationen innerhalb dieser Domänen anderer Proteine sind mit einer Vielzahl verschiedener Erkrankungen assoziiert [42].

Über Einzelkanalmessungen an ClC-0 konnte früh eine funktionelle Eigenart der Kanäle aus der CLC-Familie beschrieben werden. Ein Kanal besteht in seiner Quartärstruktur als Dimer aus zwei Molekülen [29, 31]. Innerhalb jedes Moleküls ist eine eigene Anionenpore vorhanden. In

Einzelkanalmessungen können daher drei Amplitudenniveaus unterschieden werden, abhängig von der Anzahl der offenen Poren: Geschlossen (Closed), eine offene Pore (Open 1) oder zwei offene Poren (Open 2) [30]. Die Poren können getrennt geöffnet oder geschlossen werden (Protopore-Gate). Wäre dies der einzige Mechanismus würde man aufgrund der dimeren Struktur mit zwei Anionenporen eine Verteilung der Wahrscheinlichkeiten entlang einer Binomialverteilung ($a^2+2ab+b^2$) erwarten, da sich der Kanal in vier verschiedenen Zuständen aufhalten könnte: beide Protoporen geschlossen (a^2), beide Protoporen offen (b^2) oder eine Protopore offen und die benachbarte geschlossen ($2ab$). Innerhalb einer längeren Messung trifft diese Annahme jedoch nicht zu. Es gibt Phasen mit vielen Transitionen zwischen den einzelnen Niveaus (Bursts), aber auch lange Phasen in denen die Kanäle weitestgehend geschlossen sind. Innerhalb der Bursts ist die Annahme einer binomischen Verteilung gegeben, nicht jedoch bei Betrachtung der ganzen Aufnahme inklusive der langen geschlossenen Phasen. Aufgrund dieses Verhaltens ist früh von einem zusätzlichen kooperativen Schließmechanismus ausgegangen worden (Common-Gate) [31].

Das Vorhandensein mindestens zweier Schaltprozesse zeigt sich in Ganzzell-Strömen (Whole-Cell) häufig als eine Kinetik, welche der Summe zweier Exponentialfunktionen folgt. Die zwei Zeitkonstanten unterscheiden sich meist um den Faktor 10. Die schnellere der beiden Zeitkonstanten wird hierbei häufig dem Protoporen-, die langsamere Zeitkonstante dem Common-Gate zugeschlagen, so dass häufig synonym von einem Fast- und einem Slow-Gate gesprochen wird.

CIC-2 ist ein langsam schaltender Chloridkanal

Wenige Jahre nach der Entdeckung der ersten Primärstruktur aus der CLC-Familie wurden aus menschlichen Geweben zunächst der muskuläre CIC-1 und folgend der in verschiedensten Geweben auftretende CIC-2 kloniert [46].

CIC-2 ist ein Chloridkanal, der eine langsame Aktivierung bei Spannungen negativ des Chlorid-Umkehrpotentials zeigt und einen Chlorid-Ausstrom vermittelt. Es ist kein Transport von Protonen beschrieben, so dass CIC-2 als ein reiner Chlorid-Kanal fungiert. Die charakteristische Aktivierung beruht auf der Spannungsabhängigkeit beider Schaltprozesse. Die einzelne Protopore schließt bei Spannungen oberhalb des Chlorid-Umkehrpotentials komplett und die Offenwahrscheinlichkeit des gemeinsamen Schließprozesses nimmt ebenfalls ein Minimum an. Ist der Kanal bereits aktiviert, führt eine Änderung der Spannung hin zu positiveren Werten zu einer langsamen, bis zu mehrere Sekunden andauernden, Deaktivierung des Kanals [14, 41].

Die physiologische Rolle von CIC-2 ist unklar. Frühe Studien mittels heterologer Expression in *Xenopus laevis* Oozyten beschrieben eine starke Abhängigkeit der Kanalaktivierung von hypo-osmolaren Umgebungsbedingungen [15]. Knock-Out Mäuse zeigten eine Beeinträchtigung der Flüssigkeitssekretion in Speicheldrüsen, eine Degeneration der Retina, eine Degeneration der Hoden sowie eine Leukenzephalopathie [3, 4, 33].

Die pharmakologische Charakterisierung von CIC-2 ist schwierig. Unspezifische Chloridkanalblocker wie 5-Nitro-2-(3-phenylpropylamino)benzoat (NPPB) oder 9-Anthracene-carboxylat (9-AC) inhibieren CIC-2 in Konzentrationen ab etwa 0.1mM [13]. Ein Peptid, GaTx2, blockiert CIC-2 Ströme bei einem niedrigem K_d von ca. 20 pM spezifisch, aber nur bis etwa 50% [47]. Es wurde eine Substanz – Lubiproston - beschrieben, welche eine Aktivierung von CIC-2 hervorruft [6]. Lubiproston sorgt für eine Zunahme der Chlorid- und damit auch der Flüssigkeitssekretion in Dünn- und Dickdarm und ist in den USA für die Behandlung chronischer Obstipation zugelassen.

Weitere Hinweise für die physiologische und pathophysiologische Funktion dieser Kanäle zeigen genetische Assoziationsstudien. Mehrere Punktmutationen im *CLCN2* Gen mit einer Häufung im Bereich des C-Terminus konnten so in Bezug zu idiopathisch generalisierten Epilepsien (IGE) [24, 39] und auch zu Leukenzephalopathien [8] gebracht werden. Die funktionelle Untersuchung dieser Veränderungen lieferte bislang widersprüchliche Ergebnisse: Eine Veränderung der Chloridabhängigkeit, der Zeitkonstanten von Aktivierung und Deaktivierung, des subzellulären Transportes aber auch Mutationen ohne Änderung im Vergleich zum Wild-Typ wurden beschrieben. Die Häufung von Mutationen innerhalb des C-Terminus in der Nähe beider CBS-Domänen wurde auch in Bezug zu einer Änderung der Affinität zu ATP beschrieben [42]. Eine ältere elektrophysiologische Studie zeigte aber keine Änderung des Kanalverhaltens in Gegenwart von ATP [34]. Die heterologe Überexpression von CIC-2 führt jedoch häufig zu großen Stromamplituden und deswegen einer Abnahme der intrazellulären Chloridkonzentrationen während der langen Messprotokolle. Hierdurch kann eine Schließung des CIC-2 Kanals erfolgen, welche in der Summe mit der stattfindenden Aktivierung zu einer scheinbaren Beschleunigung der Zeitkonstanten führen und damit einen Effekt durch die Bindung von ATP überdecken kann. In dieser Studie wird daher eine spezielle, mittels Tetracyclin induzierbare, Zelllinie verwendet die eine genaue Kontrolle der Expression des Kanals erlaubt.

In dieser Studie soll beschrieben werden, inwiefern eine Bindung von ATP zu einer Veränderung des Kanals führt. Aufgrund der Häufung von Punktmutationen im Bereich der Bindungsdomänen für Nukleotide soll weiterhin untersucht werden, ob diese Sequenzänderungen zu Änderungen in der Funktion des CIC-2 Kanals führen.

Zusammenfassung

Ergebnisse und Methodik

Die Ergebnisse dieser Studie sind vornehmlich mittels der Patch-Clamp Methode entstanden. Hierfür wurden transient transfizierte HEK293T Zellen in der Ganzzell-Konfiguration (Whole-Cell) oder Einzelkanäle in der Inside-Out Technik gemessen [17]. Für die Modellierung von Ganzzell-Strömen wurde ein eigenes Programm in der Programmiersprache Python entwickelt. Die genetischen Analysen für die Entdeckung der neuen Mutation R653T wurden in der Klinik für Neurologie und Epileptologie an der Universität Tübingen in Kooperation mit der Klinik für Pädiatrische Neurologie am Hopital Necker in Paris durchgeführt.

ATP-Abhängigkeit des ClC-2 Wild-Typ

Messungen in der Whole-Cell Technik zeigten sowohl ohne, als auch mit ATP die typische Aktivierung bei Spannungen negativ zum Chlorid-Umkehrpotential und die Deaktivierung bei positiveren Spannungen. Der Zeitverlauf erfolgt wegen der für CLC-Proteine typischen Aufteilung in Fast- und Slow-Gate in der Summe zweier Exponentialfunktionen. Hieraus ergeben sich je zwei Zeitkonstanten, die Aktivierung oder Deaktivierung beschreiben. Die Messung in der Gegenwart von 5 mM ATP zeigte in der Aktivierung eine deutliche Verlängerung der langsamen Zeitkonstante bei weitestgehend unveränderter schneller Zeitkonstante und eine Verlangsamung beider Prozesse in der Deaktivierung.

Diese Veränderungen führen nur zu moderaten Effekten auf den einzelnen Kanal im Gleichgewichtszustand. Mittels stationärer Rauschanalyse kann auch in Whole-Cell Strömen die Offenwahrscheinlichkeit bestimmt werden [14, 43, 48]. Die Anwendung dieser Methode ergab keine signifikante Änderung der maximalen Offenwahrscheinlichkeit bei sehr negativen Spannungen, sehr wohl aber eine kleine Verschiebung der Aktivierung hin zu positiveren Spannungen. Die Änderungen der Offenwahrscheinlichkeit über die Zeit zeigen aufgrund der langsameren Kinetik einen deutlichen Unterschied in der Gegenwart von ATP. Die zeitabhängige Offenwahrscheinlichkeit innerhalb der ersten 500 ms nach einem Spannungssprung weicht um bis zu 30% von der ohne ATP ab.

Der Effekt von ATP auf die Kinetik von ClC-2 ist konzentrationsabhängig und ließ sich mit einer apparenten Michaelis-Menten Konstante im niedrigen millimolaren Bereich beschreiben. Um zu unterscheiden, ob der Effekt von ATP durch die Bindung oder aber der Hydrolyse des Nukleotids zustande kommt wurden Experimente mit verschiedenen Substanzen durchgeführt. ATP und ADP zeigten eine signifikante, wenn auch unterschiedlich ausgeprägte Verlangsamung der Schaltkinetik. Die Applikation von AMP alleine ergab keine Veränderung der Kinetik und konnte ATP in der Ko-

Applikation auch nicht kompetitiv verdrängen. Das nicht hydrolysierbare ATP Analogon AMP-PNP zeigte eine mit ADP vergleichbare Verlangsamung. So ist davon auszugehen, dass Nukleotide nicht als Energielieferanten für die Veränderung des Kanalverhaltens benötigt werden. Weiterhin ist der Effekt durch den C-Terminus von ClC-2 vermittelt; eine Trunkation des Kanals vor der ersten CBS-Domäne zeigte – neben einer generellen Beschleunigung von Aktivierung und Deaktivierung - keine Abhängigkeit von ATP.

Einzelkanalmessungen am ClC-2 Wild-Typ

Einzelkanalmessungen können helfen Informationen über das Schaltverhalten eines Kanals zu gewinnen. Bei den Mitgliedern der CLC-Familie ist diese Technik häufig durch die kleinen Einzelkanalamplituden eingeschränkt. Durch Rauschanalysen und Einzelkanalmessungen wurde die Protoporen-Leitfähigkeit von ClC-2 bislang bei Werten zwischen 2,4 und 2,8 pS bestimmt [14, 47, 49]. In dieser Studie konnten stabile Einzelkanalmessungen bei maximal -100 mV erreicht werden, so dass Amplituden zwischen 0,24 und 0,28 pA für eine Protoporenöffnung erwartet werden. In mehreren Experimenten konnte in der Tat das typische CLC-“double barrel“ Muster mit langen Geschlossenzuständen und Bursts mit zwei Öffnungsniveaus von je 0,23 +/- 0,02 pA nachgewiesen werden. Die Offenwahrscheinlichkeiten bestätigten die Werte aus der Rauschanalyse mit einer Offenwahrscheinlichkeit von etwa 55% ohne und unter 50% in der Gegenwart von 5 mM ATP.

In Gegenwart von ATP zeigte sich ein gehäuftes Auftreten von sehr langen Geschlossenzuständen beider Poren. Dieses Ergebnis weist auf den gemeinsamen Schaltprozess als Zielstruktur für die Modulation von ClC-2 durch ATP hin.

Die Schwierigkeit in der Analyse von Einzelkanalmessungen an ClC-2 ist die kleine Amplitude. Aufgrund technischer Limitationen kann das Hintergrundrauschen nicht beliebig reduziert werden. Die vorliegenden Aufnahmen sind nur mit einer starken Filterung (Eckfrequenz 1 kHz) des Rohsignals möglich. Mit dieser Filterung konnte das vom Hersteller angegebene theoretische Limit von 55-65 fA *Root Mean Square (RMS)* Rauschen erreicht werden. Für kinetische Analysen ist dieser Wert bei einer Signalamplitude von 230 fA nicht ausreichend. Durch den nachträglich digital eingefügten Filter mit einer Eckfrequenz von 200 Hz konnte in allen Aufnahmen ein ausreichendes Signal-zu-Rausch-Verhältnis erzielt werden, die zeitliche Auflösung reduzierte sich jedoch soweit, dass Ereignisse kürzer als 1 ms nicht ausgewertet werden konnten.

Modellierung der ATP-Abhängigkeit von Whole-Cell CIC-2 Strömen

Die Messungen von Whole-Cell und Einzelkanalströmen deuten auf eine starke Beteiligung des gemeinsamen Schließprozesses in der ATP-abhängigen Regulation von CIC-2 hin. Um zu testen welche Änderungen im Schaltverhalten notwendig sind damit die gemessenen Änderungen auftreten, wurde eine Simulation der Whole-Cell-Ströme auf Ebene eines Zustandsmodells mit Ratenkonstanten durchgeführt.

Aufgrund der Spannungsabhängigkeit von CIC-2 wurden die Übergänge innerhalb des Modells aus spannungsabhängigen und -unabhängigen Ratenkonstanten zusammengesetzt. Diese Werte können in einer sogenannten Q-Matrix zusammengefasst werden und über die Eigenwerte einer Matrix-Exponentialfunktion zu zeitabhängigen Zustandswahrscheinlichkeiten aufgelöst werden [40].

Lange Einzelkanalmessungen, wie in dieser Studie vorliegend, dienen vor allem der Bestimmung von Parametern im Gleichgewichtszustand, so dass sie ungeeignet sind die kinetischen Änderungen von Aktivierung oder Deaktivierung zu erfassen. Der Vergleich der Simulation zu experimentell gewonnenen Daten erfolgte daher an Whole-Cell Messungen. Hierfür wurden 250-500 exponentiell verteilte Datenpunkte pro Testspannung mit Werten des Modells verglichen und über die Summe der quadratischen Abweichungen bewertet. In einem genetischen Algorithmus wurden zunächst zufällig verteilte Werte für die Komponenten der Ratenkonstanten eingesetzt und verglichen. Aus den besten Werten wurden für die nächste Runde des Algorithmus zufällig Parameter vertauscht und für den Rest neue Parameter zufällig hinzugefügt. So konnte üblicherweise nach mehreren tausend Runden ein Zustand erreicht werden bei dem die zufällig neu entstehenden Parameter das beste Ergebnis nicht mehr als um 0,1% verbessern konnten. Dieser Algorithmus wurde daraufhin erneut gestartet und die besten Ergebnisse jeweils verglichen bis über mehrere Neustarts keine Verbesserung erreicht werden konnte. Eine sekundäre Evaluation erfolgte durch den Vergleich der errechneten Werte für Zeitkonstanten, der ATP-Abhängigkeit und der Offenwahrscheinlichkeiten mit den experimentell gewonnenen Daten.

Aufgrund der Annahme der Unabhängigkeit von Protoporen-Gate und Common-Gate wurde die Simulation hier, wie auch in anderen Studien zuvor [41], getrennt durchgeführt. Für das Protoporen-Gate wurde von einem einfachen Zustandsmodell (Closed zu Open) ausgegangen. Das Modell für das Common-Gate wurde ausgehend von einem solchen Modell schrittweise komplizierter gestaltet um alle Änderungen hinreichend zu beschreiben.

Die Verlangsamung von CIC-2 durch ATP konnte alleine durch Veränderungen des gemeinsamen Schaltprozesses vorhergesagt werden. Ein Teil der Kanäle befindet sich in der Gegenwart von ATP in einer Dopplung der normalen Schaltprozesse, die veränderte Spannungsabhängigkeiten der Ratenkonstanten zeigen und so Aktivierung wie Deaktivierung betreffen.

Die Änderung der schnellen Zeitkonstante in der Deaktivierung ist vermutlich auf die Überlagerung sehr ähnlicher Zeitkonstanten von Protoporen- und Common-Gate zurückzuführen. Durch die Überlagerung führt eine Änderung des gemeinsamen Schließprozesses zu einer Veränderung beider Zeitkonstanten. Eine Bestimmung von Zeitkonstanten über die Summe zweier Exponentialfunktionen kann somit keine genauen Werte liefern.

Punktmutationen verändern das ATP-abhängige Schaltverhalten von CIC-2

In früheren Studien wurde eine Assoziation von Punktmutationen in CIC-2 mit Idiopathischen generalisierten Epilepsien (IGE) beschrieben. Es wurde bislang keine einheitliche Abweichung der Kanalfunktion beschrieben, so dass der Einfluss dieser Mutationen kontrovers diskutiert wurde. Viele dieser Variationen liegen im Bereich des C-Terminus und wurden daher in dieser Studie auf ihre Abhängigkeit von Nukleotiden untersucht. In Zusammenarbeit mit der Abteilung für Epileptologie der Universität Tübingen innerhalb des EPICURE Consortiums wurde zusätzlich eine neue, bislang nicht bekannte Mutation – R653T – identifiziert und getestet.

Alle krankheitsassoziierten Mutationen die in dieser Studie untersucht wurden zeigten eine Veränderung der Geschwindigkeit von Aktivierung oder Deaktivierung, so dass die Verlangsamung der Kinetik in der Gegenwart von ATP relativ zum CIC-2 Wild-Typ weniger deutlich ausgeprägt war bzw. komplett fehlte. Die Affinität für ATP zeigte sich, zumindest für die G715E Mutation, nicht wesentlich beeinträchtigt. Zwei Mutationen, welche zuvor als benigne Polymorphismen beschrieben waren [3, 7] zeigten in diesen Experimenten keine Beeinträchtigung der Kanalfunktion in der Gegenwart von ATP.

Einzelkanalmessungen bestätigten die Ergebnisse, da die besonders langen Geschlossenzustände in den krankheitsassoziierten Mutationen deutlich seltener auftraten als im WT.

Innerhalb des aufgestellten Modells ist die Ursache dieser Beschleunigung in einer Veränderung der spannungsabhängigen Ratenkonstanten zu sehen und nicht in einer geänderten Bindung von ATP an die CBS-Domänen.

Diskussion

Die hier vorliegenden Ergebnisse lassen vermuten, dass ClC-2 in einem physiologischen Umfeld eine noch langsamere Aktivierung und Deaktivierung zeigt als bislang angenommen. Bestimmte Punktmutationen sorgen aufgrund ihrer veränderten Spannungsabhängigkeit für eine Beschleunigung von Aktivierung und Deaktivierung relativ zum WT.

Es ist fraglich, ob Nukleotide eine Anpassung des Kanals an metabolischen Stress ermöglichen. Werte für die ATP Konzentrationen in Neuronen schwanken zwischen 2 und 10 mM [27, 35, 50]. ATP und ADP zeigen einen ähnlichen Effekt, so dass ein rascher Abbau von ATP zu ADP zunächst keinen Effekt hätte. Ein weiterer Abbau zu AMP würde erst zu einer Beschleunigung des Schaltverhaltens von ClC-2 führen, wenn gleichzeitig die Summe der Konzentration von ATP und ADP in niedrigere Bereiche fiele. Es ist davon auszugehen, dass die physiologische Funktion des Kanals der in Gegenwart höherer ATP Konzentrationen entspricht. Diese normale Funktion ist in den getesteten Punktmutationen nicht mehr gegeben – auch schon bei physiologischen ATP Konzentrationen.

Die Bindung von Nukleotiden an den C-Terminus von CLC Proteinen ist nicht ungewöhnlich. Für ClC-5 ist diese Bindung durch Röntgenstrukturanalysen sogar auf atomarer Ebene aufgelöst [28] und für ClC-2 mittels radiographischer Analysen nachgewiesen worden [42]. Der Bezug zwischen C-Terminus und kooperativem Gating wurde für ClC-0 und ClC-1 beschrieben [12, 18]. Die Veränderung bzw. das Fehlen der langsameren Zeitkonstante in der Aktivierung oder Deaktivierung von Ganzzellströmen wurde als Veränderung des Common-Gates aufgefasst. In ClC-0 ist das kooperative Schaltverhalten zusätzlich mit messbaren Bewegungen des C-Terminus verknüpft [5]. Röntgenstrukturanalysen an dem eukaryotischen Transporter cmClC lassen eine Interaktion des C-Terminus mit den Transmembrandomänen vermuten [10]. Für ClC-2 zeigt eine komplette Trunkation des C-Terminus nur eine Beschleunigung, nicht jedoch ein Fehlen dieser zweiten, langsamen Zeitkonstante [14], weswegen hier lediglich von einer Regulation des Common-Gates durch den C-Terminus ausgegangen werden muss.

Die hier gezeigten funktionellen Veränderungen von ClC-2 durch die Bindung von ATP lassen sich zumindest nicht alleine durch eine Änderung der Affinität erklären. Bei gleichbleibenden Parametern für die ATP-Affinität der Zeitkonstanten müssen die Konformationsänderung des C-Terminus und vermutlich ebenfalls die stattfindende Interaktion zu den Transmembrandomänen verändert sein um die beobachtete Verlangsamung hervorzurufen.

Die physiologische Relevanz dieser Veränderungen kann hier nicht abschließend geklärt werden. Die genaue Funktion von ClC-2 ist bislang nicht hinreichend bekannt. Das Vorkommen von ClC-2 Strömen in Neuronen ist bereits seit längerem bekannt [45]. Aufgrund des starken Ausstromes von Chlorid-

Ionen durch die Aktivierung bei Spannungen negativ zum Chlorid-Umkehrpotential wurde der Chlorid-Efflux lange als Hauptfunktion von CIC-2 in Neuronen angesehen [44, 45]. Dies könnte pathophysiologische Relevanz erlangen, sollte zum Beispiel nach repetitiver GABAerger Stimulation, ein zunehmender Chlorideinstrom das Chloridumkehrpotential so verschieben, dass eine weitere Stimulation mit GABA zu einem Efflux von Chlorid durch GABA_A Kanäle und damit einer exzitatorischen Wirkung führen würde. CIC-2 könnte hier durch die Differenz des Ruhemembranpotentials zum steigenden Chloridumkehrpotential aktiviert werden und einen kompensatorischen, langsamen Chloridefflux vermitteln. In den Punktmutationen könnte durch die Beschleunigung dieses Mechanismus unter Umständen eine exzitatorische Wirkung vermittelt werden [24].

Eine andere Studie kam zu der Vermutung, dass der langsam deaktivierende Strom positiv zum Chloridumkehrpotential die entscheidende Rolle für die physiologische Funktion von CIC-2 darstellt [37]. Bei der Entstehung von Aktionspotentialen soll diese lange persistierende Chloridleitfähigkeit für einen inhibierenden Effekt sorgen. In diesem Falle könnte die Beschleunigung der Deaktivierung zu einer rascheren Abnahme dieses inhibierenden Effektes und damit zu einer möglichen Hyperexzitabilität führen.

Für eine Beteiligung von CIC-2 an der Regulation des GABAergen Systems spricht, dass in Patch Clamp Experimenten an Mäusen gezeigt wurde, dass Neurone des Hippocampus eine erhöhte Dichte an CIC-2 Kanälen im Bereich des Zellsoma aufweisen [11]. Eine andere Arbeit etabliert CIC-2 als konstitutiven Hintergrundstrom welcher die Chlorid-Homöostase in GABAergen, hippocampalen Neuronen ermöglicht [38]. Die genaue Lokalisation in anderen Neuronen ist weiterhin ungeklärt.

Ein, nach der hier vorliegenden Veröffentlichung erschienenenes Manuskript hinterfragt, ob CIC-2 in menschlichen Neuronen überhaupt signifikant exprimiert wird. Aufgrund von anderen Punktmutationen im *CLCN2* Gen wurden keine epileptischen Anfälle, jedoch eine Leukenzephalopathie mit verschiedensten Symptomen beschrieben. Immunocytochemische Färbungen einzelner Hirnbereiche zeigten eine deutliche Anfärbung von glialen Strukturen, nicht jedoch von Neuronen [8]. Manche Astrozyten zeigten ebenfalls eine Koexpression mit dem Protein GlialCAM welches als β -Unterheit den CIC-2 Kanal moduliert [21]. Als pathophysiologische Ursache der Leukenzephalopathie werden Änderungen der Wasser-Homöostase diskutiert [8].

Sollte CIC-2 eine Bedeutung für die Ätiologie einer Epilepsie haben, werden Veränderungen der Funktion vermutlich nur einen Faktor darstellen. Aktuelle Studien gehen für idiopathische Epilepsien davon aus, dass häufig eine Kombination mehrerer genetischer Veränderungen zum Ausbruch der Erkrankung führt [23]. In diesem Zusammenhang kann die hier vorliegende Arbeit helfen

Veränderungen durch CIC-2 Mutationen in Zukunft gezielt in Neuronen oder Gliazellen innerhalb von Hirnschnitten zu charakterisieren um damit die Entstehung von Erkrankungen durch Veränderungen des *CLCN2* Gens zu klären.

Literaturverzeichnis

1. Accardi, A., Miller, C.: Secondary active transport mediated by a prokaryotic homologue of ClC Cl-channels. *Nature*. 427, 6977, 803–807 (2004).
2. Bateman, A.: The structure of a domain common to archaeobacteria and the homocystinuria disease protein. *Trends Biochem. Sci.* 22, 1, 12–13 (1997).
3. Blanz, J. et al.: Leukoencephalopathy upon disruption of the chloride channel ClC-2. *J. Neurosci. Off. J. Soc. Neurosci.* 27, 24, 6581–6589 (2007).
4. Bösl, M.R. et al.: Male germ cells and photoreceptors, both dependent on close cell–cell interactions, degenerate upon ClC-2 Cl⁻ channel disruption. *Embo J.* 20, 6, 1289–1299 (2001).
5. Bykova, E.A. et al.: Large movement in the C terminus of ClC-0 chloride channel during slow gating. *Nat Struct Mol Biol.* 13, 12, 1115–1119 (2006).
6. Cuppoletti, J. et al.: SPI-0211 activates T84 cell chloride transport and recombinant human ClC-2 chloride currents. *Am. J. Physiol. Cell Physiol.* 287, 5, C1173–1183 (2004).
7. D’Agostino, D. et al.: Mutations and polymorphisms of the CLCN2 gene in idiopathic epilepsy. *Neurology*. 63, 8, 1500–1502 (2004).
8. Depienne, C. et al.: Brain white matter oedema due to ClC-2 chloride channel deficiency: an observational analytical study. *Lancet Neurol.* (2013).
9. Dutzler, R. et al.: X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature*. 415, 6869, 287–294 (2002).
10. Feng, L. et al.: Structure of a Eukaryotic ClC Transporter Defines an Intermediate State in the Transport Cycle. *Science*. science.1195230 (2010).
11. Földy, C. et al.: Regulation of fast-spiking basket cell synapses by the chloride channel ClC-2. *Nat. Neurosci.* (2010).
12. Fong, P. et al.: Determinants of slow gating in ClC-0, the voltage-gated chloride channel of *Torpedo marmorata*. *Am. J. Physiol.* 274, 4 Pt 1, C966–973 (1998).
13. Furukawa, T. et al.: Characteristics of rabbit ClC-2 current expressed in *Xenopus* oocytes and its contribution to volume regulation. *Am. J. Physiol. - Cell Physiol.* 274, 2, C500–C512 (1998).
14. Garcia-Olivares, J. et al.: Gating of human ClC-2 chloride channels and regulation by carboxy-terminal domains. *J. Physiol.* 586, Pt 22, 5325–5336 (2008).
15. Gründer, S. et al.: Regions involved in the opening of ClC-2 chloride channel by voltage and cell volume. *Nature*. 360, 6406, 759–762 (1992).
16. Guzman, R.E. et al.: ClC-3 is an intracellular chloride/proton exchanger with large voltage-dependent nonlinear capacitance. *Acs Chem. Neurosci.* (2013).
17. Hamill, O.P. et al.: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch. Eur. J. Physiol.* 391, 2, 85–100 (1981).
18. Hebeisen, S. et al.: The role of the carboxyl terminus in ClC chloride channel function. *J. Biol. Chem.* 279, 13, 13140–13147 (2004).
19. Jentsch, T.J.: ClC chloride channels and transporters: from genes to protein structure, pathology and physiology. *Crit. Rev. Biochem. Mol. Biol.* 43, 1, 3–36 (2008).
20. Jentsch, T.J. et al.: Primary structure of *Torpedo marmorata* chloride channel isolated by expression cloning in *Xenopus* oocytes. *Nature*. 348, 6301, 510–514 (1990).
21. Jeworutzki, E. et al.: GlialCAM, a Protein Defective in a Leukodystrophy, Serves as a ClC-2 Cl-Channel Auxiliary Subunit. *Neuron*. 73, 5, 951–961 (2012).
22. Kieferle, S. et al.: Two highly homologous members of the ClC chloride channel family in both rat and human kidney. *Proc. Natl. Acad. Sci. U. S. A.* 91, 15, 6943–6947 (1994).
23. Klassen, T. et al.: Exome sequencing of ion channel genes reveals complex profiles confounding personal risk assessment in epilepsy. *Cell*. 145, 7, 1036–1048 (2011).
24. Kleefuß-Lie, A. et al.: CLCN2 variants in idiopathic generalized epilepsy. *Nat. Genet.* 41, 9, 954–955 (2009).

25. Koch, M.C. et al.: The skeletal muscle chloride channel in dominant and recessive human myotonia. *Science*. 257, 5071, 797–800 (1992).
26. Leisle, L. et al.: ClC-7 is a slowly voltage-gated 2Cl(-)/1H(+)-exchanger and requires Ostm1 for transport activity. *Embo J.* 30, 11, 2140–2152 (2011).
27. Merboldt, K.D. et al.: Localized 31P NMR spectroscopy of the amt human brain in vivo using stimulated-echo (STEAM) sequences. *J. Magn. Reson.* 1969. 89, 2, 343–361 (1990).
28. Meyer, S. et al.: Nucleotide recognition by the cytoplasmic domain of the human chloride transporter ClC-5. *Nat. Struct. Mol. Biol.* 14, 1, 60–67 (2007).
29. Middleton, R.E. et al.: Purification, reconstitution, and subunit composition of a voltage-gated chloride channel from *Torpedo* electroplax. *Biochemistry (Mosc.)*. 33, 45, 13189–13198 (1994).
30. Miller, C.: Open-state substructure of single chloride channels from *Torpedo* electroplax. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 299, 1097, 401–411 (1982).
31. Miller, C., White, M.M.: Dimeric structure of single chloride channels from *Torpedo* electroplax. *Proc. Natl. Acad. Sci.* 81, 9, 2772–2775 (1984).
32. Neagoe, I. et al.: The late endosomal ClC-6 mediates proton/chloride countertransport in heterologous plasma membrane expression. *J. Biol. Chem.* 285, 28, 21689–21697 (2010).
33. Nehrke, K. et al.: Loss of Hyperpolarization-activated Cl⁻ Current in Salivary Acinar Cells from *Clcn2* Knockout Mice. *J. Biol. Chem.* 277, 26, 23604–23611 (2002).
34. Niemeyer, M.I. et al.: Functional evaluation of human ClC-2 chloride channel mutations associated with idiopathic generalized epilepsies. *Physiol. Genomics*. 19, 1, 74–83 (2004).
35. Pan, J.W., Takahashi, K.: Interdependence of N-acetyl aspartate and high-energy phosphates in healthy human brain. *Ann. Neurol.* 57, 1, 92–97 (2005).
36. Picollo, A., Pusch, M.: Chloride/proton antiporter activity of mammalian CLC proteins ClC-4 and ClC-5. *Nature*. 436, 7049, 420–423 (2005).
37. Ratté, S., Prescott, S.A.: ClC-2 Channels Regulate Neuronal Excitability, Not Intracellular Chloride Levels. *J. Neurosci. Off. J. Soc. Neurosci.* 31, 44, 15838–15843 (2011).
38. Rinke, I. et al.: ClC-2 voltage-gated channels constitute part of the background conductance and assist chloride extrusion. *J. Neurosci. Off. J. Soc. Neurosci.* 30, 13, 4776–4786 (2010).
39. Saint-Martin, C. et al.: Two novel *CLCN2* mutations accelerating chloride channel deactivation are associated with idiopathic generalized epilepsy. *Hum. Mutat.* 30, 3, 397–405 (2009).
40. Sakmann, B.: Single-channel recording. Springer, New York, NY (2009).
41. De Santiago, J.A. et al.: Quantitative analysis of the voltage-dependent gating of mouse parotid ClC-2 chloride channel. *J. Gen. Physiol.* 126, 6, 591–603 (2005).
42. Scott, J.W. et al.: CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J. Clin. Invest.* 113, 2, 274–284 (2004).
43. Sesti, F., Goldstein, S.A.: Single-channel characteristics of wild-type IKs channels and channels formed with two minK mutants that cause long QT syndrome. *J. Gen. Physiol.* 112, 6, 651–663 (1998).
44. Staley, K. et al.: Alteration of GABA_A Receptor Function Following Gene Transfer of the ClC-2 Chloride Channel. *Neuron*. 17, 3, 543–551 (1996).
45. Staley, K.: The role of an inwardly rectifying chloride conductance in postsynaptic inhibition. *J. Neurophysiol.* 72, 1, 273–284 (1994).
46. Thiemann, A. et al.: A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature*. 356, 6364, 57–60 (1992).
47. Thompson, C.H. et al.: Isolation and characterization of a high affinity peptide inhibitor of ClC-2 chloride channels. *J. Biol. Chem.* 284, 38, 26051–26062 (2009).
48. Torres-Salazar, D., Fahlke, C.: Neuronal glutamate transporters vary in substrate transport rate but not in unitary anion channel conductance. *J. Biol. Chem.* 282, 48, 34719–34726 (2007).
49. Weinreich, F., Jentsch, T.J.: Pores formed by single subunits in mixed dimers of different CLC chloride channels. *J. Biol. Chem.* 276, 4, 2347–2353 (2001).
50. Younkin, D.P. et al.: Cerebral metabolic effects of neonatal seizures measured with in vivo 31P NMR spectroscopy. *Ann. Neurol.* 20, 4, 513–519 (1986).

Erklärung nach § 2 Abs. 2 Nr. 6 und 7 PromO

Ich erkläre, dass ich die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel „Funktionelle Untersuchung von Epilepsie-assoziierten Punktmutationen im CLCN2 Gen“ im Institut für Neurophysiologie unter der Leitung und Betreuung von Prof. Dr. Christoph Fahlke ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Die Gelegenheit zum vorliegenden Promotionsverfahren ist mir nicht kommerziell vermittelt worden. Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Weiterhin versichere ich, dass ich den beantragten Titel bislang noch nicht erworben habe.

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Gabriel Stölting